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Instituto Andaluz de Investigación
y Formación Agraria, Pesquera, Alimentaria
y de la Producción Ecológica
Consejería de Agricultura, Ganadería,
Pesca y Desarrollo Sostenible

IMIBIC

Caracterización fitoquímica y evaluación de la bioaccesibilidad *in vitro* y biodisponibilidad *in vivo* de cebolla negra (*Allium cepa* L.) y ajo negro (*Allium sativum* L.)

Phytochemical characterization and evaluation of the *in vitro* bioaccessibility and *in vivo* bioavailability of black onion (*Allium cepa* L.) and black garlic (*Allium sativum* L.)

Alicia Moreno Ortega

Córdoba, diciembre de 2021

INFORME DIRECTORES DE LA TESIS

TÍTULO DE LA TESIS: Caracterización fitoquímica y evaluación de la bioaccesibilidad in vitro y biodisponibilidad in vivo de cebolla negra (*Allium cepa* L.) y ajo negro (*Allium sativum* L.).

DOCTORANDA: Alicia Moreno Ortega

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

La tesis doctoral llevada a cabo por Dña. Alicia Moreno Ortega supone un avance en el estudio sobre el perfil de compuestos nutricionales y bioactivos de dos nuevos productos como la cebolla negra y el ajo negro. De igual manera, se ha profundizado en el conocimiento sobre las posibles transformaciones sufridas por sus compuestos bioactivos, compuestos fenólicos y organosulfurados, a lo largo del proceso de digestión. Para llevar a cabo esta investigación, la doctoranda ha puesto a punto metodologías analíticas como la cromatografía de líquidos acoplada a espectrometría de masas de alta resolución y ha desarrollado estudios de bioaccesibilidad *in vitro* simulando las condiciones de digestión orales, gástricas, intestinales y colónicas. Además, ha llevado a cabo un estudio clínico de intervención de ingesta aguda para la evaluación de la biodisponibilidad *in vivo* de los compuestos saludables de ambos alimentos.

Los resultados de esta investigación suponen una información valiosa para el sector agroalimentario ya que ponen de manifiesto las características funcionales de estos nuevos productos, pudiendo suponer una estrategia de venta y reclamo competitivo para ambos productos.

Asimismo, los resultados de esta investigación han dado lugar a diversas comunicaciones a congresos internacionales y nacionales y a la publicación de 4 artículos científicos en revistas internacionales de alto índice de impacto y 4 en revisión. Además, una parte de la investigación de esta tesis se ha desarrollado a través de una estancia internacional de 12 meses (tesis en régimen de co-tutela) en la "Human Nutrition Unit of Food and Drugs" de la Universidad de Parma, en Italia.

Por todo ello, se autoriza la presentación de la tesis doctoral.

LOS DIRECTORES DE LA TESIS

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TÍTULO DE LA TESIS: Caracterización fitoquímica y evaluación de la bioaccesibilidad in vitro y biodisponibilidad in vivo de cebolla negra (Allium cepa L.) y ajo negro (Allium sativum L.).

DOCTORANDA: Alicia Moreno Ortega

INFORME RAZONADO DEL TUTOR

(Ratificando el informe favorable del director. Sólo cuando el director no pertenezca a la Universidad de Córdoba).

La doctoranda ha desarrollado su tesis doctoral bajo mi tutela, realizando la caracterización de un alimento completamente nuevo, como es la cebolla negra, de la cual hasta el momento se desconocía absolutamente todo de su composición y por ende de los cambios sufridos desde el alimento de origen que es la cebolla cruda. Dado el precedente de un alimento elaborado con metodología estudiar (ajo negro) se ha realizado una análisis paralelo de sus perfiles de compuestos nutricionales y bioactivos.

Una parte de esta tesis se ha desarrollado a través de una estancia internacional de 12 meses (tesis en régimen de co-tutela) en la "Human Nutrition Unit of Food and Drugs" de la Universidad de Parma, en Italia.

La tesis ha dado lugar a las siguientes publicaciones en forma de artículo científico publicado en revista indexada en JCR:

Revista	Título	Año	Categoría	Índice de impacto	Cuartil
LWT: Food Science & Technology	Development and validation of UHPLC-HRMS methodology for the determination of flavonoids, amino acids and organosulfur compounds in black onion, a novel derived product from fresh shallot onions (*Allium cepa* var. aggregatum).	2018	Food Science & Technology	3.129	Q1 (2017): 24/133 (82.33%)
Food Chemistry	Changes in the antioxidant activity and metabolite profile of three onion varieties during the elaboration of 'black onion'.	2020	Food Science & Technology	5.399	Q1 (2019): 6/139 (96.04%)
Foods	Bioaccessibility of bioactive compounds of 'fresh garlic' and 'black garlic' through *in vitro* gastrointestinal digestion.	2020	Food Science & Technology	4.350	Q1 (2019): 27/139 (80.94%)
Foods	Changes in the Organosulfur and Polyphenol Compound Profiles of Black and Fresh Onion during Simulated Gastrointestinal Digestion.	2021	Food Science & Technology	4.350	Q1 (2019): 27/139 (80.94%)

Por todo ello, doy mi visto bueno a la presentación de la tesis doctoral.

Córdoba, 14 de octubre de 2021

Firma del responsable de línea de investigación

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PUBLICACIONES INCLUIDAS EN LA TESIS DOCTORAL

Revista	Título	Año	Categoría	Índice de impacto	Cuartil
LWT: Food Science & Technology	Development and validation of UHPLC-HRMS methodology for the determination of flavonoids, amino acids and organosulfur compounds in black onion, a novel derived product from fresh shallot onions (*Allium cepa* var. aggregatum).	2018	Food Science & Technology	3.129	Q1 (2017): 24/133 (82.33%)
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Journal of Agricultural and Food Chemistry (enviado)	*In vitro* colonic fermentation of fresh and black garlic	2021	Food Science & Technology	5.279	Q1 (2020): 24/144 (83.68%)
Food & Fuction (enviado)	Effect of *in vitro* colonic fermentation on the stability of fresh and black onion bioactives	2021	Food Science & Technology	5.396	Q1 (2020): 22/144 (85.07%)

FINANCIACIÓN

Esta tesis se ha realizado en el Departamento de Bromatología y Tecnología de los Alimentos de la Facultad de Veterinaria de la Universidad de Córdoba, en el Área de Alimentación y Salud del centro IFAPA-Alameda del Obispo (Córdoba) y en el "Human Nutrition Unit, Department of Food and Drugs" de la Universidad de Parma bajo la dirección de los Drs. José Manuel Moreno Rojas, Mª Gema Pereira Caro, Pedro Miguel Mena Parreño y Daniele del Rio.

La autora de esta tesis ha disfrutado de un contrato laboral en la Universidad de Córdoba en el marco de las Ayudas para la Formación de Profesorado Universitario (FPU) del Ministerio de Educación, Cultura y Deporte (FPU16-05881).

La presente Tesis Doctoral ha sido realizada gracias a la financiación de los siguientes proyectos de investigación:

- Caracterización de alimentos y nuevos productos elaborados: potencial saludable, organoléptico y trazabilidad alimentaria. Estrategias de diversificación y reclamo competitivo (PP.AVA.AVA201601.20) otorgado por el Instituto de Investigación y Formación Agraria y Pesquera (IFAPA) (Consejería de Agricultura, Pesca y Desarrollo Rural, Junta de Andalucía). (Investigador Principal: Dr. José Manuel Moreno Rojas).
- Caracterización, biodisponibilidad y potencial saludable de compuestos bioactivos de alimentos (PP.AVA.AVA2019.037) otorgado por el Instituto de Investigación y Formación Agraria y Pesquera (IFAPA) (Consejería de Agricultura, Pesca y Desarrollo Rural, Junta de Andalucía) y el Fondo Europeo de Desarrollo Rural (FEDER). (Investigador Principal: Dr. Víctor Ortiz Somovilla).

La autora de esta tesis ha disfrutado de una ayuda de movilidad internacional para el fomento de tesis con mención internacional o en régimen de cotutela (2019/2020) para la consecución de la tesis en régimen de cotutela con la Universidad de Parma, Italia.

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GOBIERNO DE ESPAÑA
MINISTERIO DE EDUCACIÓN, CULTURA Y DEPORTE

Andalucía se mueve con Europa

A mi padre y a mi hermana

A Rafa

“The journey doesn't end here.”

Gandalf,
El señor de los anillos: El retorno del rey

J.R.R. Tolkien

AGRADECIMIENTOS

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Llegar finalmente a este momento no ha sido nada fácil, nada tiene que ver la Ali que comenzó esta tesis con la que la termina, han sido 4 años de intensa locura, con pandemia incluida, pero como siempre digo, estoy rodeada de los mejores, lo que hace más llevadero todo y que merezca la pena. Esta tesis tiene un poquito de cada persona que ha cruzado mi vida durante estos años, gracias por haber sido un gran apoyo, inspiración y motivación.

A José Manuel, gracias por apostar por mí. Gracias por darme la oportunidad de demostrar lo que valgo, más allá de la alargada sombra que me precede y el apellido que compartimos (por casualidades de la vida). Gracias por invitarme a tu "casa" y hacerme sentir de la familia IFAPA. Gracias por hacerme un hueco y abrirme las puertas de la maravillosa, y sufrida, carrera investigadora. Eres un gran ejemplo a seguir.

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Gracias a todos.

ABREVIATURAS

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2VD	2-vinil-4H-1,3-ditiina
ABTS	Ácido 2,2-azinobis-(3-etilbenzotioazolín-6-sulfónico)
AGD	After gastric digestion (Después de la digestión gástrica)
AID	After intestinal digestion (Después de la digestión intestinal)
AMDE	Absorción, metabolismo, distribución y excreción
α	Alfa
ANOVA	Analysis of Variance (Análisis de varianza)
AOAC	Association of Analytical Communities (Asociación de Comunidades Analíticas)
AOD	After oral digestion (Después de la digestión oral)
BEDCA	Base de Datos Española de Composición de Alimentos
BG	Black garlic (Ajo negro)
BI/IB	Bioaccessibility index / Índice de bioaccesibilidad
BO	Black onion (Cebolla negra)
BOD	Before oral digestion (Antes de la digestión oral)
Caco-2	Células de adenocarcinoma de colon
DADS	Disulfuro de dialilo
DATS	Trisulfuro de dialilo
DPPH	2,2-difenil-1-picrilhidracina
DSS	Dextrano sulfato de sodio
DW	Dry weight (Peso seco)
EFSA	European Food Safety Agency (Agencia europea de seguridad alimentaria)
EPOC	Enfermedad pulmonar obstructiva crónica
FA	Ácido fórmico
FAOSTAT	Base de datos de la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO)
FC/CF	Final concentration of compound / Concentración final del compuesto
FG	Fresh garlic (Ajo fresco)
FO	Fresh onion (Cebolla fresca)
FW/PS	Fresh weight / Peso fresco
GABA	Ácido γ-aminobutírico

γ	Gamma
GS1PC	γ-Glutamil-*S*-(1-propenil)-L-cisteína
GS1PCS	γ-Glutamil-*S*-(1-propenil)-L-cisteína sulfóxido
GS2PC	γ-Glutamil-*S*-(2-propenil)-L-cisteína
GS2PCS	γ-Glutamil-*S*-(2-propenil)-L-cisteína sulfóxido
GSAC	γ-Glutamil-*S*-alil-L-cisteína
GSAk	γ-Glutamil-*S*-alqu(en)in-L-cisteína
GSAMC	γ-Glutamil-*S*-alilmercaptocisteína
GSMC	γ-Glutamil-*S*-metil-L-cisteína
GSMCS	γ-Glutamil-*S*-metil-L-cisteína sulfóxido
GSPC	γ-Glutamil-*S*-(propil)-L-cisteína
HDL	Lipoproteínas de alta densidad
HMF	Hidroximetilfurfural
HPLC-RID	High Performance Liquid Chromatography coupled to Refraction Index Detector (Cromatografía líquida de alta resolución con detector de índice de refracción)
HT-29	Células de carcinoma de colon
IC/CI	Initial concentration of compound / Concentración inicial del compuesto
IFAPA	Instituto Andaluz de Investigación y Formación Agraria, Pesquera, Alimentaria y de la Producción Ecológica
INE	Instituto Nacional de Estadística
LDL	Lipoproteínas de baja densidad
LOD/LDD	Limit of detection / Límite de detección
LOQ/LDC	Limit of quantification / Límite de cuantificación
MAPAMA	Ministerio de Agricultura, Pesca, Alimentación y Medio Ambiente de España
MSI MI level	Metabolite standards initiative (MSI) metabolite identification (MI) levels (Niveles de identificación de metabolitos de la iniciativa de estándares de metabolitos)
NASAC	N-acetil-*S*-alil-L-cisteína
Nrf2	Nuclear factor erythroid 2-related factor (Factor nuclear derivado del eritroide 2)
ORAC	Oxygen radical absorbance capacity (Capacidad de absorción de radicales de oxígeno)
OSCs	Compuestos organosulfurados

PBS	Phosphate buffer saline (Tampón fosfato salino)
PC	Principal Component (Componente principal)
PCA	Principal Components Analysis (Análisis de componentes principales)
RH/HR	Relative humidity / Humedad relativa
ROS	Especies reactivas de oxígeno
RT	Retention time (Tiempo de retención)
S1PC	trans-*S*-(1-proneil)-L-cisteína
SAC	*S*-alil-L-cisteína
SAG	*S*-alilglutatión
SAk	*S*-alqu(en)in-L-cisteína
SAMC	*S*-alilmercaptocisteína
SGF	Solución simuladora fluidos gástricos
SIF	Solución simuladora fluidos intestinales
SPMC	*S*-Propilmercapto-L-cisteína
SSF	Solución simuladora fluidos salivares
TNF-α	Factor de necrosis tumoral alfa
UHPLC-DAD	Ultra high performance liquid chromatography coupled to diodo array detector (Cromatografía líquida de ultra-alta resolución con detector de diodos en fila)
UHPLC-HRMS	Ultra high performance liquid chromatography coupled to high resolution mass spectrometer (Cromatografía líquida de ultra-alta resolución acoplada a un espectrómetro de masas de alta resolución)
UHPLC-LIT-MS	Ultra high performance liquid chromatography with linear ion trap coupled to high resolution mass spectrometer (Cromatografía líquida de ultra-alta resolución con trampa iónica lineal acomplada a un espectrómetro de masas de alta resolución)

RESUMEN

La cebolla negra es un producto análogo al ajo negro que se obtiene mediante un proceso de elaboración similar que consiste en un envejecimiento del producto. Para ello, se somete la cebolla fresca (*Allium cepa* L.) a condiciones de temperatura y humedad controladas durante un periodo de tiempo determinado. Este proceso ocasiona una serie de cambios físico-químicos en el producto final que permiten obtener un alimento con una cualidades sensoriales sustancialmente diferentes a las del bulbo inicial y con cambios importantes en su composición fitoquímica. Hasta la fecha, la cebolla negra no ha sido objeto de investigaciones a pesar del éxito que ha supuesto la comercialización del ajo negro y las propiedades beneficiosas para la salud con las que se ha relacionado. Esto hace necesaria la caracterización fitoquímica de este nuevo producto, para lo que se deben validar y optimizar los métodos de extracción y análisis, así como evaluar *in vitro* e *in vivo*, los posibles cambios producidos en el perfil de compuestos fitoquímicos durante la digestión gastrointestinal, la fermentación colónica y la metabolización y excreción de los mismos una vez son consumidos.

En esta Tesis Doctoral, se optimizaron y validaron dos métodos cromatográficos selectivos, sensibles y precisos para la identificación y cuantificación de flavonoides, compuestos organosulfurados y aminoácidos en cebolla fresca y cebolla negra, y se aplicaron para estudiar la influencia del proceso de elaboración de este producto sobre el perfil de compuestos fenólicos, compuestos organosulfurados, aminoácidos, azúcares y ácidos orgánicos a partir de tres variedades distintas de cebolla. Esto permitió concluir que el proceso de elaboración de la cebolla negra influye significativamente en el perfil de compuestos nutricionales y funcionales de la misma, así como ver que estos cambios estarán influidos por la sensibilidad de cada uno de los compuestos al tratamiento térmico, el pH, la presencia de oxígeno y la composición de la matriz.

Por otro lado, se evaluó el impacto de la digestión gastrointestinal *in vitro* sobre el perfil fitoquímico del ajo fresco, ajo negro, cebolla fresca y cebolla negra y se determinó el índice de bioaccesibilidad estos compuestos. Con ello se ha comprobado que el proceso de elaboración por el que se obtienen el ajo negro y la cebolla negra podría tener un efecto positivo en la bioaccesibilidad del perfil de compuestos organosulfurados, aunque no ocurre de la misma forma en el perfil de compuestos fenólicos.

También se estudió la evolución del perfil de compuestos fenólicos y organosulfurados durante la fermentación colónica *in vitro* del ajo fresco, ajo negro, cebolla fresca y cebolla negra, lo que permitió establecer que el proceso de elaboración del ajo negro y la cebolla negra también podría estar influenciando el metabolismo de estos a compuestos a nivel

colónico, observándose tendencias diferentes entre los productos originales y los finales.

Finalmente, se abordó la biodisponibilidad de los compuestos fenólicos y organosulfurados presentes en ajo negro y cebolla negra mediante un estudio de intervención de ingesta aguda, para lo que recogieron muestras de orina, lo que permitió situar la absorción mayoritaria de estos compuestos en intestino delgado y grueso.

ABSTRACT

Black onion is a product analogous to black garlic and is obtained by a similar production process involving the ageing of the product. The fresh onion (*Allium cepa L*.) is subjected to controlled temperature and humidity conditions for a certain period of time. This process causes a series of physico-chemical changes in the final product, resulting in a foodstuff with sensory qualities substantially different from those of the initial bulb and with important changes in its phytochemical composition. To date, black onion has not been the subject of research despite the success of the commercialisation of black garlic and the beneficial health properties with which it has been associated. This makes the phytochemical characterisation of this new product necessary, for which the methods of extraction and analysis must be validated and optimised, as well as evaluating in vitro and in vivo, the possible changes produced in the profile of phytochemical compounds during gastrointestinal digestion, colonic fermentation and their metabolisation and excretion once they are consumed.

In this Doctoral Thesis, two selective, sensitive and accurate chromatographic methods for the identification and quantification of flavonoids, organosulfur compounds and amino acids in fresh onion and black onion were optimised and validated and applied to study the influence of the processing of this product on the profile of phenolic compounds, organosulfur compounds, amino acids, sugars and organic acids from three different varieties of onion. This allowed to conclude that the processing of black onion has a significant influence on the profile of nutritional and functional compounds, and that these changes are influenced by the sensitivity of each of the compounds to heat treatment, pH, the presence of oxygen and the composition of the matrix.

On the other hand, the impact of in vitro gastrointestinal digestion on the phytochemical profile of fresh garlic, black garlic, fresh onion and black onion was evaluated and the bioaccessibility index of these compounds was determined. This showed that the production process by which black garlic and black onion are obtained could have a positive effect on the bioaccessibility of the organosulfur compound profile, although this is not the case for the phenolic compound profile.

The evolution of the phenolic and organosulfur compound profile during in vitro colonic fermentation of fresh garlic, black garlic, fresh onion and black onion was also studied, which allowed establishing that the processing of black garlic and black onion could also be influencing the metabolism of these compounds at the colonic level, with different trends observed between the original and the final products.

Finally, the bioavailability of phenolic and organosulfur compounds present in black garlic and black onion was addressed by means of an acute intake intervention study, for which

urine samples were collected, making it possible to locate the majority absorption of these compounds in the small and large intestine.

ÍNDICE

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CAPÍTULO 1: INTRODUCCIÓN

CAPÍTULO 1: INTRODUCCIÓN

El ajo y la cebolla, pertenecientes al género *Allium*, se integran en la mayor parte de los platos propios de la gastronomía de la cuenca Mediterránea. Son utilizados para sofritos, salsas, sopas o adobos, y como aderezos para multitud de platos populares característicos de la dieta mediterránea. Además, históricamente, el consumo de estos bulbos ha sido relacionado con diferentes propiedades beneficiosas para la salud, siendo utilizados en la elaboración de remedios caseros y formando parte de la mitología de diferentes culturas. En las últimas décadas, el estudio de estos vegetales ha permitido relacionar el efecto protector o preventivo del consumo regular de ajo y cebolla frente a algunas enfermedades con la presencia de compuestos de interés biológico por su acción a nivel fisiológico, destacando entre ellos, los compuestos fenólicos y organosulfurados, así como los fructooligosacáridos.

Sin embargo, el consumo de estos productos se realiza habitualmente tras algún tipo de tratamiento culinario, modificando su composición, en especial, afectando a los compuestos fitoquímicos de interés, provocando su transformación o degradación. Por otro lado, el desarrollo de la industria, la innovación y el aprovechamiento de los excedentes de producción han dado lugar a la elaboración de nuevos productos derivados de estos vegetales, como el ajo en polvo, la cebolla en polvo, ajo secado, etc. Y otros para consumo en fresco como el ajo negro y la cebolla negra, objetos centrales de esta tesis doctoral. En estos dos últimos casos concretos, el proceso de elaboración al que son sometidos el ajo y la cebolla frescos hace que se produzcan una serie de cambios a nivel físico-químico y sensorial que dan lugar a un producto con características radicalmente diferentes a los originales. Ambos productos se caracterizan por presentar un color amarronado, sabor dulce y menos pungente y una textura blanda. Además, entre los cambio a nivel físico-químico, se producen transformaciones de los compuestos de interés, (polifenoles y compuestos organosulfurados) que potencialmente podrían modificar el mecanismo de acción de estos compuestos a nivel fisiológico. Con el fin de conocer las transformaciones ocasionadas durante este proceso industrial, caracterizar los nuevos productos y conocer el potencial bioactivo de los compuestos presentes en las nuevas matrices, en los últimos años se ha visto incrementado el interés científico por el ajo negro y, más recientemente, por la cebolla negra, siendo este último el objetivo principal de la presente tesis.

1.1. Historia del género *Allium*

El origen del ajo se encuentra en Asia Central, desde donde se difunde por China y Asia Menor, hasta Mesopotamia, donde los sumerios ya empezaron a hacer uso de él para paliar diferentes enfermedades. En cuanto al origen de la cebolla, no es conocido con exactitud. Hay autores que mantienen que también proviene de Asia Central, como el ajo, otros afirman que es originaria de Asia Occidental, mientras que otros señalan que procede del norte de África (Block, 1985).

A lo largo de la historia, a estos bulbos se les han atribuido diferentes propiedades, destacando diferentes usos de estas plantas en las diferentes civilizaciones. Los sumerios (2300 a.C.) ya utilizaban el ajo, junto con la cerveza, para aliviar algunas molestias intestinales como la diarrea y el dolor de estómago. También se le atribuían propiedades para reducir la inflamación y como antiparasitario (Heinerman, 1995). Por otro lado, a la cebolla se le atribuían propiedades beneficiosas para la circulación y el corazón, y se utilizaba en el tratamiento de diversas enfermedades relacionadas con la sangre, aunque también era utilizada para el tratamiento de catarros y problemas de estómago. En el Papiro Médico de Ebers (s. XVI a.C.) se encontraron fórmulas que incluían el uso del ajo como remedio para cardiopatías, mordeduras, parásitos y contra infecciones y tumores, así como para el tratamiento de caries, picaduras y la peste (De Luis & Aller, 2008). En Egipto, a los esclavos que construían las Pirámides, se les daba de comer ajos, puerros, rábanos y cebollas ya que eran considerados vigorizantes. En muchas tumbas se podía encontrar el ajo físicamente y dibujado en madera o cerámica. También se utilizaban las cebollas en los entierros egipcios, ya que creían que su fuerte olor los devolvería a la vida. También se usaban en el proceso de momificación y se como ofrenda a los dioses.

Aristófanes y Virgilio, mencionan en sus obras las propiedades del ajo como alimento que consumían los atletas en las competiciones olímpicas y los vendimiadores para mantener sus fuerzas. En la Edad Media, el ajo era apreciado como un poderoso antídoto contra la peste. Mientras que la cebolla se consideraba un buen remedio para la tos (López Luengo, 2007). En la actualidad, se siguen atribuyendo diversas propiedades a estos alimentos formando parte de diferentes remedios caseros. Se emplea el ajo de diferentes maneras para aliviar callosidades, eccemas, verrugas e incluso la pérdida de pelo. La cebolla se utiliza para paliar dolores de garganta, sabañones, afecciones de oído, llagas, muelas, orzuelos, reuma, etc. (Torija Isasa et al., 2014)

1.2. Taxonomía del ajo y la cebolla

El género Allium se encuadra en la familia de las Liliáceas, engloba unas 600 especies, algunas de ellas con un elevado valor gastronómico, como es el caso del ajo (*Allium sativum* L.), la cebolla (*Allium cepa* L.), el puerro (*Allium porrum* L.), la cebolleta (*Allium fistolosum* L.) y la chalota (*Allium ascalonicum* L.), entre otros. La taxonomía del ajo y la cebolla se encuentra recogida en la Figura 1.

Nombre común	**Ajo**	**Cebolla**
Reino	Plantae	
División	Magnoliophyta	
Clase	Liliopsida	
Orden	Asparagales	
Familia	Amaryllidaceae	
Subfamilia	Allioideae	
Tribu	Allieae	
Género	*Allium*	
Especie	*Allium sativum* L.	*Allium cepa* L.

Figura 1.- Taxonomía del ajo y la cebolla

El ajo y la cebolla son bulbos, lo que quiere decir que la parte comestible de éstos se encuentra bajo tierra. Ambas plantas presentan raíces espesas, finas y simples que no tendrán una gran profundidad y el tallo sobresale por el centro del bulbo, rodeado por las hojas. En el caso del ajo, el bulbo es lo que comúnmente conocemos como “cabeza de ajo” y está formado por unidades más simples denominadas “dientes”, que estarán recubiertos por una fina membrana, que los separa entre ellos, así como de una membrana que envuelve el conjunto de dientes. El color de esta membrana tomará tonos desde blanquecinos a amoratados en función de la variedad. La cabeza de ajo normalmente tendrá un peso medio de entre 30 y 100 g, aunque puede llegar a duplicar este valor, y contará con entre 8 y 14 dientes.

En cuanto a la cebolla, el bulbo está formado por capas gruesas que servirán como almacenaje de sustancias que nutrirán los brotes. Estas capas tienen unas membranas muy delgadas que las recubren que servirán como base para las hojas. Dependiendo de la variedad, el bulbo de una cebolla tendrá un peso medio entre los 100 y 250 g.

1.3. Producción y consumo de ajo y cebolla

En los últimos 5 años, a nivel mundial, la producción de ajo se encuentra fundamentalmente en Asia, siendo los principales países productores China e India. Mientras que, en el caso de la cebolla, la producción se reparte fundamentalmente entre Asia y África, siendo los principales países productores China, Mali, Japón y la República de Corea. En los últimos 5 años, se han producido más de 141 millones de toneladas de ajo en el mundo y más de 23 millones de toneladas de cebolla. España es el sexto país productor de ajo del mundo, suponiendo casi el 29% de la producción europea de ajo (Figura 2). Mientras que, ocupa el puesto 25 en la producción mundial de cebolla, suponiendo más del 4% de la producción europea de cebolla (FAOSTAT, 2019).

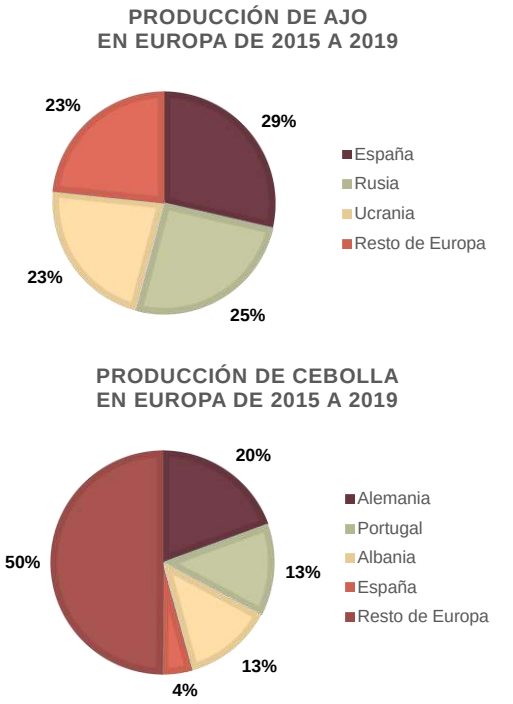

Figura 2.- Producción de ajo y cebolla en Europa desde 2015 a 2019 según FAOSTAT.

La producción en España y en Andalucía de ajo y cebolla durante el año 2019 se encuentra representada en la Figura 3. En España, los principales productores de ajo y cebolla fueron Castilla-La Mancha y Andalucía, con un 86% de la producción de ajo y un 69% de la producción de cebolla en España. Y más concretamente, en Andalucía, la producción de ajo y cebolla en 2019 se repartió entre Córdoba, Sevilla y Granada, siendo Córdoba la principal productora de ajo con más de 17 mil toneladas y la segunda provincia de mayor producción de cebolla en Andalucía con más de 36 mil toneladas (MAPA, 2019).

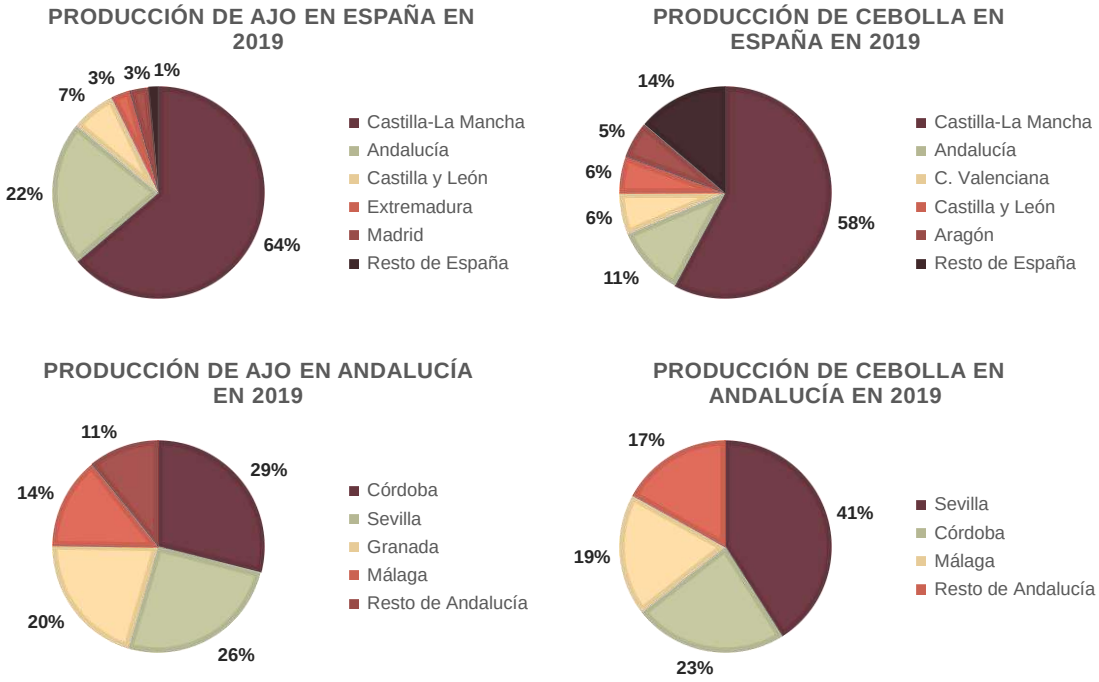

Figura 3.- Producción de Ajo y Cebolla en España y en Andalucía durante el año 2019 según MAPA (2019).

1.4. Composición nutricional del ajo y la cebolla

La Tabla 1 recoge los principales componentes nutricionales que se encuentran tanto en 100 gramos de porción comestible de ajo como de cebolla basado en las tablas de composición de alimentos de la Base de Datos Española de Composición de Alimentos (BEDCA). Los datos composicionales que se muestran en la Tabla 1 pueden presentar variaciones en función del tipo de producto, de la procedencia y del tiempo post-cosecha, ya que conforme aumenta el tiempo de almacenamiento se producen pérdidas de agua con el consecuente aumento de la concentración del resto de componentes; de la misma forma, se puede producir la pérdida de algunos compuestos como la vitamina C, que tiende a oxidarse rápidamente.

Tabla 1.- Composición nutricional del ajo (Allium sativum L.) y la cebolla (Allium cepa L.) por 100 gramos de porción comestible basada en las tablas de composición de alimentos de la Base de Datos Española de Composición de Alimentos (BEDCA)

	Ajo	**Cebolla**
100 g porción comestible		
Energía (kcal)	117	26
Proteínas (g)	3.9	1.1
Lípidos totales (g)	0.23	Traza
Hidratos de carbono (g)	24.3	5.3
Fibra (g)	1.2	1.8

Agua (g)	70.4	91.8
Vitaminas		
Vitamina B1 (mg)	0.16	0.03
Vitamina B2 (mg)	0.02	0.03
Vitamina B3 (mg equivalentes niacina)	0.27	0.4
Vitamina B6 (mg)	0.32	0.13
Vitamina B9 (µg)	4.8	7
Vitamina B12 (µg)	0	0
Vitamina C (mg)	14	6.9
Vitamina A (µg equivalentes retinol)	Traza	0
Vitamina D (µg)	0	0
Vitamina E (mg)	0.1	0.45
Minerales		
Calcio (mg)	17.8	25.4
Hierro (mg)	1.2	0.27
Yodo (µg)	4.7	8.9
Magnesio (mg)	24.1	4.2
Zinc (mg)	1.1	0.26
Sodio (mg)	19	3
Potasio (mg)	446	162
Fósforo (mg)	134	33
Selenio (µg)	0.5	1.5

Cabe destacar que el ajo y la cebolla, al tratarse de productos frescos, presentan un elevado contenido de agua (70.4 y 91.8%, respectivamente), lo que hará que presenten un valor energético moderadamente bajo, con valores en torno a las 26 kcal por cada 100g para la cebolla y a las 116 kcal por cada 100g del ajo. Las calorías de estos alimentos provienen principalmente del contenido de hidratos de carbono, fundamentalmente complejos como el almidón, siendo éstos los hidratos de carbono que la agencia europea de seguridad alimentaria (EFSA) recomienda consumir en mayor proporción en la dieta (Agostoni et al., 2010). El contenido proteico es bajo en ambas matrices y el contenido lipídico es prácticamente vestigial. En cuanto al contenido de fibra dietética del ajo y la cebolla es fundamentalmente de la fracción soluble, siendo característicos para la cebolla los fructanos (fructooligosacáridos e inulina), lo que la hace una de las principales fuentes de estos compuestos en la dieta europea (Stephen et al., 2017). Finalmente, en cuanto al contenido en vitaminas y minerales, lo más destacable es el contenido de selenio, 0.5 µg en ajo y 1.5 µg en cebolla, por su contribución al potencial antioxidante de estos productos (Põldma et al., 2011).

1.5. Compuestos fitoquímicos en ajo y cebolla

Además de los componentes nutricionales presentes en ajo y cebolla, estos alimentos son ricos en compuestos potencialmente beneficiosos para la salud, considerados como no nutrientes. Estos compuestos son denominados fitoquímicos, y concretamente en ajo y cebolla se encuentran fundamentalmente compuestos fenólicos y organosulfurados (Figura 4).

La definición de fitoquímico se ha ido perfilando durante las últimas décadas Liu y col. (2013), los define como los “compuestos bioactivos no nutritivos presentes en frutas, verduras y/o cereales relacionados con la reducción del riesgo de las principales enfermedades crónicas no transmisibles”. Thakur y col. (2020), señalan que las principales características de los fitoquímicos son las siguientes:

- Sustancias químicas vegetales no nutritivas que tienen propiedades protectoras o preventivas frente a enfermedades.
- Sustancias químicas producidas por las plantas como medio de protección frente a estreses y/o agentes externos.
- No son nutrientes esenciales y, por lo tanto, el cuerpo humano no los necesita para vivir.

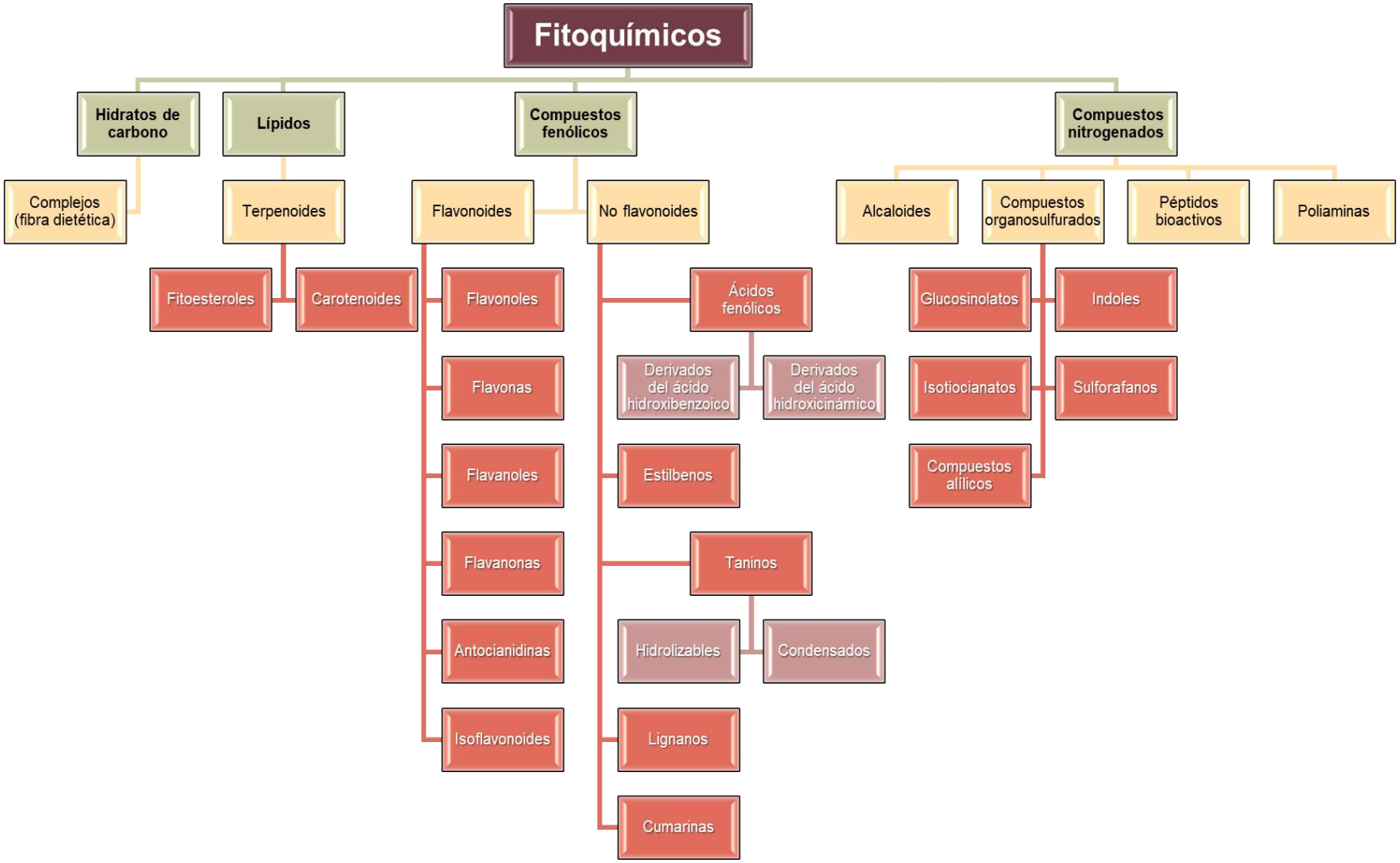

Figura 4.- Clasificación de los grupos de fitoquímicos de los alimentos según Liu et al. (2013) y Huang et al. (2016) con modificaciones (Huang et al., 2016; R. H. Liu, 2013)

1.5.1. Compuestos fenólicos en ajo y cebolla

Los compuestos fenólicos conforman un gran grupo de compuestos orgánicos naturales que se caracterizan por presentar en su estructura, al menos, un grupo funcional fenol. Han sido descritos más de 8000 compuestos pertenecientes a este grupo, presentándose fundamentalmente esterificados, unidos en formas insolubles o libres (Pandey & Rizvi, 2009). Los que se encuentran unidos en formas insolubles son aquellos que encontramos ligados a la pared celular de los vegetales formando parte de estructuras más complejas como hidratos de carbono complejos (fibra dietética) (Bravo et al., 2002).

Los compuestos fenólicos forman un grupo minoritario, que normalmente encontramos a concentraciones bajas y son denominados metabolitos secundarios, ya que, en las plantas, el metabolismo secundario tiene diversas funciones como la protección frente a agentes externos, atracción para polinizadores y dispersores de semillas, y señalización molecular (Tuladhar et al., 2021). En los alimentos suelen ser los responsables del color, el aroma y el sabor (Shahidi & Ambigaipalan, 2015).

En general, existen multitud de estudios que evalúan la capacidad de los compuestos fenólicos para ejercer un efecto beneficioso para la salud, desde el punto de vista de la prevención, protección o tratamiento de diversas enfermedades (Tomás-Barberán, 2003). De esta forma, en la actualidad se encuentran estudios que relacionan el consumo regular de polifenoles en la dieta con un efecto protector frente a diferentes tipos de cáncer, enfermedades cardiovasculares y enfermedades neurodegenerativas, entre otras, principalmente por el carácter antioxidante de estas moléculas (Cory et al., 2018; Ebrahimi & Schluesener, 2012; Khurana et al., 2013; Mileo & Miccadei, 2016; Tangney & Rasmussen, 2013; Yamagata, 2019).

A la hora de clasificar los compuestos fenólicos se atiende a diferentes criterios:

- Naturaleza química.
- Número de anillos.
- Localización de los sustituyentes.
- Esterificaciones con azúcares y/o ácidos orgánicos.

Teniendo en cuenta estos factores, los compuestos fenólicos se pueden dividir en dos grandes grupos(Figura 4): flavonoides y no flavonoides (Singla et al., 2019).

- Compuestos fenólicos flavonoides: Los flavonoides son compuestos que tienen un bajo peso molecular cuya estructura está compuesta por 3 anillos: 2 anillos fenilo (A y B, Figura 5) y un anillo heterocíclico pirano (C, Figura 5). Los

flavonoides se encuentran en la naturaleza principalmente glicosilados, es decir, unidos a un monosacárido como la glucosa, la galactosa, la xilosa, la ramnosa o la arabinosa (Amiot et al., 2016). También se pueden encontrar de forma minoritaria como agliconas o conjugados. Dentro de este grupo podemos encontramos los siguientes familias de compuestos: Flavonoles, flavonas, flavanonas, antocianidinas e isoflavonoides (Manach et al., 2004).

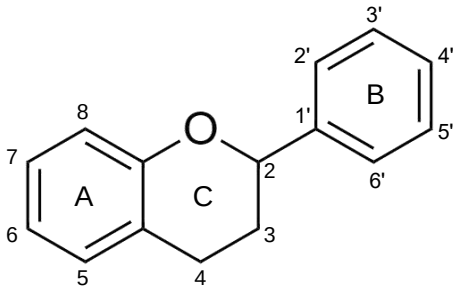

Figura 5.- Estructura química básica de los compuestos fenólicos flavonoides

- Compuestos fenólicos no flavonoides: Se trata de un grupo muy heterogéneo que comprende desde las estructuras fenólicas más simples, como los ácidos fenólicos, hasta estructuras más complejas, como los taninos. Según la estructura química que presenten, dentro de este grupo podemos encontrar los siguientes subgrupos: Fenoles simples, ácidos fenólicos, estilbenos, lignanos, taninos y cumarinas (de la Rosa et al., 2019). En la Figura 6 se encuentran representadas las estructuras químicas genéricas de los fenoles simples y los ácidos fenólicos principales: ácidos hidroxibenzoicos y ácidos hidroxicinámicos.

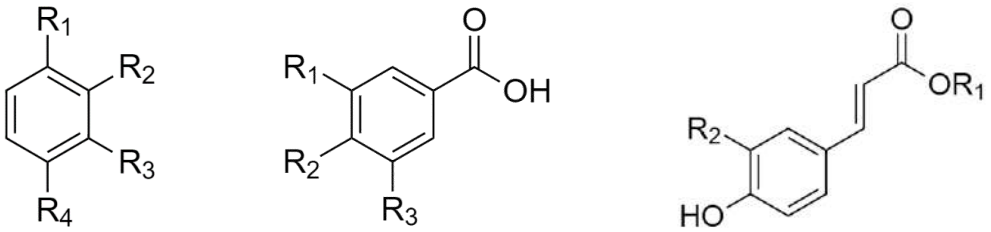

Figura 6.- Estructuras químicas básicas de algunos compuestos fenólicos no flavonoides. De izquierda a derecha: fenoles simples, ácidos hidroxibenzoicos y ácidos hidroxicinámicos

En ajo y cebolla los grupos fenólicos mayoritarios descritos son los ácidos fenólicos, los flavanoles, los flavonoles y las flavonas. En las Figuras 7 y 8 se encuentran recogidas las estructuras químicas de los principales compuestos fenólicos en ajo y cebolla, respectivamente.

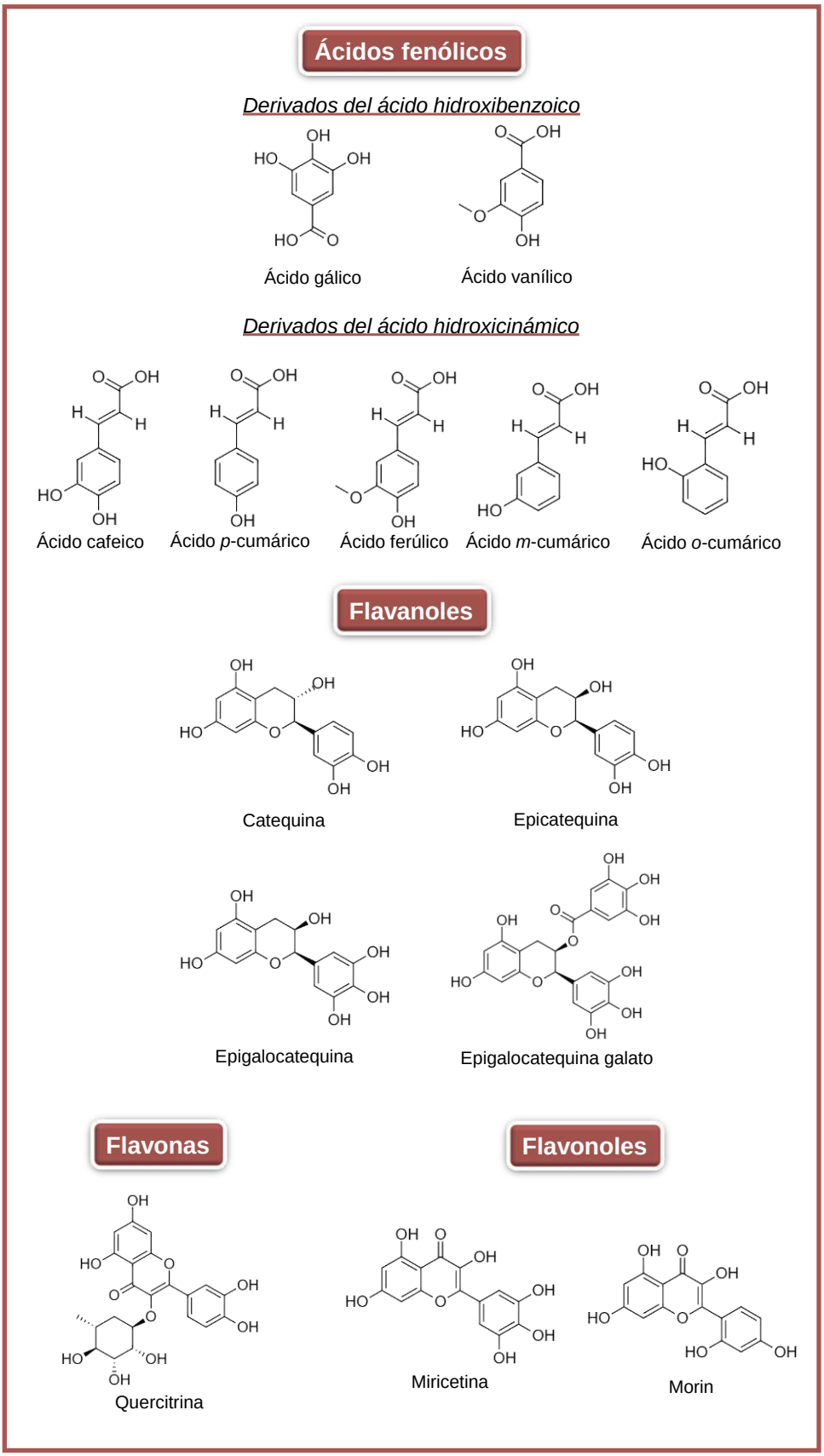

Figura 7.- Estructuras químicas de los compuestos fenólicos del ajo (J. S. Kim et al., 2013; Moreno-Ortega, Pereira-Caro, Ordóñez, Moreno-Rojas, et al., 2020)

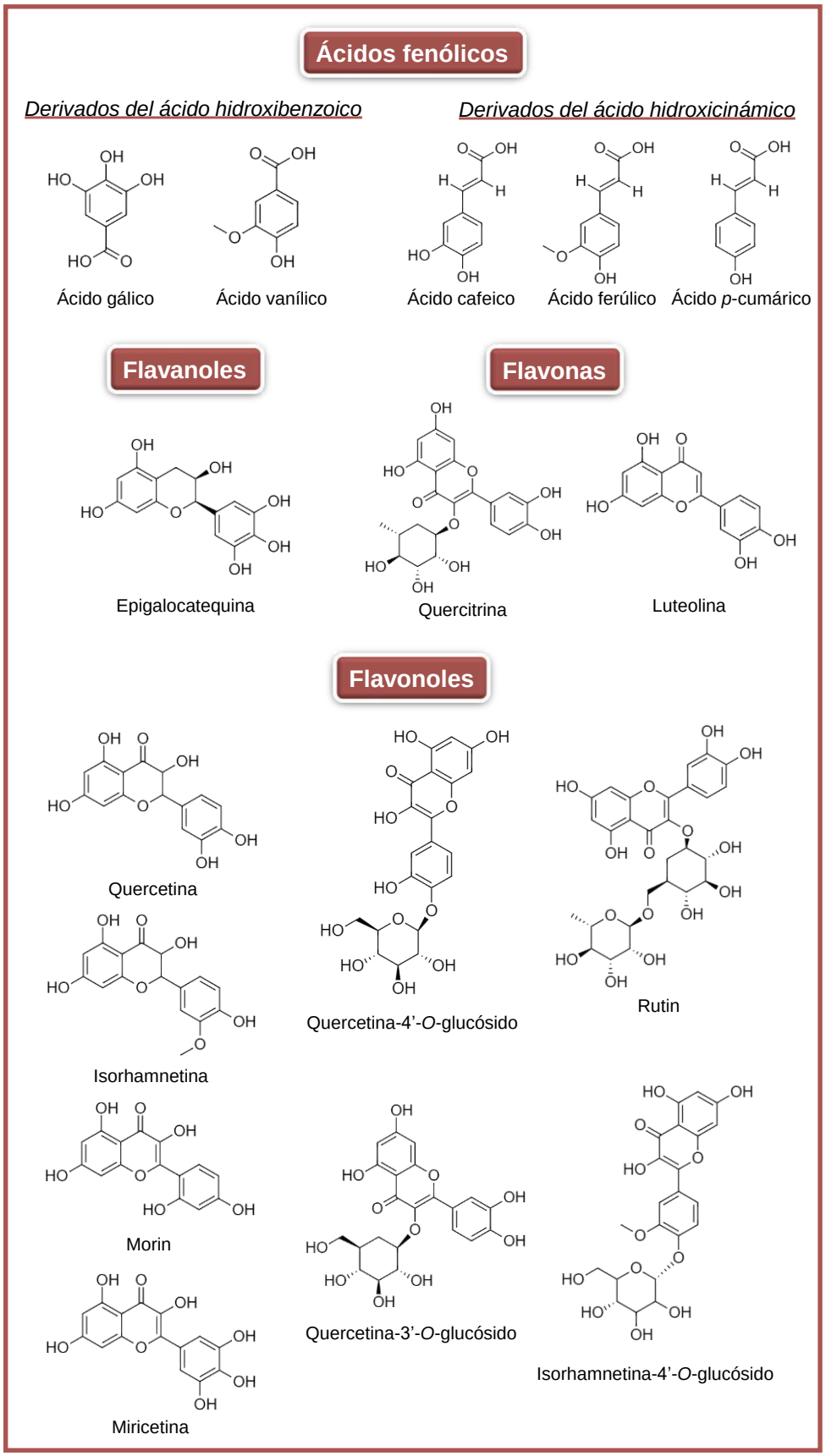

Figura 8.- Estructuras químicas de los compuestos fenólicos de la cebolla (Moreno-Ortega et al., 2021; Moreno-Ortega, Pereira-Caro, Ordóñez, Muñoz-Redondo, et al., 2020; Moreno-Rojas et al., 2018)

1.5.2. Compuestos organosulfurados en ajo y cebolla

Muchos de los compuestos que contienen azufre en su estructura son considerados tóxicos, sin embargo, existe una pequeña cantidad de compuestos azufrados en la naturaleza que reportan un beneficio para la salud tras su ingesta. Los compuestos azufrados que se consumen en mayor cantidad son los aminoácidos que contienen este átomo en su estructura, como es el caso de la cisteína, la metionina, la taurina y la homocisteína. Por otro lado, también se puede encontrar el azufre en la dieta a partir de los sulfitos y los sulfatos que se incorporan a algunos alimentos como aditivos alimentarios. Y finalmente, se encuentran los compuestos organosulfurados, presentes naturalmente en algunos productos vegetales, como el ajo y la cebolla. Estos compuestos son responsables de su olor y el sabor característico (Kohlmeier, 2015).

Además, estos compuestos organosulfurados han sido relacionados con diferentes efectos beneficiosos para la salud como propiedades antioxidantes, antiinflamatorias, antibacterianas, antihipertensivas, antihiperlipidémicas, antidiabéticas o anticancerígenas, a través de diversos mecanismos celulares (Walag et al., 2020). Estas propiedades ponen de manifiesto que el consumo regular de estos compuestos pueden tener un efecto preventivo y/o protector frente a diversas patologías como enfermedades neurodegenerativas, cardiovasculares, algunos tipos de cáncer o el síndrome metabólico (Lea, 1996; Miękus et al., 2020; Ruhee et al., 2020; Zhai et al., 2018).

Los compuestos organosulfurados van a clasificarse atendiendo al grupo funcional que contiene el átomo de azufre (Dini, 2018) (Figura 9):

- Tioéteres, tioésteres y tioacetales: Son compuestos cuya estructura está caracterizada por la unión de carbonos al átomo de azufre (C-S-C).
- Tioles, disulfuros y polisulfuros: El azufre se encuentra formando parte del grupo tiol (SH).
- Sulfóxides, sulfonas, tiosulfinatos y tiosulfonatos: El azufre se encuentra en su estructura como parte del grupo sulfóxido (SO).
- Sulfamidas: Son compuestos que contienen en su estructura la unión de nitrógeno y azufre.
- *S*-Nitrosotioles o tionitritos: Son compuestos que contienen un grupo nitroso (NO) unido al átomo de azufre de un grupo tiol.
- Glucosinolatos, isotiocianatos e indoles: Los glucosinolatos son β-tioglucósidos N-hidroxisulfatos con una cadena lateral R y una fracción de β-d-glucopiranosa ligada al azufre. Los isotiocianatos se forman por la degradación de los glucosinolatos con cadenas alifáticas o aromáticas

mientras que los indoles se forman por la degradación de los glucosinolatos que poseen cadenas indol.

- Sulfonios, oxosulfonios e iluros de tiocarbonil: Los sulfonios y los oxosulfonios son iones de azufre cargados positivamente, cuya desprotonación de las sales da lugar a la formación de iluros.

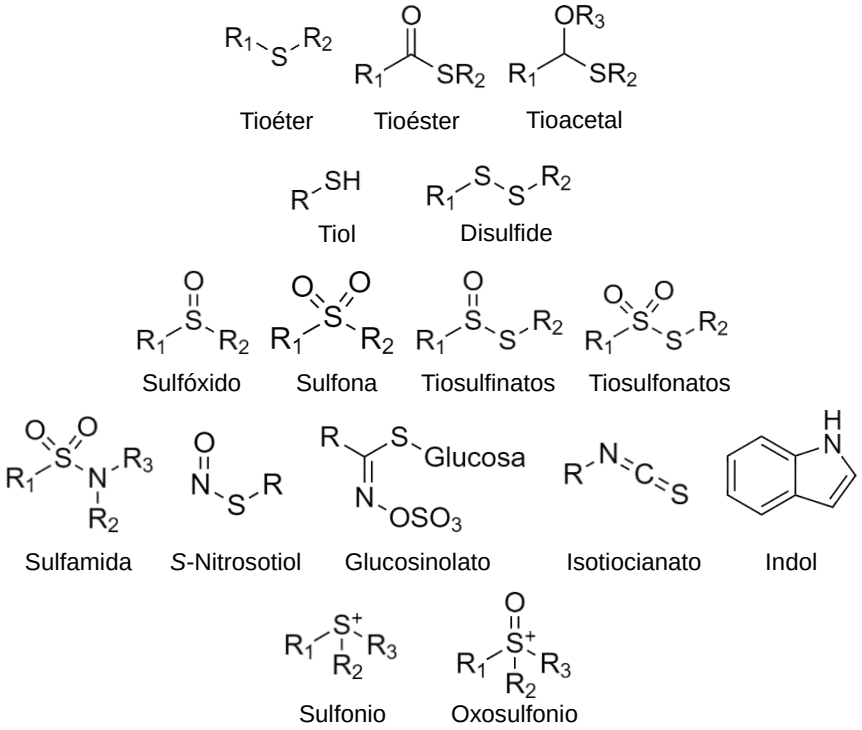

Figura 9.- Estructuras químicas generales de los diferentes tipos de compuestos organosulfurados

Los compuestos organosulfurados característicos del género *Allium* poseen una estructura en la que se encuentra el azufre unido a un grupo cianato (R-N=C=O) en forma cíclica o no cíclica (Barba, 2017). Entre ellos, destacan los derivados *S*-alqu(en)il-L-císteina y los derivados de γ-glutamil-*S*-alqu(en)il-L-císteina. Estos compuestos están formando parte del citosol de las células vegetales mientras que la enzima aliinasa se encuentra en las vacuolas. Cuando se produce una lesión en los tejidos del bulbo, como puede ser el corte, la trituración o la masticación, la aliinasa entra en contacto con los compuestos organosulfurados y comienza la degradación de estos a ácidos sulfénicos. Sin embargo, los ácidos sulfénicos tienen muy baja estabilidad química, por lo que terminarán transformándose en tiosulfinatos (Figura 10) (Poojary et al., 2017; Yoshimoto & Saito, 2019).

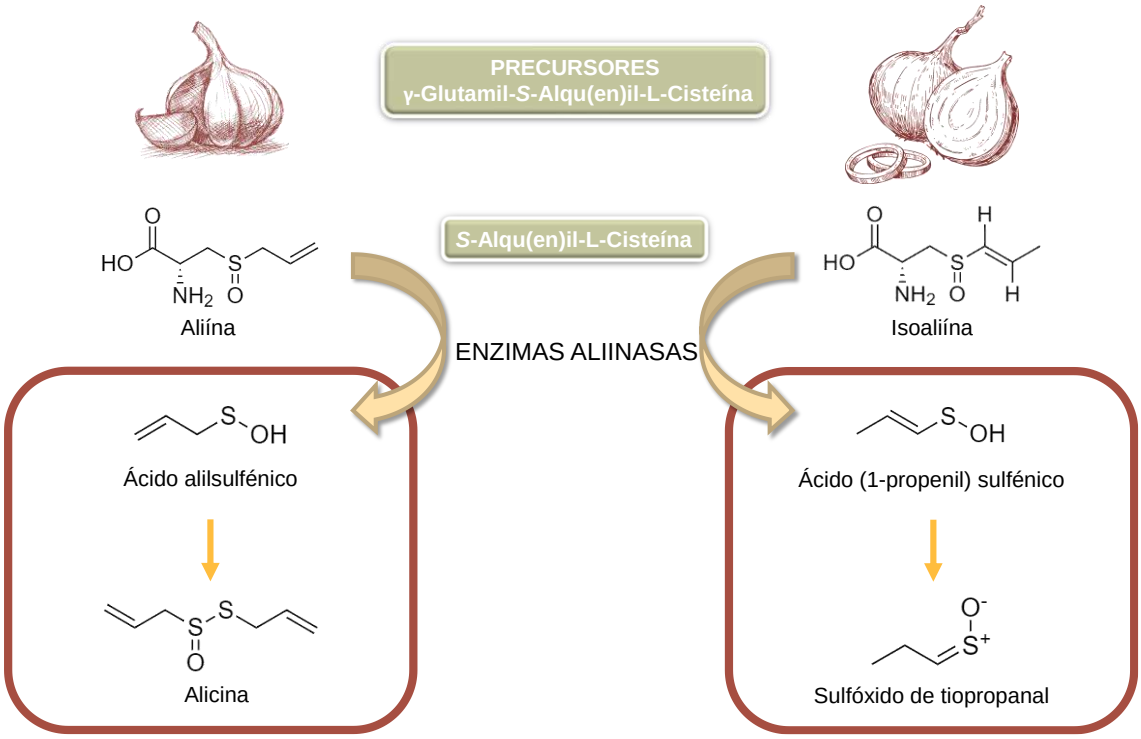

Figura 10.- Formación de compuestos por la acción de la enzima aliinasa en ajo y cebolla basado en las rutas propuestas por (Leontiev et al., 2018; Sato & Matsui, 2012)

Las Figuras 11 y 12 recogen las estructuras químicas de los principales compuestos organosulfurados identificados en ajo y en cebolla, respectivamente.

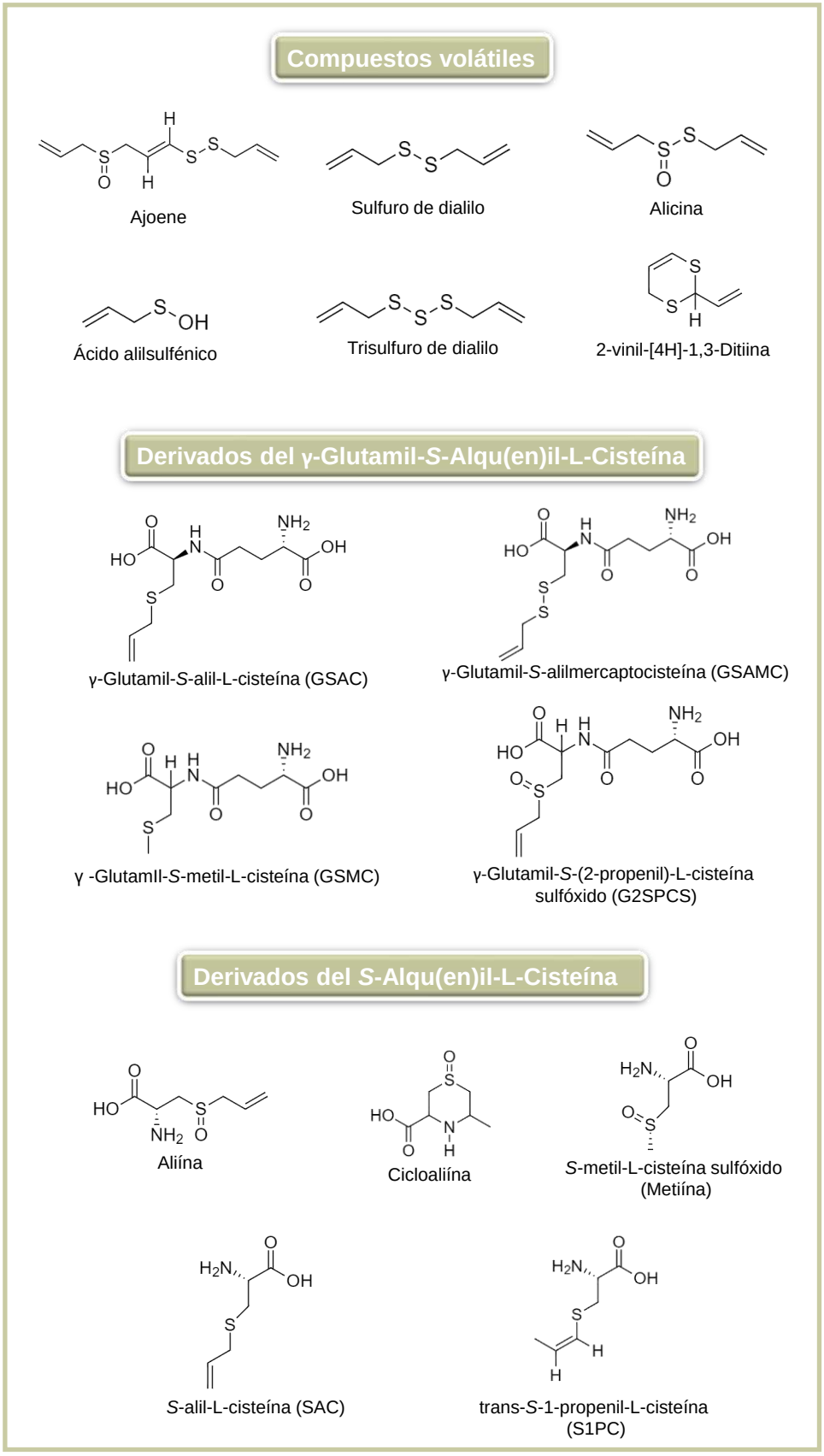

Figura 11.- Estructuras químicas de los principales compuestos organosulfurados del ajo (Keusgen, 2011; Moreno-Ortega, Pereira-Caro, Ordóñez, Moreno-Rojas, et al., 2020)

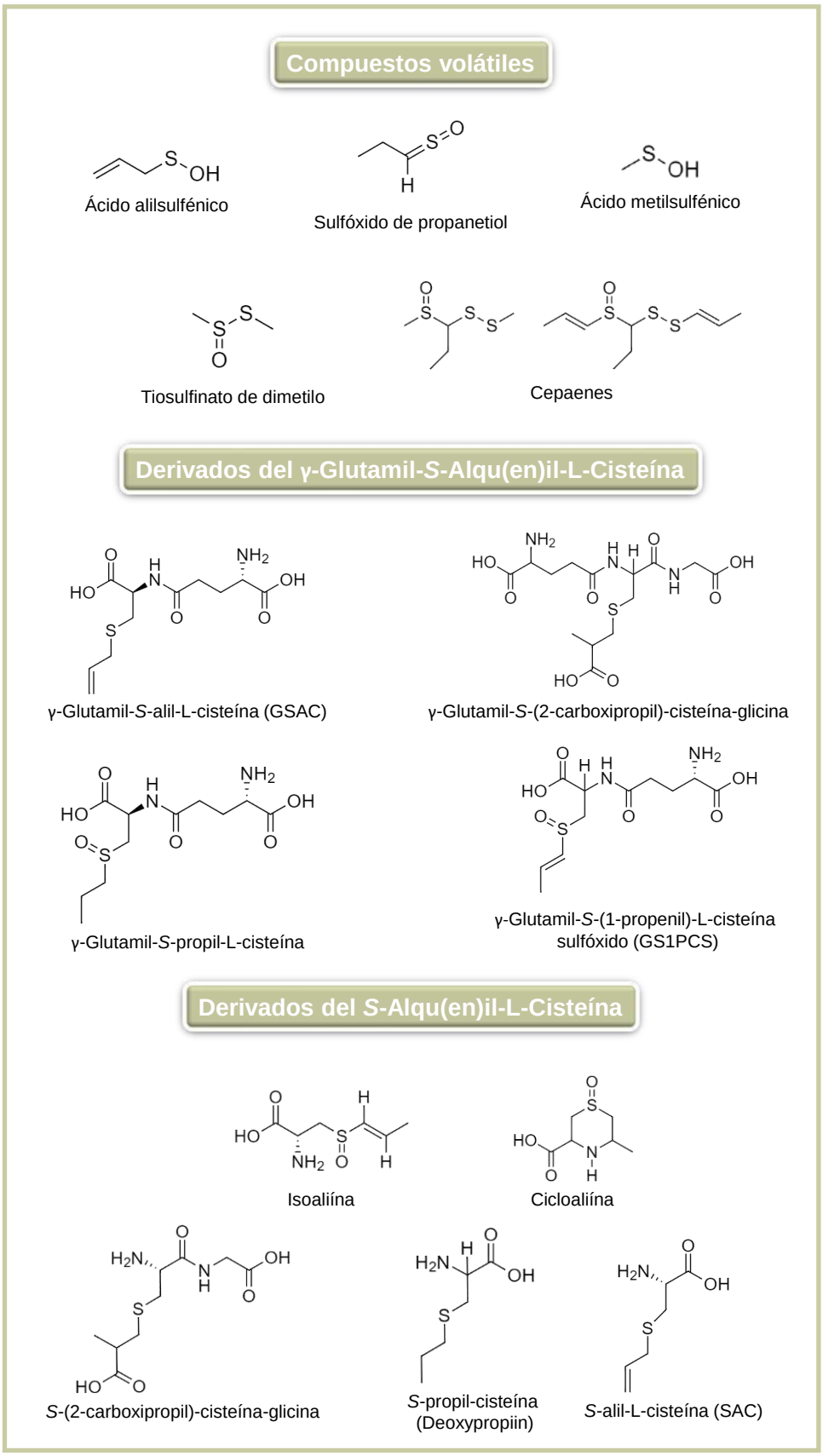

Figura 12.- Estructuras químicas de los principales compuestos organosulfurados de la cebolla (Keusgen, 2011; Moreno-Ortega et al., 2021; Moreno-Ortega, Pereira-Caro, Ordóñez, Muñoz-Redondo, et al., 2020)

1.6. Propiedades saludables relacionadas con el consumo de ajo y cebolla

Los vegetales del género *Allium* han sido considerados alimentos con propiedades beneficiosas para la salud para diferentes culturas a lo largo de la historia, como se ha mencionado anteriormente. La investigación del efecto del consumo de ajo y cebolla, así como la exposición a los polifenoles, compuestos organosulfurados y otros fitoquímicos presentes en estas matrices han sido objeto de numerosos estudios a lo largo de los años (Asemani et al., 2019; Hertog et al., 1992; Schwingshackl et al., 2016). Según los datos recogidos en la Web of Science, se han publicado más de 8.700 artículos en relación con el género *Allium*, entre ellos, más de 178 revisiones. El interés científico por los productos de este género ha ido aumentando a lo largo de los años, desde las 118 publicaciones que se hicieron en 1999 hasta las 472 publicaciones que se hicieron durante el año 2020. España se encuentra entre los 10 países que más publicaciones ha realizado sobre el género *Allium*, encabezando esta lista India, Estados Unidos y China.

Muchas de estas investigaciones se han dirigido a la obtención de información sobre los efectos saludables del consumo regular de las plantas del género *Allium,* especialmente ajo y cebolla, así como descubrir los mecanismos de acción de los componentes presentes en ellos (Ansary et al., 2020; Karavelioğlu & Hoca, 2021; Rana et al., 2011; Shang et al., 2019; Zhao et al., 2021).

1.6.1. Enfermedades neurodegenerativas

Las enfermedades neurodegenerativas hacen referencia a una serie de dolencias que principalmente afectarán a las neuronas, algunas de las más conocidas son Alzheimer, Parkinson, Huntington, entre otras. Se han realizado estudios en ratas con extracto de ajo que han demostrado disminuir el contenido de plomo en sangre y en cerebro, mostrando un efecto preventivo frente a la apoptosis inducida por plomo en las neuronas (Ebrahimzadeh-Bideskan et al., 2016). Además, se ha visto mejorada la memoria de ratas diabéticas que han sido tratadas con un extracto de ajo en etanol, por un aumento de las ATPasas y la glutamina sintasas en el hipocampo (Semuyaba et al., 2017).

Respecto a la cebolla, se han llevado a cabo estudios en ratas con diferentes extractos de cebolla en los que se ha observado una mejora en la disfunción cognitiva, el aprendizaje y los problemas de memoria, así como en las funciones de crecimiento cognitivo en ratas que presentaban lesiones cerebrales (Nosrani et al., 2021; Singh et al., 2020; Tamtaji et al., 2017).

1.6.2. Cáncer

El cáncer, como lo define el instituto nacional del cáncer, es el nombre que recibe “un conjunto de enfermedades relacionadas. En todos los tipos de cáncer, algunas de las células del cuerpo empiezan a dividirse sin detenerse y se diseminan a los tejidos del derredor.”

La evaluación del potencial efecto preventivo o protector del ajo y/o sus componentes se realiza principalmente mediante estudios *in vitro* con modelos celulares. Diferentes estudios han demostrado que el consumo regular de ajo y, por ende, de los fitoquímicos presentes en éste, pueden ejercer un efecto protector frente a algunos tipos de cáncer como el colorrectal, el de pulmón, el gástrico o el de vejiga. El efecto protector podrán ejercerlo a diferentes niveles: regulando el metabolismo de sustancias carcinógenas, inhibiendo el crecimiento o la proliferación de las células cancerosas, induciendo la apoptosis de las células cancerosas, reduciendo la angiogénesis, disminuyendo la invasión o la migración de las células tumorales o, incluso, paliando o aliviando los efectos secundarios de la terapias contra el cáncer (Bagul et al., 2015; Gatt et al., 2015; X. Jiang et al., 2017; Kaschula et al., 2016; S.-S. Shin et al., 2017; W. Wang et al., 2015; Xu et al., 2013).

También se han llevado a cabo estudios *in vitro* que relacionan el consumo de cebolla con un efecto protector frente algunos tipos de cáncer actuando a diferentes niveles: inhibiendo la proliferación de las células tumorales en líneas celulares de glioblastoma, cáncer de mama y de colon, induciendo la apoptosis de células cancerosas en cáncer de útero, de colon y osteosarcoma, evitando el desarrollo de tumores o inhibiendo la metástasis en osteosarcoma y cáncer de pulmón (Fredotović et al., 2017, 2020; Fujiwara et al., 2016; Murayyan et al., 2017). Por otro lado, se ha relacionado con un efecto protector en pacientes con cáncer de mama sometidos a quimioterapia, donde se ha visto que mejora la hiperglicemia y la resistencia a la insulina (Ko et al., 2018).

1.6.3. Diabetes Mellitus tipo 2

Respecto a la diabetes, se han realizado estudios *in vivo* en ratas y en humanos donde se ha observado un efecto antidiabético del consumo de ajo. El estudio en ratas diabéticas mostró cómo el consumo de ajo mejoró el peso, la concentración de glucosa en sangre y mostraba un efecto protector frente a la retinopatía diabética. Por otro lado, el estudio en humanos mostró una reducción del contenido de hemoglobina glucosilada y fructosamina, los cuáles son parámetros que proporcionan información sobre el grado de compensación metabólica en pacientes con diabetes mellitus tipo 2 (J. Wang et al., 2017).

Se han hecho estudios en ratas a las que se les inducía diabetes por acción de la estreptomicina en las que el consumo de cebolla en la dieta se ha relacionado con una disminución del estrés oxidativo, una mejora en la hiperglucemia, una reducción de la nefropatía diabética o un efecto protector frente a los efectos secundarios de la diabetes en el sistema reproductor (Fallah et al., 2017; Pradeep & Srinivasan, 2017a, 2017b, 2018). También se ha asociado a su consumo un efecto antidiabético, esto es debido a que es capaz de inhibir la absorción de carbohidratos, lo que atenúa el aumento de los niveles de glucosa en sangre en ratas (Pradeep et al., 2019). En un estudio en humanos con cebolla roja, se ha observado que el consumo de cebolla mejora los marcadores de la resistencia a la insulina (Ebrahimi-Mameghani et al., 2018).

1.6.4. Enfermedades cardiovasculares

El consumo de ajo ha sido relacionado con una reducción de la presión arterial, del colesterol total, de los triglicéridos y otros factores de riesgo relacionados con enfermedades cardiovasculares. Se han llevado a cabo numerosos estudios en ratas que han demostrado que el consumo de ajo o de los componentes presentes en este son capaces de reducir los niveles plasmáticos de colesterol total, lipoproteínas de baja densidad (LDL) y triglicéridos, así como reducir la hipertrofia cardiaca inducida o proteger la función cardiaca mediante diferentes mecanismos enzimáticos (Khatua et al., 2017; Sohn et al., 2012; Sultana et al., 2016; Supakul et al., 2013). También ha mostrado la capacidad de inhibir la actividad de la enzima convertidora de la angiotensina, disminuyendo la presión arterial (Asdaq & Inamdar, 2010). Adicionalmente, se ha hecho un estudio en humanos que presentaban dislipidemia diabética en los que se observó una disminución de los niveles plasmáticos de colesterol total y de LDL y un aumento de la concentración de lipoproteínas de alta densidad (HDL) en sangre (Yeh & Liu, 2001) con el consumo de ajo.

Al igual que ocurre con el ajo, la cebolla también actúa a diferentes niveles mostrando un efecto protector frente a las enfermedades cardiovasculares. Se han realizado estudios *in vivo* en ratones y ratas que han relacionado el consumo de extracto de cebolla o de cebolla en polvo con una disminución de los niveles plasmáticos de colesterol total, LDL y triglicéridos, mostrando índices aterogénicos más bajos y un menor riesgo cardiaco (Colina-Coca et al., 2017; H. Kang et al., 2016).

1.6.5. Enfermedades digestivas

Las enfermedades digestivas son trastornos relacionados con cualquier tejido u órgano que encontremos incluido en el sistema digestivo, como pueden ir desde el estreñimiento, la celiaquía, hasta las úlceras gástricas, problemas hepáticos, etc.

En un estudio con pacientes con infección por *Helicobacter pylori* se observó que el consumo de ajo crudo disminuyó la actividad de la ureasa bacteriana, cuya actividad es tóxica para las células del epitelio gástrico, y redujo el tiempo de permanencia de *H. pylori* en el estómago (Zardast et al., 2016). Por otro lado, se trataron ratas con extracto de ajo y col, lo que mejoró el pH de los jugos gástricos y redujo la duración de la úlcera gástrica (Ben Hadda et al., 2014).

Por otro lado, se han realizado estudios in vivo en ratas y conejos para evaluar la acción del ajo tras inducir un daño con aloxano y con tetracloruro de carbono (CCl_4), respectivamente. El aloxano es un tóxico cuya acción destruye las células β-pancreáticas provocando una caída de la insulina en sangre y una hiperglucemia, induciendo una diabetes que provoca lesiones moleculares y celulares en diferentes tejidos y órganos. En ratas, se observó que la administración de extracto de ajo disminuyó el daño hepático inducido por el aloxano, así como mejoró los parámetros bioquímicos hepáticos (creatinina, urea y transaminasas) (Aprioku & Amah-Tariah, 2017). Por otra parte, el CCl_4 es un modelo utilizado para inducir daño hepático, el ajo mostró un efecto protector frente a una lesión aguda de hígado inducida por este tóxico (Naji et al., 2017).

El consumo de cebolla se ha podido relacionar también con una reducción de la severidad de la colitis inducida en ratones mediante dextrano sulfato de sodio (DSS) (Hong & Piao, 2018; Khajah et al., 2019), así como ha aumentado la actividad enzimática de la microbiota intestinal en ratas con dietas ricas en grasas (S. ur Rahman et al., 2017). También se han realizado estudios *in vivo* en modelos animales en los que el consumo de cebolla produce un efecto protector frente a la hepatotoxicidad de ciertos compuestos como la doxorrubicina o el CCl_4 (Mete et al., 2013). Así como en ratas con dietas ricas en grasas y azúcares, la cebolla disminuye la esteatosis hepática y la expresión de citoquinas como el factor de necrosis tumoral (TNF-α) (Emamat et al., 2018).

1.6.6. Enfermedades renales

Las enfermedades renales hacen referencia al grupo de trastornos que pueden afectar al correcto funcionamiento de los riñones, entre las más habituales encontramos los cálculos renales, infecciones, lesiones renales agudas y síndrome nefrótico.

Se han llevado a cabo estudios con ratas a las que se les administra extracto de ajo y éste ha mostrado un efecto protector frente al estrés oxidativo que puede llegar a provocar lesiones renales (Miltonprabu et al., 2017; Nasiri et al., 2016).

Por otro lado, estudios en ratas diabéticas han demostrado que el consumo regular de cebolla produce un efecto beneficioso ya que mejora la lesiones renales asociadas a la diabetes. Este hecho puede explicarse debido, fundamentalmente, a los efectos hipolipidémicos y antioxidantes que se atribuyen a su consumo (Babu & Srinivasan, 1999). También el consumo de cebolla se ha asociado con una mejora en las alteraciones histopatológicas del riñón inducidas por la estreptomicina en ratas (Abouzed et al., 2018).

1.6.7. Enfermedades respiratorias

Las enfermedades respiratorias son aquellas que afectan a los pulmones y/o al resto de partes del sistema respiratorio. Entre las enfermedades respiratorias más comunes se encuentra el asma, la fibrosis pulmonar, la neumonía y la enfermedad pulmonar obstructiva crónica (EPOC).

El consumo de cebolla ha sido asociado con la mejora de diferentes patologías de naturaleza respiratoria como el asma. Estudios en modelos animales con extractos de cebolla ha demostrado que su administración en ratas puede reducir el efecto patológico de la nicotina en el pulmón, así como en ratas asmáticas se ha visto una disminución de la respuesta traqueal, es decir, de la contracción de la tráquea durante el ataque de asma inducido (Ghorani et al., 2018; Zaki, 2019).

1.7. Ajo negro y cebolla negra

A pesar de todas las propiedades beneficiosas para la salud asociadas al consumo de estos productos en fresco, su ingesta se produce principalmente tras algún tratamiento culinario. En este sentido, la pungencia y el picor que los caracteriza se atenúa con estos procesos, lo que puede conllevar la pérdida de algunos de los compuestos de interés previamente descritos. De esta forma, surge en la industria alimentaria el ajo negro, como un producto nuevo que permitiría un mayor consumo de este bulbo debido principalmente a sus características organolépticas, menor pungencia y mayor dulzor.

Aunque el ajo y la cebolla presenten un amplio abanico de efectos saludables, su consumo en fresco no está muy extendido debido fundamentalmente a sus características organolépticas, sobre todo en el caso del ajo. Por ello una forma más interesante de consumo es en forma de ajo negro, que se obtiene a partir de ajo fresco tras un proceso de fermentación en el que no intervienen bacterias ni levaduras, sino condiciones controladas y adecuadas de temperatura y humedad.

Figura 13.- Ajo negro y cebolla negra

El origen del ajo negro es incierto, aunque su consumo está más extendido en Asia, donde se comercializa desde hace más de una década. El interés en este producto radica en unos estudios que se realizaron en células cancerosas de ratón, observándose una reducción de éstas al usar ajo negro que no se observaba con ajo fresco (Kimura et al., 2017). En la actualidad, la producción y consumo de ajo negro se ha extendido a todo el mundo.

En cuanto a la cebolla negra, se estima que su origen puede encontrarse también en Asia ya que se trata de un producto análogo al ajo negro del cuál encontramos una patente coreana en 2008 (WON, 2008). Sin embargo, ha sido una empresa cordobesa, más concretamente de Montalbán, la que a través de su conocida marca La Abuela Carmen®, ha sido pionera en su producción y comercialización a nivel nacional e internacional.

En la actualidad, tanto el ajo negro como la cebolla negra son considerados productos gourmet, formando parte de elaboraciones de alta cocina. Además, son productos que han aumentado mucho su valor en el mercado respecto a sus productos frescos, encontrando que el precio medio del ajo y la cebolla fresca ronda los 0.5 €/kg mientras que el ajo negro y la cebolla negra rondan los 4 €/kg. La elaboración de ajo negro y cebolla negra ha permitido hacer uso de los excedentes de producción de ajo fresco y cebolla fresca, permitiendo una comercialización a un precio mayor y permitiendo obtener beneficio de estos excedentes. Por otro lado, también es un método que aumenta la vida útil de los productos, por lo que permite un mayor tiempo de comercialización que el producto original, que es un producto estacionario y perecedero.

1.7.1. Proceso de elaboración del ajo negro y la cebolla negra

El ajo negro y la cebolla negra son productos derivados del ajo fresco (*Allium sativum* L.) y la cebolla fresca (*Allium cepa* L.), respectivamente, los cuáles se obtienen tras un proceso industrial que somete el producto fresco a condiciones de temperatura y humedad controladas durante un periodo de tiempo determinado. Este proceso es comúnmente conocido en el sector como fermentación, aunque en él no participen microorganismos ni aditivos, de hecho, se trata más bien de un proceso de envejecimiento del producto fresco. Este tratamiento provoca una serie de reacciones y cambios físico-químicos en el ajo y la cebolla que darán lugar tanto a la transformación sus propiedades organolépticas como de los compuestos de interés o fitoquímicos previamente descritos.

En literatura se pueden encontrar publicaciones de diferentes autores que han descrito el proceso de elaboración del ajo negro, estableciendo finalmente unas condiciones de temperatura entre los 40 y 90ºC con una humedad relativa de entre el 80 y el 95% y durante periodos de tiempo de entre 10 y 40 días (Ahmed & Wang, 2021; Jang et al., 2008; O.-J. Kang, 2016; Y.-M. Lee et al., 2009).

En cambio, el proceso de elaboración de la cebolla negra, al ser un producto de explotación relativamente más reciente, se encuentra descrito en menor medida. Encontramos una patente coreana en 2008 que describe el proceso de envejecimiento de la cebolla fresca en 3 partes, un primer tratamiento con temperaturas entre los 50 y los 120ºC durante entre 24 y 72 horas. Tras esto, se lleva a cabo el proceso de envejecimiento en el que se tratará la cebolla a 60-80 ºC durante entre 1 y 15 días. Posteriormente, se aplica un proceso de secado a baja temperatura que consistirá en un tratamiento a 40-60 ºC durante 24-72 horas (WON, 2008). Sin embargo, Chung et al. (2011) en su estudio sobre el perfil de glucósidos de la quercetina en cebolla fresca y cebolla negra reporta un proceso de elaboración de la cebolla negra con temperaturas alrededor de los 60ºC y una humedad relativa del 90% durante 30 días.

Recientemente, el grupo IFAPA a través del personal de su centro en Palma del Río se encargó de optimizar el proceso de elaboración tanto para el ajo negro como para la cebolla negra (Toledano Medina et al., 2016). En este sentido, el ajo negro se obtendría sometiendo el ajo fresco a temperaturas de entre 70 y 80 ºC con una humedad relativa entre 85 y 90% durante entre 20 y 45 días. Mientras que la cebolla negra se obtendría al someter la cebolla fresca a temperaturas entre los 65 y 70 ºC con una humedad relativa entre el 90 y el 95% durante 28 días, tras esto se sometería a una desecación durante 24 horas a 50 ºC.

1.7.2. Cambios durante el proceso de elaboración del ajo negro y cebolla negra

Los cambios que se producen en la composición química del ajo fresco durante el proceso de elaboración del ajo negro han sido estudiados en los últimos años por diferentes autores, sin embargo, no existe información hasta el momento de cómo afecta este proceso al perfil de compuestos saludables tanto de ajo como de la cebolla.

Durante el proceso de elaboración del ajo negro se producen cambios composicionales que darán lugar a una propiedades organolépticas diferentes, encontrando un producto con una menor pungencia, un sabor más dulce, una textura más blanda y un color amarronado-negro (Ríos-Ríos et al., 2019).

Como se ha mencionado anteriormente, la aliína es el principal compuesto organosulfurado presente en el ajo fresco, responsable de su olor y sabor pungente. Este compuesto es sustrato de una enzima presente en el ajo llamada aliinasa que reacciona con ella cuando el ajo es cortado o machacado, ya que se produce la rotura de las paredes celulares. Esta reacción produce alicina, otro compuesto responsable de esta pungencia. Durante el proceso de elaboración del ajo negro, la enzima aliinasa se inactiva por la temperatura del tratamiento y el periodo de tiempo prolongado de éste. Sin embargo, durante este proceso, se producirá la transformación del aliína y la alicina, en otros compuestos, fundamentalmente *S*-alil-cisteína (SAC), siendo este el principal compuesto organosulfurado del ajo negro y disminuyendo la pungencia en el nuevo producto (Figura 14) (Ahmed & Wang, 2021).

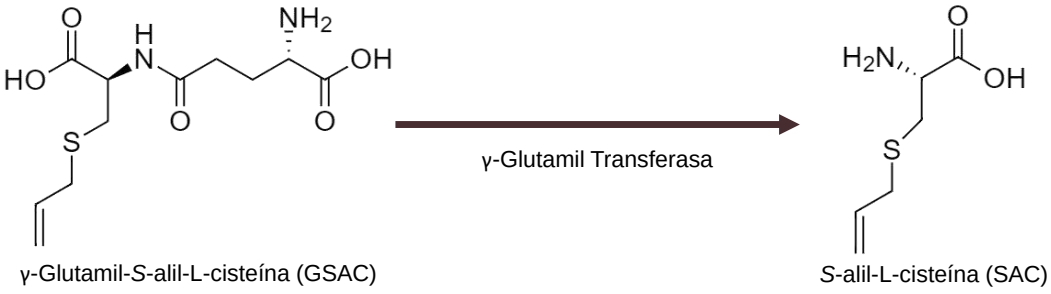

Figura 14.- Esquema de la transformación del GSAC a SAC por acción de la enzima γ-glutamiltransferasa.

Además, durante el proceso de elaboración se va a producir la hidrólisis de los azúcares más complejos, entre ellos, la sacarosa, dando lugar a un aumento significativo del contenido de fructosa y glucosa, lo que hace que el ajo negro presente un sabor marcadamente dulce. El color más oscuro se debe a las reacciones de Maillard y de caramelización, que se producen entre los azúcares y los aminoácidos cuando están sometidos a temperatura durante un determinado periodo de tiempo (Yuan et al., 2016).

Además, durante el proceso de elaboración del ajo negro se producirán cambios en otros compuestos como el hidroximetilfurfural (HMF) siendo uno de los principales responsables de la actividad antioxidante de este producto (Zhang et al., 2016). Mientras que el ácido cítrico será el ácido orgánico mayoritario en el ajo negro, siendo el responsable principal de la disminución del pH en el producto final (Bae et al., 2014).

También se ha demostrado que el ajo negro presenta un mayor contenido de sólidos solubles, acidez, concentración de polifenoles totales y actividad antioxidante respecto al ajo fresco (Toledano Medina et al., 2016).

Del nuevo producto, cebolla negra, no hay aun estudios que describan su composición en compuestos organosulfurados y su concentración. Dado el éxito del ajo negro, cabe enfatizar la importancia de la caracterización del nuevo producto “cebolla negra” con objeto de aumentar su reclamo frente al consumidor.

1.7.3. Propiedades beneficiosas para la salud del ajo negro

Dado los cambios a nivel composicional del nuevo producto, ajo negro, y las propiedades saludables atribuidas al producto original, no tardaron en comenzar las investigaciones dirigidas a estudiar el potencial efecto beneficioso para la salud del consumo regular de ajo negro.

A día de hoy, encontramos algunas publicaciones científicas que evalúan el papel de los fitoquímicos presentes en el ajo negro en la prevención y tratamiento de diferentes enfermedades, sin embargo, no existen hasta la fecha estudios que evalúen los posibles efectos positivos de los fitoquímicos propios de la cebolla negra debido a que es un producto relativamente nuevo.

1.7.3.1. Enfermedades neurodegenerativas

Se ha relacionado el consumo de ajo negro y SAC como un potencial agente protector frente a la neurodegeneración, concretamente frente a la ocasionada por enfermedades como el Alzheimer, el Parkinson y la isquemia cerebral. En estudios sobre el Alzheimer se ha visto cómo el ajo negro puede prevenir el deterioro cognitivo ejerciendo un efecto neuroprotector con el que evita la muerte neuronal causada por la isquemia y mejora la retención de la memoria y la capacidad de aprendizaje (Jeong et al., 2013). Por otro lado, en estudios sobre el Parkinson, aunque aún son limitados, se ha visto que la administración continuada de SAC mejora significativamente la peroxidación lipídica, la producción de especies reactivas de oxígeno (ROS), la pérdida de dopamina y el déficit de locomoción (Rojas et al., 2011). Además, también se han llevado a cabo estudios sobre la isquemia cerebral en los que se ha observado que el SAC puede atenuar el

daño oxidativo y mejorar el déficit neurológico en ratas con isquemia cerebral focal. El tratamiento con SAC redujo significativamente el tamaño de la lesión isquémica y disminuyó la pérdida neuronal (Ashafaq et al., 2012). También se ha observado que el extracto de ajo negro y el SAC tienen la capacidad de inducir neuroprotección mediante el control de la formación de ROS (Gomez et al., 2019). De momento, los estudios que existen se han realizado en modelos celulares y animales (ratas y ratones), por lo que aún hay que llevar a cabo estudios que permitan observar los efectos del consumo regular de ajo negro, y, por tanto, de sus compuestos fitoquímicos frente a estas enfermedades neurodegenerativas en humanos.

1.7.3.2. Cáncer

Existen un importante número de estudios que relacionan el potencial anticancerígeno del consumo de ajo negro debido a los efectos que pueden ejercer los compuestos que se encuentran formando parte de su composición, pero todos ellos exclusivamente en modelos celulares. Entre ellos, hablamos de un efecto antiinflamatorio, antioxidante, antiproliferativo, antiagiogénico o induciendo la apoptosis. El compuesto aislado de mayor interés con potencial anticancerígeno es la *S*-alilmercaptocisteína (SAMC), la cual ha mostrado acción protectora frente al cáncer de próstata, de hígado, de tiroides, de vejiga y de ovario, mediante diferentes mecanismos que provocan la reducción de la inducción de la apoptosis, la inhibición del crecimiento de las células cancerígenas, la inhibición de la invasión y la migración de las células cancerosas y/o la inhibición de la proliferación y la metástasis de las células tumorosas (Chu et al., 2006; Hu et al., 2011; Y. Liu et al., 2015; Tong et al., 2014; J. Wu et al., 2016). También se han llevado a cabo estudios con el ajo negro, el cual ha mostrado potencial anticancerígeno frente al cáncer de colon, de estómago, de mama y de pulmón, siendo capaz de inhibir el crecimiento de las células cancerígenas (Dong et al., 2014; X. Wang et al., 2012; Yang et al., 2013). Adicionalmente, se ha observado la capacidad que tiene para inhibir la metástasis y el desarrollo del tumor en cáncer de estómago y de colon, así como un efecto potenciador de un fármaco empleado en quimioterapia en cáncer de mama (Alkreathy et al., 2020; Jikihara et al., 2015; D.-Y. Shin et al., 2010).

1.7.3.3. Diabetes Mellitus y Obesidad

El consumo de ajo negro ha sido relacionado con un efecto anti-diabético, encontrando diferentes estudios que evalúan su acción sobre los marcadores habituales de esta enfermedad como el peso corporal, la glucosa en sangre, el colesterol sérico, los triglicéridos o la fructosamina, los cuáles se han visto disminuidos en diferentes estudios en ratas y ratones (Seo et al., 2009; Thomson et al., 2016). Por otro lado, también se ha

encontrado una relación entre el consumo del ajo negro y una disminución en los marcadores metabólicos relacionados directamente con la obesidad, como son el peso corporal, la masa del tejido adiposo, los triglicéridos, el colesterol total y el malondialdehído plasmático en ratones obesos que presentaban una dieta rica en grasas (Chang et al., 2017; I. Kim et al., 2011).

1.7.3.4. Enfermedades cardiovasculares

El papel del ajo negro para reducir el riesgo de padecer una enfermedad cardiovascular estará relacionado con tres marcadores: la agregación plaquetaria, la hipertensión y la aterosclerosis. Varios estudios han demostrado la relación entre el consumo de ajo negro y la inhibición de la agregación plaquetaria, lo cual puede estar relacionado con una disminución del riesgo de sufrir un infarto de miocardio o un accidente cerebrovascular (K. Rahman & Billington, 2000; Steiner & Li, 2001). Por otro lado, se ha visto que el ajo negro también disminuye la hipertensión arterial e inhibe la acción de la enzima convertidora de angiotensina, la cual es responsable de la transformación de la angiotensina I en angiotensina II, un vasoconstrictor y estimulante de la producción de aldosterona que provoca una retención de sodio y un aumento de la presión sanguínea (Castro et al., 2010; Riet et al., 2015). Además, el ajo negro también ha sido relacionado con los principales factores relacionados con la formación de placas ateroescleróticas, como son la masa grasa y el colesterol plasmático. En diferentes estudios se ha visto cómo el consumo regular de ajo negro es capaz de reducir o inhibir la progresión de la aterosclerosis mediante diferentes mecanismos (Budoff et al., 2009; Morihara et al., 2016; Wlosinska et al., 2020).

1.7.3.5. Enfermedades digestivas

Se ha visto que el consumo de ajo tiene un efecto beneficioso frente a diferentes patologías relacionadas con el sistema digestivo como puede ser motilidad gastrointestinal disminuida, estreñimiento, reflujo gastroesofágico, úlcera gástrica, daño gástrico e intestinal y enfermedades relacionadas con el hígado. Diversos estudios en modelos animales han observado como el ajo negro favorece la motilidad intestinal, así como tiene un ligero efecto laxante y tiene un efecto protector frente al reflujo esofágico (Chen et al., 2018; K. J. Kim et al., 2019; Xin et al., 2014). También se ha observado que previene la aparición de úlceras inducidas por indometacina y restaura el daño causado en la mucosa gástrica por este mismo agente, así como la reducción del daño intestinal inducido en el intestino delgado (Badr & Al-Mulhim, 2014; El-Ashmawy et al., 2016; Yüncü et al., 2006). Adicionalmente, el consumo de ajo negro se encuentra relacionado con un disminución del estrés oxidativo, de la inflamación y de las lesiones

en el hígado, demostrando un efecto hepatoprotector (G. Jiang et al., 2021; H. S. Lee et al., 2016; D.-Y. Shin et al., 2010).

Por otro lado, el ajo negro también ha sido relacionado con un significativo potencial para tratar diferentes enfermedades inflamatorias. Se han realizado estudios con ajo negro y con el 5-hidroximetilfurfural, aislado de éste, en modelos celulares, observándose en todos ellos efectos antiinflamatorios mediante diferentes mecanismos relacionados con la reducción de los mediadores inflamatorios, como las citoquinas (M. J. Kim et al., 2014; Kong et al., 2019).

1.7.3.6. Enfermedades renales

Los estudios que relacionan la acción del ajo negro con enfermedades renales son limitadas y han sido realizadas exclusivamente en modelos animales. No obstante, se ha observado cómo el ajo negro es capaz de prevenir la nefrotoxicidad inducida por un antibiótico y presenta un efecto protector frente a la nefropatía diabética por su acción antiglicante e hipolipemiante (Maldonado et al., 2003; Shiju et al., 2013). Por otro lado, el ajo negro es potencialmente capaz de reparar el daño causado en el riñón por un fitotóxico, así como puede mejorar una lesión renal aguda inducida por un antibiótico (Albrakati, 2020; T. W. Lee et al., 2019).

1.8. Evaluación de la bioaccesibilidad y la biodisponibilidad

Los fitoquímicos son compuestos bioactivos, los cuáles deben alcanzar el torrente sanguíneo para poder ejercer su acción en el tejido diana, lo que es conocido como bioactividad. Sin embargo, para que esta bioactividad llegue a su consecución, el compuesto en cuestión debe ser bioaccesible y biodisponible.

La biodisponibilidad puede ser definida de diversas maneras, pero en general, este concepto hace referencia a los compuestos obtenidos a partir de la matriz ingerida que alcanzan el sistema circulatorio para su distribución hacia los tejidos diana, de forma que estos compuestos se encuentren biológicamente disponibles para ejercer la acción beneficiosa para la salud. Las rutas habituales de los compuestos fitoquímicos de la dieta incluyen la ingestión, es decir, la digestión y el transporte a través del epitelio gastrointestinal antes de su llegada al sistema circulatorio (Epriliati & Ginjom, 2012).

La biodisponibilidad consistirá en la fracción de la cantidad total del compuesto ingerida que consigue alcanzar el tejido diana. En este sentido, un compuesto debe recorrer las siguientes etapas tras su ingestión para considerarse biodisponible (Aggett, 2010):

1. Bioaccesibilidad: Es la fracción de un compuesto que es liberado de la matriz alimentaria durante la digestión, en el tracto gastrointestinal, lo que hace que se encuentre disponible para ser absorbido en el intestino (Pellegrini et al., 2017; Rein et al., 2013).
2. Permeabilidad intestinal: Fracción de un compuesto que es capaz de atravesar el epitelio intestinal y llegar a la circulación portal hepática.
3. Metabolismo: Porción de un metabolito que llega al hígado sin ser metabolizado y que alcanzará la circulación sistémica. Esta será denominada la fracción biodisponible.
4. Tejido u órgano diana: Deposición, uso metabólico y funcional en el tejido diana.
5. Excreción: Se realizará por vía urinaria o fecal.

La biodisponibilidad de un compuesto dependerá de su estructura química, así como de la matriz alimentaria en la que se encuentre (Selby-Pham et al., 2017). Por otro lado, también dependerá de factores intrínsecos del individuo como el sexo, la edad, el estado nutricional y la etapa de la vida (Heaney, 2001). Debido a la variabilidad que pueden ocasionar estos factores, la mayoría de las definiciones de biodisponibilidad asumen que la fracción biodisponible del compuesto es aquella que es absorbida tras la digestión gastrointestinal y la acción de la microbiota colónica.

En esta memoria, nos centraremos en las experimentaciones llevadas a cabo durante la presente Tesis Doctoral, es decir, la evaluación de la bioaccesibilidad *in vitro*, evaluación de la influencia de la microbiota intestinal durante la fermentación *in vitro* y la evaluación de la biodisponibilidad in vivo.

1.8.1. Evaluación de la bioaccesibilidad y de la influencia de la microbiota intestinal

Existen métodos *in vivo* que permiten evaluar la bioaccesibilidad en humanos con el inconveniente de que requieren procedimientos muy invasivos o tecnologías muy punteras que encarecen mucho la experimentación (Sullivan et al., 2014). También se han llevado a cabo estudios *in vivo* en modelos animales, sin embargo, estos implican procedimientos quirúrgicos o la muerte del animal, considerándose cuestionable su uso, desde un punto de vista ético, para evaluar el proceso de digestión de un alimento en humanos. Es debido a esto que en las últimas décadas se están empleando métodos *in vitro* para llevar a cabo este tipo de estudios.

La digestión *in vitro* puede realizarse siguiendo dos metodologías: métodos estáticos o métodos dinámicos (Didier Dupont & Mackie, 2015). Los métodos dinámicos consisten en un sistema que va a permitir simular tanto los aspectos bioquímicos como los

mecánicos de una digestión gastrointestinal de forma lo más realista posible, respetando escrupulosamente los tiempos de digestión. Estos métodos son caros de instalar y de mantener, complejos y no accesibles para la mayoría de laboratorios (D. Dupont et al., 2018). Por otro lado, encontramos los métodos estáticos, más baratos y simples, en los que se trata de mantener la relación correcta entre la cantidad de alimento y la de enzimas y electrolitos, llevándose a cabo controles de pH para cada una de las fases de la digestión (Dima et al., 2020). Estos modelos han sido ampliamente utilizados en estudios para evaluar compuestos en alimentos y fármacos (Berthelsen et al., 2019; Juániz et al., 2017). El principal inconveniente de este sistema radica en que a lo largo de los años se han empleado diferentes protocolos para la realización de las digestiones *in vitro*, lo que complica la comparación de resultados entre estudios de diferentes laboratorios. Es por ello que surge la red internacional INFOGEST que trata de establecer un único protocolo para estandarizar las condiciones empleadas a la hora de llevar a cabo un estudio de digestibilidad *in vitro*, y recientemente se ha actualizado, presentando el INFOGEST 2.0 (Brodkorb et al., 2019), cuyo diagrama de flujo se representa en la Figura 15.

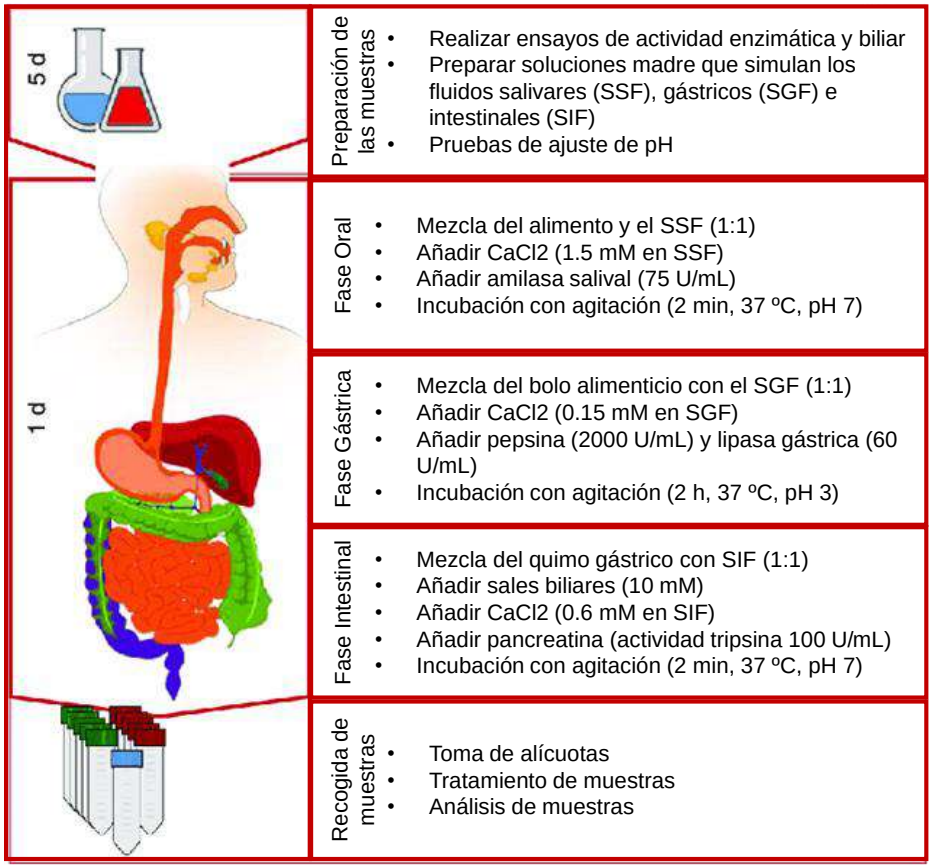

Figura 15.- Adaptación del diagrama de flujo del método de digestión gastrointestinal in vitro de INFOGEST 2.0 (Brodkorb et al., 2019)

Los métodos de digestión *in vitro* permitirán determinar la bioaccesibilidad de los compuestos identificados, cuantificados y monitoreados durante el proceso digestivo. La bioaccesibilidad se evalúa mediante el índice de bioaccesibilidad, que se calcula siguiendo la siguiente fórmula:

$$Índice\ de\ Bioaccesibilidad\ (IB) = \frac{CF\ (Concentración\ final\ del\ compuesto)}{CI\ (Concentración\ inicial\ del\ compuesto)} \times 100$$

CF hace referencia a la concentración del compuesto tras la digestión gastrointestinal in vitro, mientras que CI hace referencia a la concentración del compuesto antes del proceso digestivo.

Sin embargo, en los últimos años se ha puesto en valor el papel de la microbiota colónica en la metabolización de los fitoquímicos, y es que las bacterias intestinales participan en la degradación de los compuestos, siendo capaces de hidrolizar glucósidos, glucurónidos, amidas, sulfatos, ésteres, etc. (Pereira-Caro et al., 2015; Rechner et al., 2004). Es por ello que se considera que la microbiota colónica tiene un papel fundamental en la regulación de los efectos beneficiosos para la salud que pueden ejercer los fitoquímicos (Cassidy & Minihane, 2017; Marhuenda-Muñoz et al., 2019). De hecho, a nivel colónico se pueden ver afectadas la absorción, la biodisponibilidad y la actividad biológica de los compuestos ingeridos, ya que pueden ser metabolizados por la microbiota dando lugar a nuevos compuestos que pueden presentar también una reconocida bioactividad (Aura, 2008; Ludwig et al., 2013). En este sentido, se ha demostrado que los catabolitos microbianos muestran propiedades bioactivas diferentes a las de los compuestos originales (Duda-Chodak et al., 2015; Makarewicz et al., 2021; Verzelloni et al., 2011). No tener en cuenta el metabolismo colónico a la hora de evaluar la bioactividad y biodisponibilidad de un compuesto limita la información que se obtiene a los compuestos originales que se encuentran en los alimentos, sin evaluar los catabolitos que pueden formarse a este nivel.

Para la evaluación del metabolismo de los compuestos de interés a nivel colónico se pueden encontrar metodologías *in viv*o e *in vitro*, siendo los métodos *in vivo* con humanos los que proporcionan la información de mayor relevancia desde un punto de vista fisiológico. A pesar de ello, los estudios *in vitro* continúan siendo clave para evaluar componentes o alimentos específicos, así como para hacer estudios preliminares. Las modelos de fermentaciones colónicas *in vitro* pueden ser: fermentaciones por etapas o fermentaciones en sistemas continuos (Nissen et al., 2020). Los modelos de fermentaciones basados en sistemas continuos son más próximos a las condiciones fisiológicas, permiten una mejor representación de las comunidades microbianas intestinales, se pueden mantener durante periodos de tiempo más largos y de forma automática imita las condiciones fisiológicas de las diferentes partes del colon. Las

limitaciones que presenta este modelo son las dimensiones necesarias para implantar este sistema y que solo es capaz de estudiar una única muestra cada vez, requiriendo un mes para evaluar esta muestra entre la preparación del sistema y el desarrollo de la fermentación colónica (Fehlbaum et al., 2015).

Por otro lado, se encuentran los modelos basados en fermentaciones por etapas, los cuáles no requieren de instalaciones especiales, permiten evaluar múltiples muestras en el mismo experimento y necesitan menores tiempos de experimentación, normalmente 24-48h (Ordoñez-Díaz et al., 2020; Pereira-Caro et al., 2021). Es un buen sistema para llevar a cabo unas primeras aproximaciones hacia la metabolización de los componentes de un alimento (Venema & Van Den Abbeele, 2013). Sin embargo, al igual que ocurría con las digestiones gastrointestinales *in vitro*, la diversidad de protocolos empleados para llevar a cabo las fermentaciones colónicas *in vitro* produce una variabilidad en los resultados obtenidos por diferentes investigadores, lo que dificulta la comparación de éstos. Recientemente, Pérez-Burillo y col. (2021) han propuesto un protocolo para estandarizar las condiciones de realización de las fermentaciones colónicas *in vitro* por etapas donde se emplearán heces humanas para la obtención de las colonias microbianas intestinales y se trabajará con alimentos previamente digeridos mediante una digestión gastrointestinal *in vitro*, preferiblemente, siguiendo el protocolo de INFOGEST 2.0 antes mencionado. El diagrama de flujo del protocolo propuesto por este grupo de investigadores viene representado en la Figura 16.

Etapa 1: Recoger la muestra fecal. Recogida y almacenamiento de las muestras fecales de los voluntarios sanos.

Etapa 2: Preparación de material y reactivos. Preparación de la solución tampón fosfato, solución de peptona, solución reductora, solución de resazurina y medio de cultivo. Esterilización del material y los reactivos.

Etapa 3: Preparación de las muestras.
Pesar el residuo sólido obtenido tras el proceso de digestión *in vitro*.
Añadir el 10% del sobrenadante del proceso de digestión i*n vitro*.

Etapa 4: Preparación solución fecal.
Preparación del inóculo con un 32% de las heces en 0.1 M de tampón fosfato a pH 7.

Etapa 5: Fermentación colónica
Añadir la solución fecal y el medio de cultivo en condiciones anaeróbicas y asépticas.
Anotar el volumen final.
Incubar durante 20 h a 37 ºC en agitación.

Etapa 6: Muestreo y almacenamiento.
Detener la fermentación y mantener en tubos en hielo durante 15 min. Almacenar muestras.

Etapa 7: Procesamiento de muestras
Tratamiento de las muestras para el posterior análisis.

Figura 16.- Adaptación del diagrama de flujo del proceso de fermentación colónica in vitro propuesto por Pérez-Burillo y col. (2021)

1.8.2. Evaluación de la biodisponibilidad

A la hora de evaluar la biodisponibilidad de un compuesto o alimento se pueden emplear tanto modelos *in vivo* como modelos *in vitro*. La estrategia ideal sería la realización de estudios clínicos en humanos, no obstante, son estudios que necesitan mucha organización, complejos y requieren una significativa inversión tanto económica como técnica. Debido a esta casuística, se suelen emplear modelos *in vitro* o *in vivo* con animales de experimentación, a pesar de las limitaciones que tienen estos modelos a la hora de extrapolar resultados.

Los modelos *in vitro* empleados para la evaluación de la biodisponibilidad de compuestos están basados en membranas artificiales o en el uso de cultivos celulares. Lo más habitual es el empleo de cultivos celulares, ya que presentan muchas ventajas respecto al resto de modelos de experimentación: son más baratos, requieren menos tiempo y no es necesaria la aprobación por un comité ético. Estos cultivos celulares pueden contener solo un tipo de células o varias, en cuyo caso se denominan co-cultivos. Las células que suelen utilizarse para estos modelos son las Caco-2, células de adenocarcinoma de colon, esto se debe a que las células del epitelio gastrointestinal humano no son capaces de formar monocapas celulares en condiciones *in vitro*, las

cuáles son necesarias para simular el epitelio intestinal. Las Caco-2 presentan una morfología y una funcionalidad muy similar a la de un enterocito sano, además son capaces de formar una monocapa celular que simulará el epitelio intestinal humano. Los estudios con estas líneas celulares reportan información consistente que permite tener resultados previos a estudios *in vivo* (Glahn, 2009; T. Wu et al., 2017). Sin embargo, en la búsqueda de desarrollar sistemas que simulen más fielmente la situación real en el intestino, se están llevando a cabo estudios con co-cultivos celulares que combinan las Caco-2 y las HT29-MTX. Estas últimas son células de carcinoma de colon humano cuya particularidad radica en la capacidad que tienen para secretar mucina, proteína que se encuentra recubriendo el epitelio intestinal. Existen modelos celulares que combinan las Caco-2 con otras líneas celulares o que incorporan diferentes solutos, variando en función del objetivo de estudio del experimento (Lozoya-Agullo et al., 2017; Mahler et al., 2009). El estudio en modelos celulares se centrará en la etapa de permeabilidad intestinal de todo el proceso de biodisponibilidad de un compuesto.

A pesar de la versatilidad y los avances en las metodologías *in vitro*, los modelos *in vivo* animales siguen siendo los que proporcionan una información más próxima a lo esperable en humanos. En este sentido, los estudios con ratas y ratones son los más empleados, tomando muestras de sangre a diferentes tiempos tras una única ingesta del alimento o el compuesto de interés, muestras de orina y de heces (Oliveira et al., 2013; Pereira-Caro et al., 2018).

Por último, se encuentran los estudios clínicos en humanos, denominados estudios de intervención y consisten en “la aplicación de una intervención nutricional, aguda o crónica, en condiciones controladas para medir un efecto biológico”. Existen diferentes tipos de estudios de intervención en nutrición en función de la metodología (controlados o no controlados), de la información que posea el sujeto y el investigador (abierto, simple ciego, doble ciego), de la aleatorización del estudio (aleatorizado o no aleatorizado) y del diseño del estudio (comparativo o cruzado). En estos estudios se suelen establecer criterios de inclusión que permiten obtener resultados de poblaciones generales o específicas (estudios exclusivos con mujeres, con embarazadas, con pacientes con cáncer, etc.). Para la evaluación de la biodisponibilidad de un compuesto de un alimento o de la dieta, tras el estudio de intervención se tomarán muestras de sangre, de orina y/o heces a diferentes tiempos tras la ingesta del alimento o dieta en cuestión, para posteriormente ser analizados (Mullen et al., 2004, 2006; Pereira-Caro et al., 2016).

1.8.3. Estudios de bioaccesibilidad y biodisponibilidad en ajo, cebolla, ajo negro y cebolla negra

Actualmente, los compuestos fenólicos conforman uno de los grupos de compuestos fitoquímicos más ampliamente estudiados, lo que incluye aspectos tales como su bioaccesibilidad y biodisponibilidad, tanto de forma aislada como formando parte de diferentes matrices alimentarias (Almeida et al., 2018; Angelino et al., 2017; Del Rio et al., 2010; Lafay & Gil-Izquierdo, 2007; Nardini et al., 2002; Pereira-Caro et al., 2014). En este sentido, también se han llevado a cabo estudios que evalúan la bioaccesibilidad y la biodisponibilidad de los compuestos fenólicos del ajo y, mayoritariamente, de la cebolla. Ceccanti y col. (2020) llevaron a cabo un estudio con dos variedades de ajo (*Allium sativum* y *Allium ampeloprasum* var*. holmense*) de la Toscana italiana, con el objetivo de comparar el perfil fitoquímico y sensorial de ambos durante la digestión gastrointestinal, proponiendo el ácido 4-hidroxibenzoico-4-*O*-glucósido, el 5-nonadecilresocinol y el triptófano como biomarcadores del consumo de *Allium ampeloprasum* var*. holmense* (Ceccanti et al., 2021). Por otro lado, diferentes autores han llevado a cabo estudios sobre el impacto de la digestión gastrointestinal sobre el perfil de compuestos fenólicos de la cebolla. Shim y col. (2011) mostraron que la miricetina fue el flavonoide que presentó el mayor índice de bioaccesibilidad tanto en cebolla roja como amarilla, mientras que Fernández-Jalao y col. (2021) reportaron que la quercetina-7,4'-diglucósidos y la quercetina-3,4'-diglucósido presentaron los índices de bioaccesibilidad más altos tras la digestión gastrointestinal *in vitro* (Fernández-Jalao et al., 2021; Shim et al., 2011). También se ha evaluado cómo afectan los métodos de cocinado a la bioaccesibilidad de estos compuestos de la cebolla, observándose que el horneado y el asado aumentan la bioaccesibilidad de los compuestos fenólicos de la cebolla amarilla y la roja, siendo la quercetina-3-*O*-hexósido-4'-*O*-hexósido el compuesto más abundante y con mayor índice de bioaccesibilidad (Cattivelli et al., 2021).

Respecto a los estudios de biodisponibilidad, Mullen y col. (2004), llevaron a cabo la identificación de los metabolitos de la quercetina en plasma y orina tras la ingesta de cebolla frita por individuos sanos, observándose principalmente conjugados glucurónidos y sulfatados de la quercetina y la isorhamnetina en estas muestras (Mullen et al., 2004). Por otro lado, se han determinado los metabolitos mayoritarios tras la ingesta de cebolla frita, encontrando en plasma la quercetina-3'-sulfato y la quercetina-3-glucurónido, mientras que en orina, la quercetina glucurónido y la quercetina sulfato fueron los mayoritarios (Mullen et al., 2006). También se han realizado estudios que han

comparado la suplementación con quercetina y el consumo de la quercetina propia de la cebolla, mostrando que la matriz alimentaria tiene un efecto positivo en la biodisponibilidad de este compuesto (Kashino et al., 2015; Shi & Williamson, 2015).

La información relacionada con la determinación de la bioaccesibilidad y la biodisponibilidad de los compuestos organosulfurados presentes en ajo y cebolla es mucho más limitada. Fundamentalmente, se han llevado a cabo estudios que evalúan estos aspectos en ajo y como compuestos aislados, sin embargo, la mayor parte de las investigaciones se han llevado a cabo mediante análisis por cromatografía de gases, determinando el perfil de compuestos volátiles (Qin et al., 2020; Rosen et al., 2000; Scheffler et al., 2016, 2019a, 2019b; Verhagen et al., 2001). Torres-Palazzolo y col. (2021) concluyeron que los compuestos organosulfurados presentes naturalmente en el ajo son más estables que los aislados durante la digestión gastrointestinal *in vitro*, ejerciendo la matriz un efecto protector frente a la acción digestiva (Torres-Palazzolo et al., 2021). Por otro lado, Nagae y col. (1994) evaluaron la farmacocinética del S-alil-L-cisteína (SAC) en ratas, ratones y perros, observando que la mayor parte es excretada en orina en forma N-acetilada y como SAC, mostrando una biodisponibilidad de entre el 87 y el 103% (Nagae et al., 1994). De la misma forma, Ichikawa y col. (2006) administraron cicloaliína a ratas por vía intravenosa, siendo ésta rápidamente excretada en la orina, mientras que, si se administraba vía oral, aparecía rápidamente en plasma. Finalmente observaron que este compuesto es reducido a ácido (3R,5*S*)-5-metil-1,4-tiazano-3-carboxílico por la microbiota intestinal, disminuyendo la biodisponibilidad de la cicloaliína (Ichikawa et al., 2006). No obstante, es necesario que llevar a cabo estudios que permitan ampliar conocimientos en cuanto a estos compuestos, sus rutas metabólicas y sus posibles efectos beneficiosos para la salud.

Hasta la fecha, tras una profunda revisión bibliográfica, hemos podido concluir que no existen estudios que evalúen la bioaccesibilidad y la biodisponibilidad de los compuestos fitoquímicos de interés del ajo negro y la cebolla negra, por lo que se considera necesario determinar el impacto de la digestión gastrointestinal, la fermentación colónica y el metabolismo sobre éstos, de tal forma que permita obtener más información sobre la evolución del perfil de estos compuestos y su potencial bioactividad.

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CAPÍTULO 2: OBJETIVOS

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Un grupo de alimentos importante y de alto interés nutricional, dada su composición en componentes bioactivos, son los del género *Allium*, entre los que destaca el ajo (*Allium sativum* L.) y la cebolla (*Allium cepa* L.), siendo considerados alimentos con características terapéuticas excepcionales. En este sentido, son bien conocidas las excelentes cualidades del ajo y la cebolla, pudiendo destacar una asociación entre el consumo de ajo y cebolla y el descenso del riesgo de padecer algunos tipos de cáncer.

Gran parte de los beneficios asociados al consumo de estos alimentos se deben a la presencia de los llamados compuestos organosulfurados que son a su vez los responsables del aroma, los olores y sabores característicos del ajo y cebolla, así como las posibles molestias estomacales asociadas con su ingesta.

Sin embargo, aunque el ajo y la cebolla presenten un amplio abanico de efectos saludables, su consumo en fresco no está muy extendido debido fundamentalmente sus características organolépticas, sobre todo en el caso del ajo. Por ello una forma más interesante de consumo es en forma de ajo negro o cebolla negra, productos que se obtienen a partir de ajo fresco o cebolla fresca, respectivamente, tras un proceso de fermentación en el que no intervienen bacterias ni levaduras, sino condiciones controladas y adecuadas de temperatura.

Del nuevo producto, cebolla negra, no hay aun estudios que describan su composición en compuestos organosulfurados y su concentración. Dado el éxito del ajo negro, cabe enfatizar la importancia de la caracterización de este nuevo producto con objeto de aumentar su reclamo frente al consumidor. Además, es muy importante conocer la absorción, metabolismo, distribución y excreción (AMDE) de los compuestos bioactivos de estos alimentos con el fin de entender las funciones biológicas sobre la salud humana. En este sentido, existen pocos trabajos que describan la AMDE de los compuestos organosulfurados cuando éstos se consumen a través de ajo negro, y ninguno cuando se consume cebolla negra.

Por todo ello, el **objetivo general** que se persigue en la presente Tesis Doctoral es la caracterización fitoquímica de un nuevo producto transformado derivado de la cebolla fresca (*Allium cepa* L.), denominado cebolla negra. Así como la evaluación de la bioaccesibilidad *in vitro* y biodisponibilidad *in vivo* de los compuestos biosaludables

identificados en cebolla negra y su análogo, el ajo negro, en comparación con sus matrices originales, cebolla fresca y ajo fresco (*Allium sativum* L.). Este objetivo general se concreta en los siguientes **objetivos específicos**:

Objetivo 1: Optimizar y validar una metodología para la caracterización del perfil de compuestos fitoquímicos (compuestos fenólicos, aminoácidos y compuestos organosulfurados) y la capacidad antioxidante de la cebolla blanca de partida y de su producto elaborado (cebolla negra) en tres variedades distintas de cebollas. Así como la evolución del perfil de estos compuestos durante el proceso de elaboración de la cebolla negra (ya optimizado en investigaciones previas).

Objetivo 2: Estimar la bioaccesibilidad de los compuestos bioactivos del ajo fresco y el ajo negro, así como de la cebolla fresca y la cebolla negra mediante estudios de estabilidad gastrointestinal *in vitro*.

Objetivo 3: Evaluar el impacto de la fermentación colónica *in vitro* sobre los compuestos bioactivos del ajo fresco, el ajo negro, la cebolla fresca y la cebolla negra.

Objetivo 4: Determinar el potencial saludable de ajo negro y cebolla negra mediante el estudio de su metabolismo y biodisponibilidad en voluntarios sanos. Estudio post-prandial.

Objetivo 5: Aprendizaje de técnicas de manejo de líneas celulares Caco-2 y co-cultivos Caco-2 y HT29 para evaluar la biodisponibilidad metabolismo y bioactividad de los compuestos bioactivos del ajo negro y la cebolla negra en líneas celulares Caco-2.

CAPÍTULO 3

CAPÍTULO 3: RESULTADOS OBJETIVO 1

Artículo 1

Development and Validation of UPHLC-HRMS Methodology for the Determination of Flavonoids, Amino Acids and Organosulfur Compounds in Black Onion, a Novel Derived Product from Fresh Shallot Onions (*Allium cepa* var. *aggregatum*)

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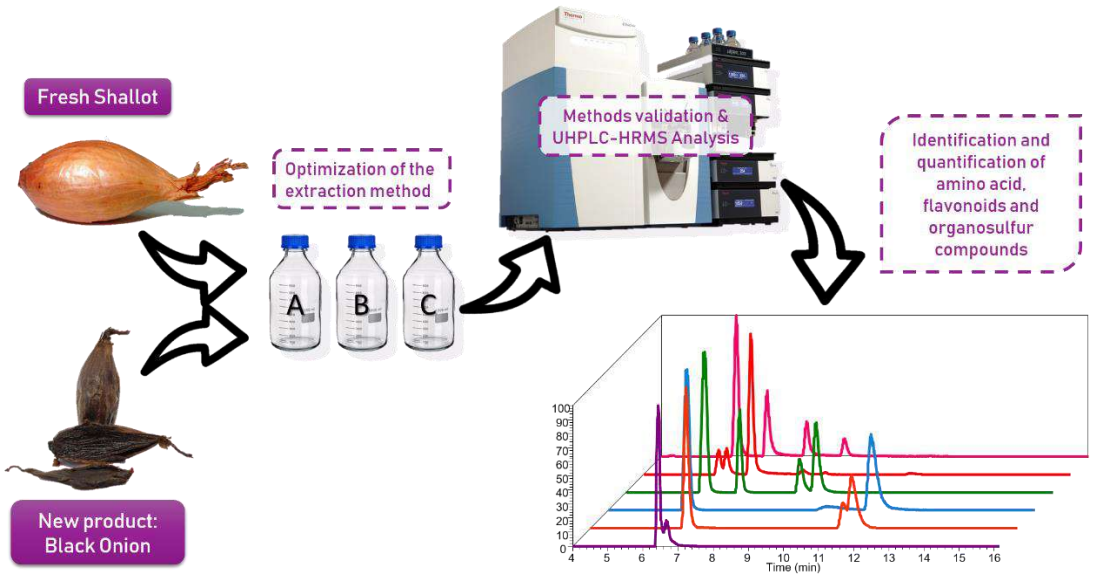

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Development and validation of UHPLC-HRMS methodology for the determination of flavonoids, amino acids and organosulfur compounds in black onion, a novel derived product from fresh shallot onions (*Allium* cepa var. *aggregatum*)

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HPLC-HRMS method validation

ABSTRACT

Black onion, a new derived product from fresh onion, has been developed by processing (aging) fresh shallot onion in a temperature- and humidity-controlled room without using any artificial additives. The aim of this study was to adapt, optimize and validate two ultra-high-performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) methodologies for the determination of flavonoids, amino acids and organosulfur compounds in black onion. UHPLC-HRMS methods involving RP-C18 and HILIC columns were adapted and validated in terms of specificity, linearity, limit of detection (LD) and quantification (LQ), precision inter- and intra-day, recovery and matrix effect. Linearity ranged from 0.012 to 12.5 ngμL^{-1} and from 0.1 to 75 ngμL^{-1} for flavonoid and amino acids and organosulfur compounds, respectively. LD varied from 0.004 to 0.06 ngμL^{-1} and LQ from 0.012 to 0.2 ngμL^{-1}. The intra-day and inter-day precision for all compounds were less than 15% and the recovery ranged from 69 to 106%. The matrix effect ranged from 80 to 114% for flavonoids, amino acids and organosulfur compounds. The described methods were successfully applied for the correct separation and determination of 53 compounds in black onion. These results establish the value of these new two UHPLC-HRMS protocols in providing detailed compound profiles of black onion, highlighting their potential applicability to similar vegetables.

1. Introduction

Onion (*Allium cepa* L.) is one of the most important bulb crops and is commonly used as food, spice and medicinal plant almost worldwide. With an estimated global production rate of 5.7 Mt per year (http://faostat.fao.org, 2016), onion is the first most produced bulb crops in Spain, and together with garlic, is the most consumed bulb vegetable either fresh or after processing into various (cooked) products. Epidemiological and clinical studies have reported that the consumption of *Allium* vegetables such as onion, garlic and leek protects against the development of metabolic diseases such as diabetes (Akash, Rehman, & Chen, 2014) or cardiovascular diseases (Bahadoran, Mirmiran, Momenan, & Azizi, 2017). Besides, its consumption is associated with a reduce risk of developing diverse types of cancers including stomach, colorectal (Nicastro, Ross, & Milner, 2015) and breast cancer (Pourzand et al., 2016), playing a significant role in human nutrition. From a nutritional point of view, free proteogenic amino acids account for 5–7% dry weight of an onion bulb with arginine and glutamine being the most abundant ones. In addition, onion is characterized by its high levels of health-promoting constituents comprising flavonoids and a huge variety of sulfur-containing compounds which accounted to its well-known nutritional properties (Böttcher, Krähmer, Stürtz, Widder & Schulz, 2017).

Nowadays, the food industry is looking for new foodstuffs with added value and functionality. Recently, a derived product from onion, black onion, is gaining great popularity among the Spanish consumers. This new product is made by processing (aging) fresh shallot onion in a temperature- and humidity-controlled room without using any artificial

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additives. This manufacturing process led to colour changes from white onion to black onion, and to improve organoleptic properties, also increasing the fruit-like sweetness of the final product. These changes are linked to substantial changes on chemical composition during the heating process of onion. However, the scientific data in relation to this issue is scare. Previous studies on an analogous product called "black garlic" showed that the profiles of bioactives compounds in that product increased after the heating process of the raw garlic (Jung et al., 2014). Therefore, this new product not only has a great commercial value by its culinary use, but it could also be used as functional food.

To date, the analysis of the primary and secondary metabolites in foods is challenging due to their different structure, distributions and concentrations in plants vary greatly and the limitation of commercially available reference standards. Several techniques based on high-performance liquid chromatography coupled to mass spectrometry (LC-MS) offer versatile tools for addressing the identification and quantification of a wide number of compounds in onion samples. For instance, Soininen et al. (2014) analysed by LC-MS and NMR the composition of flavonols, free amino acids and organic acids in different Allium species. Other authors characterized the flavonol profile of several onion varieties by LC-DAD-ESI-MS-MS analysis (Bonaccorsi, Caristi, Gargiulli, & Leuzzi, 2008) and identified a complete profiling of polar and semi-polar onion metabolites including fructooligosaccharides, proteinogenic amino acids, peptides, S-substituted cysteine conjugates, flavonoids and saponins by LC-ESI-QTOFMS (Böttcher, Krahmer, Stürt, Widder, & Schulz, 2017). Moreover, an entire set of sulfur-containing onion metabolites in onion has been determined by RP-LC-ESI-Fourier transform ion cyclotron resonance mass spectrometry in conjunction with ^{13}C labelling (Nakabayashi et al., 2013).

To the best of our knowledge, no data on the fully characterization on primary (amino acids) and secondary metabolites (phenolic compounds and sulfur-containing compounds) of black onion have been reported. This study aims to identify and quantify these compounds in black onion, a new product derived from fresh shallot onion, by the optimization and validation of the extraction procedure as well as two rapid analytical UHPLC-HRMS methodologies.

2. Material and methods

2.1. Chemicals

Formic acid (FA), LC-MS grade acetonitrile, LC-MS grade methanol, ammonium acetate, ammonium formate, the reference compounds quercetin (95%), rutin (94%), isorhamnetin (99%), quercetin-3-*O*-glucoside (98%), kaempferol-3-*O*-rutinoside (98%), luteolin (98%), apigenin (95%) and the amino acids leucine (98%), isoleucine (98%), phenylalanine (98%), tryptophan (98%), methionine (98%), valine (98%), proline (99%), tyrosine (98%), alanine (98%), threonine (98%), glycine (99%), glutamic acid (99%), glutamine (99%), serine (99%), asparagine (98%), lysine (98%), histidine (99%), ornithine (99%), aspartic acid (98%), arginine (98%) and gamma-aminobutyric acid (GABA) (98%) and the organosulfur compounds alliin (95%) and s-allyl-L-cysteine (SAC) (95%) were purchased from Sigma-Aldrich (Madrid, Spain). All the standards used were not further purified. Deionized water was used throughout the analytical analyses.

2.2. Materials and sample preparation

Two kg of fresh shallot onions and black onions were obtained from a local supplier which provided authenticated fresh and black onion for the study. Both fresh and black onions were randomized and 0.5 kg was first frozen under liquid nitrogen to avoid enzymatic activity, then lyophilized and grinded afterwards to a final particle size of 10 μm using a mixer mill equipment (Retsch GmbH, Haan, Germany) and stored at −80 °C until analysis.

2.3. Extraction method

The optimization of the extraction of fresh and black onions were performed using three different solvents: deionized water:methanol (20:80, v/v) acidified with 1% formic acid (A), deionized water:methanol (50:50, v/v) acidified with 1% formic acid (B) and deionized water:acetonitrile (20:80, v/v) acidified with 1% formic acid (C). The extraction method was as follow: 0.5 g of fresh or black onion lyophilized and grinded was mixed with 5 mL of solvent (A), (B) or (C) for 2 min at room temperature and the mixture was sonicated for 15 min and then centrifuged at 4900 rpm for 15 min. The supernatant was collected and residues were re-extracted twice using 5 mL of the same solvent by following the same protocol described previously. All the supernatants were pooled and frozen at −80 °C until UHPLC-HRMS analysis.

2.4. UHPLC-HRMS analysis

Identification and quantification of flavonoids, amino acids and organosulfur compounds in fresh and black onion extracts were carried out using an UHPLC-PDA-MS mass spectrometer system (Thermo Scientific, San José, CA, USA) comprising of a UHPLC pump, a PDA detector scanning from 200 to 600 nm, and an autosampler operating at 4 °C (Dionex Ultimate 3000 RS, Thermo Corporation).

2.4.1. Analysis of flavonoids

Separation of flavonoids was performed on a 100 × 2.1 mm i. d. 1.8 μm Zorbax SB-C18 RRHD column (Agilent, Santa Clara, CA) preceded by a guard pre-column of the same stationary phase and maintained at 40 °C. The mobile phases, A: acidified water 1% formic acid and B: acetonitrile, were pumped at a flow rate of 0.15 mL min^{-1} with a 33 min gradient starting in 3% B and maintained during 1 min, then rising 60% B in 24 min, maintained during 3 min and then rising 70% B in 5 min. After that, the column was equilibrated to the previous conditions within 5 min.

After passing through the flow cell of the PDA detector the column eluate went directly to an Exactive Orbitrap mass spectrometer (Thermo Scientific, San José, CA) fitted with a Heated Electrospray Ionization Probe (HESI) operating in negative ionization mode for the determination of flavonoids. Full scans were recorder in *m/z* range from 100 to 1000 with a resolution of 50.000 Hz and with a full AGC target of 100000 charges, using 2 microscans. Analyses were also based on scans with in-source collision-induced dissociation (CID) at 25.0 eV. MS experiment condition with HESI in negative ionization mode was: (i) capillary temperature was 275 °C, the heater temperature was 100 °C, the sheath gas was 19 units, the auxiliary gas was 15 units, and the spray voltage was 4.0 kv.

Quality control samples (QC) were applied to assess and ensure the analytical process. The QC samples, consisting of a pool of all fresh or black onion samples, were injected regularly throughout the run. Data acquisition and processing were carried out using Xcalibur 3.0 software (Thermo Scientific, San José, CA).

2.4.2. Analysis of amino acids and organosulfur compounds

Separations of amino acids and organosulfur compounds in fresh and black onion extracts were based on a 2.1 × 150 mm ACQUITY UPLC 1.7 μm BEH amide column (equipped with a ACQUITY UPLC BEH amide 1.7 μm van-guard pre-column) (Waters, Spain) which was maintained at 35 °C and eluted using two mobile phases: A: deionized water with 5 mM of ammonium acetate, 5 mM ammonium formate and 1% formic acid and B: acetonitrile, over the course of 20 min at 0.4 mL min^{-1}. The gradient started with 5% of A rising 10% A in 0.5 min, then rising 30% A in 8 min following 46% of A after 4.5 min and finally return to 5% A in 3 min and maintained during 4 min to equilibrate the column to the initial conditions.

After passing through the flow cell of the PDA detector the column

Table 1
Recovery (%) of flavonoids, amino acids and organosulfur compounds from fresh onion and black onion using three different extraction solvents. Solvent A: deionized water:methanol (20:80, v/v) with 1% formic acid; solvent B: deionized water:methanol (50:50, v/v) with 1% formic acid and solvent C: deionized water:acetonitrile (20:80, v/v) with 1% formic acid.

	Compounds	Fresh Shallot Onion[a]			Black Onion[a]		
		Solvent A	Solvent B	Solvent C	Solvent A	Solvent B	Solvent C
Flavonoids	Quercetin	90.2a	91.7a	80.8a	109.5a	103.0a	80.0b
	Rutin	80.0a	83.7a	62.4b	83.7a	57.5b	64.1c
	Isorhamnetin	96.8a	86.5a	75.7a	88.2a	64.8b	52.7c
	Luteolin	83.9a	82.8a	76.8a	90.7a	108.6a	88.3a
	Apigenin	93.3a	81.7a	83.5a	94.2a	83.0a	94.3a
Amino Acids	Leucine	95.2a	97.6a	65.3b	101.3a	101.0a	65.5b
	Isoleucine	92.1a	96.1a	68.9b	110.6a	98.8b	46.9c
	Phenylalanine	96.6a	100.7a	67.3b	101.6a	103.0a	80.2b
	Tryptophan	89.1a	89.4a	55.4b	73.2a	73.4a	< 20b
	Methionine	98.3a	90.0a	65.1b	78.2a	79.0a	< 20
	Valine	93.8a	95.1a	66.6b	108.2a	98.7b	30.4c
	Proline	94.0a	93.2a	63.8b	107.2	98.0a	< 20b
	Tyrosine	94.2a	97.4a	64.2b	103.7a	97.2a	< 20b
	Alanine	89.7a	102.0a	77.5b	103.4a	98.3b	77.6c
	Threonine	94.9a	97.3a	65.5b	106.6a	99.2a	< 20b
	Glycine	92.1a	92.2a	< 20b	106.7a	98.6a	< 20b
	Glutamic Acid	93.5a	96.7a	57.3b	105.8a	95.9b	< 20c
	Glutamine	94.6a	97.4a	52.2b	104.8a	108.9a	< 20b
	Serine	96.8a	98.1a	48.5b	104.5a	99.2b	< 20c
	Asparagine	95.1a	96.3a	56.7b	103.1a	100.5b	< 20c
	Lysine	99.7a	97.9a	< 20b	105.0a	83.9b	< 20c
	Histidine	110.9a	106.9a	< 20b	105.9a	89.4b	< 20c
	Ornithine	93.4a	80.0a	< 20b	104.5a	74.5b	< 20c
	Aspartic Acid	95.3a	96.3a	65.5b	100.6a	90.9a	< 20b
	Arginine	97.1a	98.7a	< 20b	103.3a	91.2b	< 20c
	GABA	99.1a	96.7a	75.6b	107.7a	101.4b	84.7c
Organosulfur Compounds	Alliin	98.2a	99.0a	< 20b	96.0a	103.0a	< 20b
	SAC	97.1a	101.0a	< 20b	70.8a	74.7a	40.0b

[a] Different letters in a row denote significant differences ($p < 0.05$) among the three solvents used (one-way ANOVA followed by Tukey test).

eluate went directly to an Exactive Orbitrap mass spectrometer (Thermo Scientific, San José, CA) fitted with a Heated Electrospray Ionization Probe (HESI) operating in positive ionization mode for the determination of amino acids and organosulfur compounds. Full scans were recorder in *m/z* range from 100 to 1000 with a resolution of 50.000 Hz and with a full AGC target of 100000 charges, using 2 microscans. Analyses were also based on scans with in-source collision-induced dissociation (CID) at 25.0 eV. MS experiment condition with HESI in positive ionization mode was: (i) capillary temperature was 300 °C, the heater temperature was 150 °C, the sheath gas was 30 units, the auxiliary gas was 25 units, and the spray voltage was 3.5 kv. Quality control samples (QC) were also applied for the analysis of amino acids and organosulfur compounds as described previously.

2.4.3. Identification of flavonoids, amino acids and organosulfur compounds

Targeted identifications of phenolic compounds and amino acids and organosulfur compounds were achieved as follows: i) by comparing the exact mass and the retention time with available standards, ii) in the absence of standards, compounds were tentatively identified by comparing the theoretical exact mass of the molecular ion with the measured accurate mass of the molecular ion and searched against metabolite databases including Metlin, Phenol Explorer and more general chemical databases such as PubChem and ChemSpider. Metabolites having molecular masses within the pre-specified tolerance (≤5 ppm) of the query masses are retrieved from these databases. Quantification of phenolic compounds, amino acids and organosulfur compounds were carried out by selecting the theoretical exact mass of the molecular ion by reference to standard curves prepared in diluted fresh and black onion extracts. In absence of reference compounds, they were quantified by reference to the calibration curve of a closely related parent compound.

2.5. Method validation

The method was fully validated for specificity, linearity, limit of detection (LOD) and quantification (LOQ), intra-day (repeatability) and inter-day precision and matrix effects, according to the FDA guidelines (FDA, 2015).

Linearity was assessed in reference compounds comprising 7 flavonoids, 21 amino acids and 2 organosulfur compounds by preparing individual stock solutions of all of them. Thus, flavonoids were diluted in methanol, while amino acids and organosulfur compounds were diluted in acidified deionized water (1% of FA). The stock solutions of flavonoids, amino acids and organosulfur compounds were diluted and pooled to obtain standard solutions at a final concentration of 200 μM of each compound. A total of eight working solutions with concentrations ranging from 0.01 to 12.5 mgL^{-1} for flavonoids, and between 0.09 and 50 $mg\,L^{-1}$ and 0.1–75 $mg\,L^{-1}$ for amino acids and organosulfur, respectively, were prepared. Calibration curve were prepared using pure solvent (methanol or acidified deionized water) and diluted matrices (fresh and black onion extracts), by triplicate, for matrix effect evaluation.

2.6. Statistical analysis

Results are expressed as means ± standard deviations (SD) of three measurements for the analytical determination. Multiple comparisons were carried out using one-way ANOVA, followed by Tukey test. The level of significance was established at $p < 0.05$. The statistical software SPSS Statistic Program (v. 22) was used.

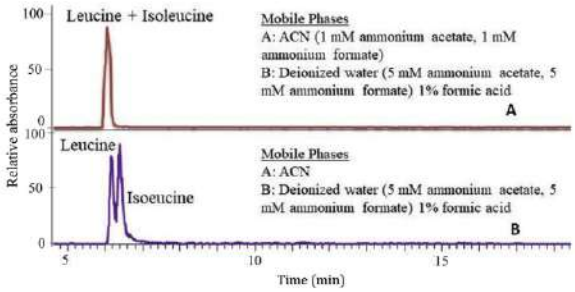

Fig. 1. Representative UHPLC-HILIC-HRMS chromatogram separation of leucine and isoleucine amino acids by using as organic mobile phase A) acetonitrile with 1 mM ammonium acetate and 1 mM ammonium formate or B) acetonitrile; and as aqueous mobile phase 1% formic acid with 5 mM ammonium acetate and 5 mM ammonium formate.

3. Results and discussion

3.1. Optimization of the extraction method

In this study, 5 flavonoids, 21 amino acids and 2 organosulfur compounds were selected to perform the optimization of the extraction method from fresh and black onion, while 7 flavonoids, 21 amino acids and 2 organosulfur compounds were used for the validation study. These compounds are commercially available and they were selected based on their previous identification in onion samples (Böttcher et al., 2017; Juániz et al., 2016).

Recovery (%) of the selected compounds as representative components of flavonoids, amino acids and organosulfur compounds in black and fresh onion using different extraction solvents are shown in Table 1. The recovery rate was calculated using three different extraction solvents. These solvents are commonly used for the extraction of primary and secondary metabolites from food matrices (Nakabayashi et al., 2013; Sharma, Assefa, Ko, Lee, & Park, 2015). For that, fresh and black onion samples were spiked with 10 μg all the standards and then were submitted to extraction in duplicate (before and after spiked) using three different solvents. Fresh and black onion samples were also submitted to extraction and either diluted and injected directly (blank samples) or spiked with a mixture of analytes (after-spiked). The recovery was calculated as the ratio between the areas of each analyte recorder in before-spiked samples minus the endogenous analytes in the matrix, divided by the area of each analyte recorder in after-spiked samples minus the endogenous analytes in the matrix, and expressed as percentage.

Thus, solvent A and B gave yields between 80.0 and 109.5% for flavonoids, showing no significant differences between these solvents used with the exception of rutin and isorhamnetin which had recovery rates less than 80% using solvent B in black onion matrix, (Table 1). In case of amino acids and organosulfur compounds, there were not significant differences using solvents A or B yielding recoveries between 80 and 111% in fresh onion matrix. However, some amino acids in black onion extraction using solvent B showed recovery values below 80%, such as tryptophan (73.4%), ornithine (74.5%) and methionine (79%) (Table 1). Recovery rates below 80% were found for an important number of tested compounds using solvent C. Based on these results, the subsequence steps for the method validation and quantification were done using as extraction solvent A which is a mixture of deionized water and methanol (20:80, v/v) acidified with 1% formic acid.

3.2. Development of UHPLC-HRMS methods

The UHPLC-HRMS analytical method using HILIC column were developed and optimized to determine free amino acids and organosulfur compounds in fresh and black onion. Modifications in the amount of ion-pairing reagent of the mobile phases (ammonium acetate and ammonium formate) and in the elution gradient, key factors for a good peak separation, were performed to obtain better peak resolution of underivatized amino acids in onion matrixes. A clear example of that is the better separation of the isomers leucine and isoleucine (Fig. 1) achieved by using as mobile phase A: 100% acetonitrile, and as mobile phase B: 1% acidified deionized water with 5 mM ammonium acetate and 5 mM ammonium formate. The use of ammonium salts in the mobile phase also increase MS signal and peak shape, without affecting the sensitivity of the MS detector. In addition, the gradient was optimized to obtain the best resolution and the shortest run time. It is noteworthy that high-resolution MS (HRMS) used in this study could avoid the risk of inaccurate measurements caused by unresolved background interferences in complex matrices such as black onion.

Table 2
Summary of validation parameters for 7 flavonoids in fresh shallot onion and black onion.

Compounds	Linear Range (ng μL^{-1})	Slope	Intercept	R^{2a}	LOD (ng μL^{-1})^b	LOQ (ng μL^{-1})^b	Intra-day Precision^c		Inter-day Precision^c		Matrix Effects^d
							L1	L2	L1	L2	
Fresh Shallot Onion											
Quercetin	0.012–12.5	25402373	22555261	0.9923	0.004	0.012	1.7	2.5	8.5	5.5	93
Rutin	0.012–12.5	18055622	3113609	0.9975	0.004	0.012	1.2	2.5	1.9	2.2	98
Isorhamnetin	0.012–12.5	62416301	17835161	0.9942	0.004	0.012	1.3	1.6	9.3	5.6	110
Quercetin-3-*O*-glucoside	0.024–12.5	12372182	5040922	0.9909	0.007	0.024	0.7	1.5	7.6	7.1	98
Kaempferol-3-*O*-rutinoside	0.012–6.3	43785782	5867651	0.9927	0.004	0.012	0.8	3.2	5.5	5.8	104
Luteolin	0.012–12.5	53512956	5382539	0.9972	0.004	0.012	3.7	3.8	13.1	11.1	100
Apigenin	0.012–12.5	44384929	7064976	0.9838	0.004	0.012	1.0	1.8	8.5	4.6	106
Black Onion											
Quercetin	0.012–6.3	30312758	7307594	0.9936	0.004	0.012	1.0	0.5	5.7	2.7	111
Rutin	0.012–12.5	16459260	1921621	0.9942	0.004	0.012	1.0	2.0	4.9	6.3	89
Isorhamnetin	0.012–12.5	55821170	13023749	0.9936	0.004	0.012	0.4	0.8	4.8	4.7	98
Quercetin-3-*O*-glucoside	0.012–12.5	10673405	81671	0.9985	0.004	0.012	1.0	0.5	8.1	4.1	85
Kaempferol-3-*O*-rutinoside	0.012–6.3	37877605	2631519	0.9960	0.004	0.012	0.8	1.0	5.5	5.8	90
Luteolin	0.012–12.5	49094166	4207330	0.9956	0.004	0.012	1.5	2.7	9.5	7.7	92
Apigenin	0.012–12.5	44197434	14094745	0.9907	0.004	0.012	0.7	0.8	7.4	5.4	106

[a]R^2: Coefficient of determination. [b]LOD: limit of detection. [b]LOQ: limit of quantification. [c]Intra- and inter-day precision correspond to RSD (%) of the injection of fresh and black onion extracts spiked with standards at a final concentration of L1 (0.78 ng μL^{-1}) and L2 (6.25 ng μL^{-1}). [d]Matrix effect is expressed as percentage.

Table 3
Summary of validation parameters for 21 amino acids and 2 organosulfur compounds in fresh shallot onion and black onion.

Peak	Compounds	Linear Range (ng μL^{-1})	Slope	Intercept	R^{2a}	LOD (ng μL^{-1})[b]	LOQ (ng μL^{-1})[b]	Intra-day Precision[c]		Inter-day Precision[c]		Matrix Effects[d]
								L1	L2	L1	L2	
	Amino acids	*Fresh Shallot Onion*										
11	Phenylalanine	0.10–50.0	1663641	691526	0.9980	0.03	0.10	2.6	2.5	13.9	8.7	105
12	Leucine	0.10–50.0	3245297	1434703	0.9971	0.03	0.10	1.7	2.4	5.6	8.2	83
13	Tryptophan	0.10–50.0	572486	−544643	0.9922	0.03	0.10	5.3	2.3	15.2	13.9	85
14	Isoleucine	0.10–50.0	4537671	1065457	0.9988	0.03	0.10	2.2	0.4	5.8	7.7	88
15	Methionine	0.10–50.0	1867736	−694741	0.9991	0.03	0.10	12.1	3.2	6.5	12.8	86
16	GABA	0.10–50.0	3175583	−953869	0.9993	0.03	0.10	6.9	2.9	11.0	9.2	92
17	Valine	0.10–50.0	1993399	1066633	0.9966	0.03	0.10	4.8	2.2	5.6	8.1	94
18	Proline	0.04–50.0	9080102	−530778	0.9997	0.01	0.04	11.5	3.0	11.4	12.9	106
19	Tyrosine	0.10–50.0	377503	−76068	0.9994	0.03	0.10	3.7	1.7	7.6	12.5	87
20	Alanine	0.10–50.0	1077476	−369036	0.9986	0.03	0.10	3.9	2.5	8.9	7.1	92
21	Threonine	0.10–50.0	820067	−158642	0.9996	0.03	0.10	2.9	0.6	4.3	7.9	93
22	Glycine	0.20–50.0	271977	−109487	0.9984	0.06	0.20	8.0	7.9	7.6	11.9	93
23	Glutamic acid	0.10–50.0	542528	303417	0.9991	0.03	0.10	1.9	2.1	5.5	10.0	87
24	Glutamine	0.10–50.0	496591	24948	0.9976	0.03	0.10	1.8	2.0	5.4	13.3	89
25	Serine	0.10–50.0	378872	99659	0.9981	0.03	0.10	1.9	2.6	5.3	9.9	91
26	Asparagine	0.10–50.0	605553	11650	0.9995	0.03	0.10	1.1	1.8	2.0	9.5	84
27	Aspartic acid	0.10–50.0	200135	−136497	0.9936	0.03	0.10	6.4	13.6	11.9	8.4	90
28	Arginine	0.10–50.0	1787725	326578	0.9995	0.03	0.10	0.7	1.5	6.8	7.2	106
29	Lysine	0.10–12.5	592527	−69029	0.9943	0.03	0.10	2.0	2.3	1.4	9.4	90
30	Ornithine	0.10–12.5	233713	−89535	0.9973	0.03	0.10	1.6	3.9	11.8	10.0	101
31	Histidine	0.10–25.0	536300	−675110	0.9900	0.03	0.10	6.1	0.7	1.8	2.2	88
Organosulfur compounds												
36	Alliin	0.1–75.0	460250	−418104	0.9992	0.03	0.10	5.2	3.5	6.6	5.7	106
	SAC	0.1–75.0	966119	47217	0.9999	0.03	0.10	1.5	4.5	13.4	11.4	102
	Amino acids	*Black Onion*										
11	Phenylalanine	0.10–50.0	1587579	1316871	0.9946	0.03	0.10	2.9	2.5	7.0	8.3	100
12	Leucine	0.10–12.5	3157625	490523	0.9980	0.03	0.10	1.2	2.0	12.1	6.6	81
13	Tryptophan	0.20–50.0	678836	−286383	0.9992	0.06	0.20	13.4	6.5	13.1	13.9	101
14	Isoleucine	0.10–50.0	4389002	1466016	0.9994	0.03	0.10	0.9	1.9	4.1	5.3	85
15	Methionine	0.10–50.0	1734599	−557069	0.9987	0.03	0.10	13.8	10.1	9.0	15.6	80
16	GABA	0.10–50.0	3264382	−1618886	0.9983	0.03	0.10	0.9	2.5	2.9	8.2	95
17	Valine	0.10–25.0	2348189	701542	0.9982	0.03	0.10	2.6	1.6	8.2	7.8	111
18	Proline	0.04–20.0	9510223	100806	0.9995	0.01	0.04	3.2	2.5	4.2	13.5	111
19	Tyrosine	0.10–50.0	348298	−17300	0.9999	0.03	0.10	1.3	1.2	9.0	10.3	80
20	Alanine	0.10–50.0	1205573	−458759	0.9985	0.03	0.10	1.9	1.5	6.7	7.9	103
21	Threonine	0.10–50.0	876703	−244956	0.9989	0.03	0.10	2.3	1.2	4.7	7.3	99
22	Glycine	0.10–50.0	261732	−84802	0.9991	0.03	0.10	3.5	1.1	4.7	6.8	89
23	Glutamic acid	0.10–50.0	544661	73654	0.9994	0.03	0.10	1.7	2.4	1.0	11.2	87
24	Glutamine	0.10–25.0	438271	−63229	0.9996	0.03	0.10	6.0	9.7	10.9	4.2	92
25	Serine	0.10–50.0	415478	36839	0.9993	0.03	0.10	1.7	1.4	3.3	9.3	100
26	Asparagine	0.10–50.0	671316	−46139	0.9994	0.03	0.10	2.8	1.0	6.1	8.6	94
27	Aspartic acid	0.10–50.0	226205	−145560	0.9929	0.03	0.10	3.0	8.5	14.2	5.8	102
28	Arginine	0.10–25.0	1487466	−832105	0.9922	0.03	0.10	2.3	1.0	10.9	8.0	88
29	Lysine	0.10–25.0	516008	−61100	0.9986	0.03	0.10	1.2	1.5	1.9	9.8	85
30	Ornithine	0.20–12.5	235670	−92026	0.9982	0.06	0.20	5.2	5.4	9.2	11.1	102
31	Histidine	0.20–25.0	586845	−1905888	0.9934	0.06	0.20	7.3	2.4	10.8	4.0	97
Organosulfur compounds												
36	Alliin	0.10–10.0	427961	−169143	0.9972	0.03	0.10	3.8	4.6	2.5	2.7	95
	SAC	0.10–10.0	869774	−232084	0.9981	0.03	0.10	2.0	13.8	7.3	6.6	114

[a]R^2: Coefficient of determination. [b]LOD: limit of detection. [b]LOQ: limit of quantification. [c]Intra- and inter-day precision correspond to RSD (%) of the injection of fresh and black onion extracts spiked with standards at a final concentration of L1 (0.1 ng μL^{-1}) and L2 (0.5 ng μL^{-1}). [d]Matrix effect is expressed as percentage. [b] GABA: gamma aminobutyric acid.

3.3. Method validation

3.3.1. Specificity, linearity, limit of detection and limit of quantification

Specificity was assessed as ppm deviation comparing mass error between the predicted *m/z* and observed *m/z* (FDA, 2015). As shown in Table S1, S2 and S3 (Supplementary Information), the ppm derivations obtained were < 5 ppm in all instances and are therefore considered as an acceptable level of mass accuracy. In addition, the retention time of each analyte was compared in blank solvent (methanol or distilled water) and in different matrices (fresh or black onion) previously spiked with standards. The relative standard deviation (RSD) was in all cases below 0.5% for 50 consecutive injections.

Linearity was assessed for 7 flavonoids, 21 amino acids and 2 organosulfur compounds and prepared in methanol/acidified water and in each matrix (fresh and black onion). Results of the linear regression analysis and the coefficient of determination (R^2) of flavonoids and amino acids and organosulfur compounds are shown in Table 2 and Table 3, respectively. Acceptable fitting was estimated by using the coefficient of determination (R^2). For all compounds, R^2 were above 0.983, showing acceptable linear relation between the range of concentration assayed and the detector response. Calibration curves were not force to pass through the origin. The limit of detection and limit of quantification of each compound in each matrix were determined by injecting consecutive dilutions of a working solution in either fresh onion extract or black onion extract until the S/N ratio of each compound showed a signal-to-noise (S/N) ratio ≥ 3 and ≥ 10, respectively. As shown in Table 2, the limits of detection in both onion matrices ranged from 0.004 to 0.007 ng μL^{-1} and the limits of quantification

Table 4
Concentrations (μg g FW $^{-1}$) of individual flavonoids presented in fresh and black onion. Data is expressed as mean values ± SDV (n = 3).

Peak	Compound	Fresh Shallot (μg g FW^{-1})	Black Onion (μg g FW^{-1})
1	Quercetin-7,4′-*O*-diglucoside	42.0 ± 4.0	nd
2	Quercetin-3,4′-*O*-diglucoside	21.0 ± 1.0	nd
3	Rutin	0.8 ± 0.1	nd
4	Isorhamnetin-3,4-diglucoside	1.7 ± 0.2	nd
5	Quercetin-3-*O*-glucoside	2.2 ± 0.3[a]	1.3 ± 0.1[b]
6	Quercetin-4-*O*-glucoside	39.0 ± 1.0[a]	6.4 ± 0.3[b]
7	Isorhamnetin-4′-*O*-glucoside	3.7 ± 0.3	nd
8	Quercetin	87.0 ± 6.0[b]	144.0 ± 2.0[a]
9	Luteolin	1.3 ± 0.2[a]	1.0 ± 0.1[a]
10	Isorhamnetin	1.0 ± 0.2[a]	0.6 ± 0.1[b]
	Total Flavonoids	**199.7 ± 13.1[a]**	**153.3 ± 2.6[b]**

[a] Different letters in a row denote significant difference ($p < 0.05$) between fresh and black onion. One-way ANOVA followed by Tukey test was performed to evaluate significant differences ($p < 0.05$).

range from 0.012 to 0.024 ng μL^{-1} for flavonoids. Regarding amino acids, the limits of detection ranged from 0.01 to 0.06 ng μL^{-1} and the limits of quantification from 0.04 to 0.20 ng μL^{-1} (Table 3), in keeping with previously published data using HILIC coupled to MS analysis in wine, honey and apple juice (Gökmen, Serpen & Mogol, 2012) and in fruit juices (Guo et al., 2013). For organosulfur compounds, the limit of detection and quantification were 0.03 ng μL^{-1} and 0.1 ng μL^{-1}, respectively.

3.3.2. Intra- and inter-day precision

The intra-day precision (repeatability) was checked by measuring two different levels of concentration, one near the LOQ (L1) and other at higher concentration [5 × LOQ (L2)] in diluted fresh and black onion extract and injected five times successively. The relative standard deviation for flavonoids in fresh shallot onion ranged from 0.7 to 3.7% for the level of concentration L1 (0.78 ngμL^{-1}) and from 1.5 to 3.8% for the level of concentration L2 (6.25 ngμL^{-1}) (Table 2), while in black onion matrix, the relative standard deviation (RSD) for flavonoids ranged from 0.4 to 1.5% for L1 (0.78 ngμL^{-1}) and from 0.5 to 2.7% for L2 (6.25 ngμL^{-1}) (Table 2). For amino acids, the RSD at concentration L1 (0.1 ngμL^{-1}) ranged from 0.7 to 12.1% and L2 (0.5 ngμL^{-1}) from 0.4 to 13.6% in fresh shallot onion while in black onion the RSD ranged from 0.9 to 13.8% and 1.0–10.1% at L1 and L2, respectively (Table 3). The RSD for organosulfur compounds ranged from 1.5 to 5.2% and 3.5–4.5% in fresh onion at concentration L1 and L2, respectively; while in black onion the RSD values ranged from 2 to 3.8% and 4.6–13.8% for L1 and L2, respectively.

The inter-day precision was evaluated in five different days using the same procedure described above for the intra-day precision. The values obtained in fresh onion for flavonoids ranged from 1.9 to 13.1% for the level of concentration L1 and from 2.2 to 11.1% for the level of concentration L2 (Table 2), while in black onion matrix, the values ranged from 4.8 to 9.5% for L1 and from 2.7 to 7.7% for L2 (Table 2). For amino acids, the RSD values ranged from 2.0 to 15.2% at L1 and from 2.2 to 13.9% at L2 in fresh shallot onion, while in black onion the RSD values ranged from 1.0 to 14.2% and 2.7–15.6% at L1 and L2, respectively (Table 3). The RSD values for the inter-day precision for organosulfur compounds ranged from 6.6 to 13.4% and 5.7–11.4% in fresh onion at concentration L1 and L2, respectively; while in black onion the RSD values ranged from 4.6 to 13.8% and 2.5–7.3% for L1 and L2, respectively. The results of the repeatability and precision of most metabolites are in line with those proposed by the FDA (FDA, 2015) (RSD < 15%).

3.3.3. Matrix effects

Matrix effects (ME) were evaluated by comparing the slope of calibration curves prepared in fresh and black onion extracts and the standard curves prepared in methanol with 1% FA for flavonoids or acidified water for amino acids and organosulfur compounds, according to the following equation:

$$ME = \frac{\text{Slope of calibration curve prepared in fresh or black onion}}{\text{Slope of calibration curve prepared in solvent}} x\ 100$$

Matrix effect variations are indicative of the susceptibility of the ESI source to matrix composition and, as result, it is possible to observe ion suppression (values of matrix effect less than 100%) or ion enhancement (values of matrix effects higher than 100%).

The matrix effect varied among the different analytes (Tables 2 and 3). With respect to flavonoids, it ranged from 93 to 110% in fresh onion and from 85 to 111% in black onion. Amino acids and organosulfur compounds showed values of matrix effect ranged between 83 and 106% in fresh onion and between 80 and 114% in black ones. These values of matrix effect determined for all compounds either in fresh or black onion matrices were less than 20% and therefore were considered acceptable for the detection and further quantification of these compounds by UHPLC-HRMS (Feliciano, Mecha, Bronze, & Rodriguez-Mateos, 2016; Gasperotti, Masuero, Guella, Mattivi, & Vrhovsek, 2014).

3.4. Identification and quantification of flavonoids, amino acids and organosulfur compounds in fresh and black onion

3.4.1. Flavonoids

A total of 10 flavonoids were identified in black or fresh onions through their mass spectrometric characteristics and compared with data reported in literature. The basis of the identification and the UHPLC-HRMS traces of flavonoids are shown in Table S1 (Supplementary Information), and are detailed as follows:

Peaks **1** and **2** has been identified as quercetin-7,4′-diglucoside and quercetin-3,4′-diglucoside respectively based on their accurate masses at *m/z* 625.1410 and taking into account previous reported data (Fattorusso, Iorizzi, Lanzotti & Taglialatela-Scafati, 2002; Soininen et al., 2014).

Quercetin-3-*O*-glucoside and rutin (peaks **3** and **5**, respectively) were identified by its retention time and MS characteristics in accordance with those of the authentic standards. Additionally, peaks **8**, **9** and **10** were identified as quercetin, luteolin and isorhamnetin based on their similarities of retention time and their MS characteristics with authentic standards.

The MS data also confirmed the presence of peak **4** at m/z 639.1570, consistent with isorhamnetin-diglucoside. The identification of this compound as isorhamnetin-3,4′-*O*-diglucoside is in agreement with a previous work who studied the phenolic compound content of shallot onion (Bonaccorsi et al., 2008).

Further, the MS analyses confirmed the presence of quercetin and isorhamnetin hexosides (peaks **6** and **7**, respectively). These compounds, tentatively identified as quercetin-glucoside and isorhamnetin-glucoside, respectively, have been previously reported in shallot onion (Bonaccorsi et al., 2008; Fattorusso, Iorizzi, Lanzotti, & Tagliatela-Scafati, 2002).

Table 4 summarizes the concentrations of the phenolic compounds in fresh and black onion. Free quercetin (144 μg g^{-1} FW) was the main flavonoid detected in black onion, representing 89.6% of the total flavonoids detected in black onion, with the remaining 10.4% consisting of four minor components such as quercetin-3-*O*-glucoside, quercetin-4-*O*-glucoside, luteolin and isorhamnetin. The major flavonoids in fresh onion were free quercetin (87 μg g^{-1} FW), quercetin-4-*O*-glucoside (39.4 μg g^{-1} FW) and two quercetin-diglucoside isomers

Table 5
Concentrations (μg g FW $^{-1}$) of individual amino acids and organosulfur compounds presented in fresh shallot and black onion. Data is expressed as mean values ± SDV (n = 3).

Peak	Compounds	Fresh Shallot (μg g FW-1)	Black Onion (μg g FW^{-1})
Amino Acids			
11	Phenylalanine	28.4 ± 3.2^{b}	85.0 ± 3.1^{a}
12	Leucine	100.0 ± 11.0^{b}	148.0 ± 5.3^{a}
13	Tryptophan	87.1 ± 8.3^{a}	2.2 ± 0.2^{b}
14	Isoleucine	25.0 ± 2.1^{b}	92.2 ± 10.0^{a}
15	Methionine	7.3 ± 1.1	nd
16	GABA	15.4 ± 1.0^{a}	10.1 ± 1.4^{b}
17	Valine	53.2 ± 6.0^{b}	83.4 ± 6.3^{a}
18	Proline	17.1 ± 2.0^{a}	12.9 ± 1.1^{a}
19	Tyrosine	151.0 ± 16.2^{b}	193.3 ± 13.2^{a}
20	Alanine	28.4 ± 2.4^{b}	179.7 ± 12.2^{a}
21	Threonine	39.0 ± 4.0^{b}	63.7 ± 5.1^{a}
22	Glycine	12.2 ± 2.0^{b}	88.8 ± 3.0^{a}
23	Glutamic acid	313.1 ± 31.0^{a}	106.3 ± 9.0^{b}
24	Glutamine	504.4 ± 45.0^{a}	3.1 ± 0.6^{b}
25	Serine	28.9 ± 1.0^{b}	49.3 ± 3.1^{a}
26	Asparagine	331.0 ± 24.0^{a}	142.0 ± 8.2^{b}
27	Aspartic acid	50.0 ± 2.2^{b}	102.0 ± 10.0^{a}
28	Arginine	1299.1 ± 40.0^{a}	629.9 ± 40.0^{b}
29	Lysine	402.9 ± 31.0^{a}	128.4 ± 8.2^{b}
30	Ornithine	36.0 ± 3.3^{a}	26.1 ± 1.1^{b}
31	Histidine	75.0 ± 3.1^{a}	24.0 ± 1.1^{b}
	Total Amino Acids	**3604.5 ± 239.9^{a}**	**2170.4 ± 142.2^{b}**
Organosulfur compounds			
32	*S*-(*S*-propyl)cysteine	6.6 ± 2.2^{a}	5.5 ± 0.3^{a}
33	*S*-(*S*-1-propenyl)cysteine	4.1 ± 0.2^{a}	2.4 ± 0.1^{b}
34	*S*-propyl-cysteine sulfoxide (Propiin)	9.6 ± 0.6^{b}	12.2 ± 0.9^{a}
35	*S*-(2-carboxypropyl)cysteine	4.2 ± 0.2^{a}	2.9 ± 0.2^{b}
36	*S*-(2-propenyl)cysteine sulfoxide (Alliin)	184.0 ± 20.1	nd
37	γ –Glutamyl-*S*-(*S*-propyl)cysteine-glycine	9.6 ± 0.6	nd
38	γ –Glutamyl-*S*-(*S*-1-propenyl)cysteine	4.1 ± 0.1^{b}	6.2 ± 0.6^{a}
39	γ –Glutamyl-*S*-(*S*-1-propenyl)cysteine-glycine	6.6 ± 0.5^{a}	2.5 ± 0.1^{b}
40	γ –Glutamyl-*S*-(propyl)cysteine	8.8 ± 0.8	nd
41	γ –Glutamyl-*S*-(1-propenyl)cysteine	311.1 ± 24.0	nd
42	*S*-(2-carboxypropyl)cysteine-glycine	102.3 ± 11.1^{a}	3.2 ± 1.3^{b}
43	(*S*-(*E*)-(1-propenyl)cysteine sulfoxide (Isoalliin)	131.0 ± 20.0^{b}	1584.0 ± 66.4^{a}
44	γ –Glutamyl-*S*-(*S*-methyl)cysteine-glycine	4.0 ± 0.2^{a}	3.1 ± 0.2^{b}
45	*S*-methyl-cysteine sulfoxide (Methiin)	29.2 ± 4.4^{a}	5.6 ± 0.4^{b}
46	*S*-methylcysteine (Deoxymethiin)	7.6 ± 0.6	nd
47	γ –Glutamyl-*S*-(2-carboxypropyl)cysteine-glycine	221.1 ± 21.3	nd
48	γ –Glutamyl-*S*-(2-carboxypropyl)cysteine glycine hexoside	3.6 ± 0.1	nd
49	γ –Glutamyl-*S*-propylcysteine sulfoxide	34.0 ± 5.0	nd
50	γ -Glutamyl-*S*-(1-propenyl)cysteine sulfoxide or γ -Glutamyl-*S*-(2-propenyl)cysteine sulfoxide	1387.2 ± 115.2^{a}	120.1 ± 12.3^{b}
51	3-Methyl-1,4-thiazane-5-carboxylic acid sulfoxide (Cycloalliin)	43.1 ± 4.0^{a}	8.5 ± 1.1^{b}
52	γ -Glutamyl-*S*-methylcysteine sulfoxide	14.4 ± 0.8^{a}	7.9 ± 0.3^{b}
53	γ -Glutamyl-*S*-methylcysteine	17.0 ± 0.8^{a}	8.1 ± 0.6^{b}
	Total Organosulfur Compounds	**2543.2 ± 232.8^{a}**	**1772.2 ± 84.8^{b}**

[a] Different letters in a row denote significant difference between fresh and black onion. One-way ANOVA followed by Tukey test was performed to evaluate significant differences ($p < 0.05$).

(63 μg g^{-1} FW) which comprised 94.6% of the total flavonoids. Conversely, rutin, isorhamnetin-3,4′-diglucoside, quercetin-3-*O*-glucoside, isorhamnetin-4′-*O*-glucoside, luteolin and isorhamnetin were quantified as minor components. The values of free quercetin in fresh onion, although slightly lower, are in keeping with earlier studies who reported concentrations of quercetin in commercial onions ranging from 185 to 634 μg g^{-1} FW (Crozier, Lean, McDonald, & Black, 1997) and 284–486 μg g^{-1} FW (Hertog, Hollman, & Katan, 1992). This variation is due to differences in cultivars, maturity stages, origin places, harvest seasons or environmental conditions. Indeed, Fattorusso et al., 2002 reported the presence of high amounts of free quercetin and isorhamnetin and their glycosides: quercetin-4-glucoside, quercetin-diglucoside in shallot onion.

In general, the flavonoid content varied significantly among black (153.3 μg g^{-1} FW) and fresh shallot onion (199.7 μg g^{-1} FW), indicating potential flavonoid losses during the black onion manufactured processes. Special attention should be given to individual compounds such as free quercetin which is found in significantly higher quantity in black onion (144 μg g^{-1} FW) compared with fresh onion (87 μg g^{-1} FW), probably from the thermal degradation of quercetin-diglucosides present in fresh onions and which are not detected in black onion.

3.4.2. Free amino acids and organosulfur compounds

A total of 21 free amino acids and 22 organosulfur compounds, including S-substituted cysteine derivatives, were identified and quantified in fresh and black onions (Table 5). The UHPLC-HRMS characteristics of each free amino acid and organosulfur compound are shown in Tables S2 and S3 (Supplementary Information) together with their UHPLC-HRMS traces (Figs. S2 and S3, Supplementary Information).

The basis of the identification of amino acids was achieved by co-

chromatography with reference compounds and their fragmentation profiles upon low collision energy and by reference to properties reported in previous related publications. Peaks **11** to **31** (Table S2, Supplementary Information) corresponded to all the amino acids and were identified compared with authentic standards. Further, peaks **32** to **53** (Table S3, Supplementary Information), which corresponded to the organosulfur compounds, were identified in fresh and black onion extracts on the basis of the data from previous related publications (Arnault et al., 2003; Böttcher et al., 2017; Kubec & Dadakova, 2009; Nakabayashi et al., 2013).

Fresh onion showed significant higher concentrations of amino acids and organosulfur compounds (3.60 mg g^{-1} FW and 2.54 mg g^{-1} FW, respectively) compared with that in black onion (2.17 mg g^{-1} FW and 1.77 mg g^{-1} FW, respectively). Among them, arginine, glutamine, glutamic acid, lysine, tyrosine, asparagine and leucine together with γ-glutamil-*S*-(propenyl)cysteine sulfoxide and γ-glutamil-*S*-(1-propenyl)-cysteine were the predominant amino acids and organosulfur compounds in fresh onion, in keeping with previous published data (Kubec et al., 2009), while arginine, leucine, isoleucine, tyrosine, alanine and asparagine along with isoalliin are dominating in black onions. The abundance occurrence of isoalliin in black onion could be due to the enzymatic activity of the cysteine sulphoxidelyase activated during the heating process of fresh onions (Starkenmann, Niclass, & Troccaz, 2011) and it could be a precursors of a wide range of sensory-active and health-beneficial compounds of black onion.

4. Conclusions

Two selective, sensitive, and precise UHPLC-HRMS methods were successfully optimized and validated to identify and quantify phenolic compounds, amino acids and organosulfur compounds in black and fresh onions, allowing the determination of 53 primary and secondary metabolites in both types. These methodologies are successful to analyse individual flavonoids, amino acids as well as organosulfur compounds in onion matrices showing good separation between compounds and highest limits of detection and quantification for the tested reference standards, without time consuming pre-treatment techniques involving complex extraction methods, clean-up steps and derivatization processes which leads to derivative instability, side reaction and reagent interferences prior to the analysis. Moreover, these results give a detailed profile of potential bioactive metabolites in black onion, a novel derived product from fresh onion, highlighting the large difference on the chemical composition between fresh and black onion due to the influence of the heating process involve in the production of black onion.

Abbreviations used

UHPLC-HRMS: ultra-high-performance liquid chromatography coupled to high resolution mass spectrometry, FA: formic acid.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.lwt.2018.07.032.

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SUPPLEMENTARY INFORMATION

Development and Validation of UPHLC-HRMS Methodology for the Determination of Flavonoids, Amino Acids and Organosulfur Compounds in Black Onion, a Novel Derived Product from Fresh Shallot Onions (*Allium* cepa var. *aggregatum*)

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Table S1. HPLC-HRMS-Based Identifications of Flavonoids in Fresh and Black Onions.

Peak	RT	Compound	Chemical Formula [*m/z*]-	Experimental mass [*m/z*]-	δ (ppm)	MSI MI level[a]
1	14.9	Quercetin diglucoside I	C27H29O17	625.141	-0.68	2
2	15.2	Quercetin diglucoside II	C27H29O17	625.141	-0.66	2
3	15.3	Rutin	C27H30O16	609.146	0.42	1
4	15.6	Isorhamnetin-3,4´-diglucoside	C28H31O17	639.157	-0.78	2
5	16.8	Quercetin-3-*O*-glucoside	C21H19O12	463.088	-0.39	1
6	18.1	Quercetin-4-*O*-glucoside	C21H19O12	463.088	1.08	2
7	18.7	Isorhamneti-4´-*O*-glucoside	C22H21O12	477.104	0.85	2
8	21.2	Quercetin	C15H10O7	301.036	0.75	1
9	23.4	Luteolin	C15H10O6	285.040	0.58	1
10	23.9	Isorhamnetin	C16H12O7	315.051	-0.99	1

[a]Metabolite standards initiative (MSI) metabolite identification (MI) levels (Summer et al., 2007). Reference compounds were available for all compounds identified at MSI MI level 1

Table S2. HPLC-HRMS-Based Identifications of Amino Acids in Fresh and Black Onions.

Peak	RT	Compound	Chemical Formula $[M-H]^-$	Experimental Mass	δ (ppm)	MSI MI level[a]
11	6.18	Phenylalanine	C9H11NO2	166.0862	-2.24	1
12	6.20	Leucine	C6H13NO2	132.1029	0.06	1
13	6.29	Tryptophan	C11H12N2O2	205.0971	-1.86	1
14	6.41	Isoleucine	C6H13NO2	132.0102	0.13	1
15	6.75	Methionine	C5H11NO2S	150.0583	-0.87	1
16	6.81	GABA (gamma aminobutyric acid)	C4H9NO2	104.0706	1.61	1
17	7.07	Valine	C5H11NO2	118.0862	0.10	1
18	7.10	Proline	C5H9NO2	116.0706	-0.11	1
19	7.27	Tyrosine	C9H11NO3	182.0811	-2.14	1
20	8.20	Alanine	C3H7NO2	90.0549	3.97	1
21	8.54	Threonine	C4H9NO3	120.0655	0.26	1
22	8.74	Glycine	C2H5NO2	76.0396	6.04	1
23	8.98	Glutamic acid	C5H9NO4	148.0604	-1.67	1
24	9.21	Glutamine	C5H10N2O3	147.0764	-2.12	1
25	9.27	Serine	C3H7NO3	106.0498	1.73	1
26	9.41	Asparagine	C4H8N2O3	133.0607	0.43	1
27	9.76	Aspartic acid	C4H7NO4	134.0447	-0.33	1
28	11.38	Arginine	C6H14N4O2	175.1189	-2.93	1
29	11.81	Lysine	C6H14N2O2	147.1128	-1.77	1
30	11.92	Ornithine	C5H12N2O2	133.0972	-0.89	1
31	11.93	Histidine	C6H9N3O2	156.0767	-1.93	1

[a]Metabolite standards initiative (MSI) metabolite identification (MI) levels (Summer et al., 2007). Reference compounds were available for all compounds identified at MSI MI level 1

Table S3. UHPLC-HRMS-Based Identifications of Organosulfur Compounds in Fresh Shallot and Black Onion.

Peak	RT	Compound	Formula [M-H]$^-$	Experimental Mass	δ (ppm)	MSI MI level[a]
32	5.45	*S*-(*S*-propyl)cysteine	C6H13NO2S2	196.04605	0.62	2
33	5.48	*S*-(*S*-1-propenyl)cysteine	C6H11NO2S2	194.03040	-0.70	2
34	7.59	*S*-propyl-cysteine sulfoxide (Propiin)	C6H13NO3S	180.06889	2.46	2
35	7.59	*S*-(2-carboxypropyl)cysteine	C7H13NO4S	208.06381	1.32	2
36	7.68	*S*-(2-propenyl)cysteine sulfoxide (Alliin)	C6H11NO3S	178.05324	2.63	1
37	7.68	γ –Glutamyl-*S*-(*S*-propyl)cysteine-glycine	C13H23N3O6S2	382.11010	0.76	2
38	7.71	γ –Glutamyl-*S*-(*S*-1-propenyl)cysteine	C11H18N2O5S2	323.07299	-0.25	2
39	7.76	γ –Glutamyl-*S*-(*S*-1-propenyl)cysteine-glycine	C13H21N3O6S2	380.09445	0.35	2
40	8.11	γ –Glutamyl-*S*-(propyl)cysteine	C11H20N2O5S	293.11657	-0.11	2
41	8.14	γ –Glutamyl-*S*-(1-propenyl)cysteine	C11H18N2O5S	291.10092	0.01	2
42	8.37	*S*-(2-carboxypropyl)cysteine-glycine	C9H16N2O5S	265.08527	-0.01	2
43	8.40	(*S*-(*E*)-(1-propenyl)cysteine sulfoxide (Isoalliin)	C6H11NO3S	178.05324	2.57	2
44	8.58	γ –Glutamyl-*S*-(*S*-methyl)cysteine-glycine	C11H19N3O6S2	354.07880	0.17	2
45	9.04	*S*-methyl-cysteine sulfoxide (Methiin)	C4H9NO3S	152.03759	4.55	2
46	9.29	*S*-methylcysteine (Deoxymethiin)	C4H9NO2S	136.04268	0.11	2
47	9.30	γ –Glutamyl-*S*-(2-carboxypropyl)cysteine-glycine	C14H23N3O8S	394.12786	0.37	2
48	10.20	γ –Glutamyl-*S*-(2-carboxypropyl)cysteine glycine hexoside	C20H33N3O13S	556.18069	-0.29	2
49	10.46	γ –Glutamyl-*S*-propylcysteine sulfoxide	C11H20N2O6S	309.11148	0.36	2
50	10.58	γ -Glutamyl-*S*-(1-propenyl)cysteine sulfoxide or γ -Glutamyl-*S*-(2-propenyl)cysteine sulfoxide	C11H18N2O6S	307.09583	-0.12	2
51	10.58	3-Methyl-1,4-thiazane-5-carboxylic acid sulfoxide (Cycloalliin)	C6H11NO3S	178.05324	3.12	2
52	11.65	γ -Glutamyl-*S*-methylcysteine sulfoxide	C9H16N2O6S	281.08018	0.56	2
53	11.65	γ -Glutamyl-*S*-methylcysteine	C9H16N2O6S	281.08018	0.56	2

[a]Metabolite standards initiative (MSI) metabolite identification (MI) levels (Summer et al., 2007). Reference compounds were available for all compounds identified at MSI MI level 1.

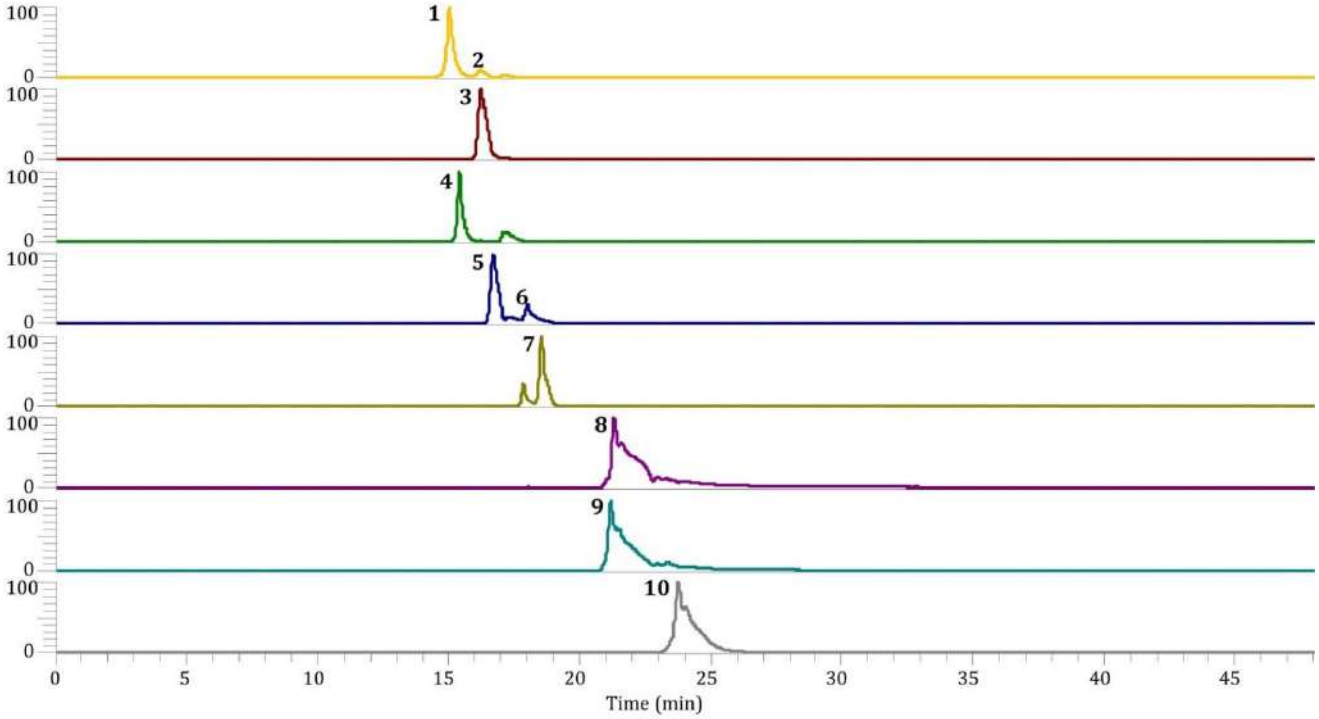

Figure S1. HPLC-HRMS chromatograms of the metabolites 1 to 10. For peak identification see Table S1.

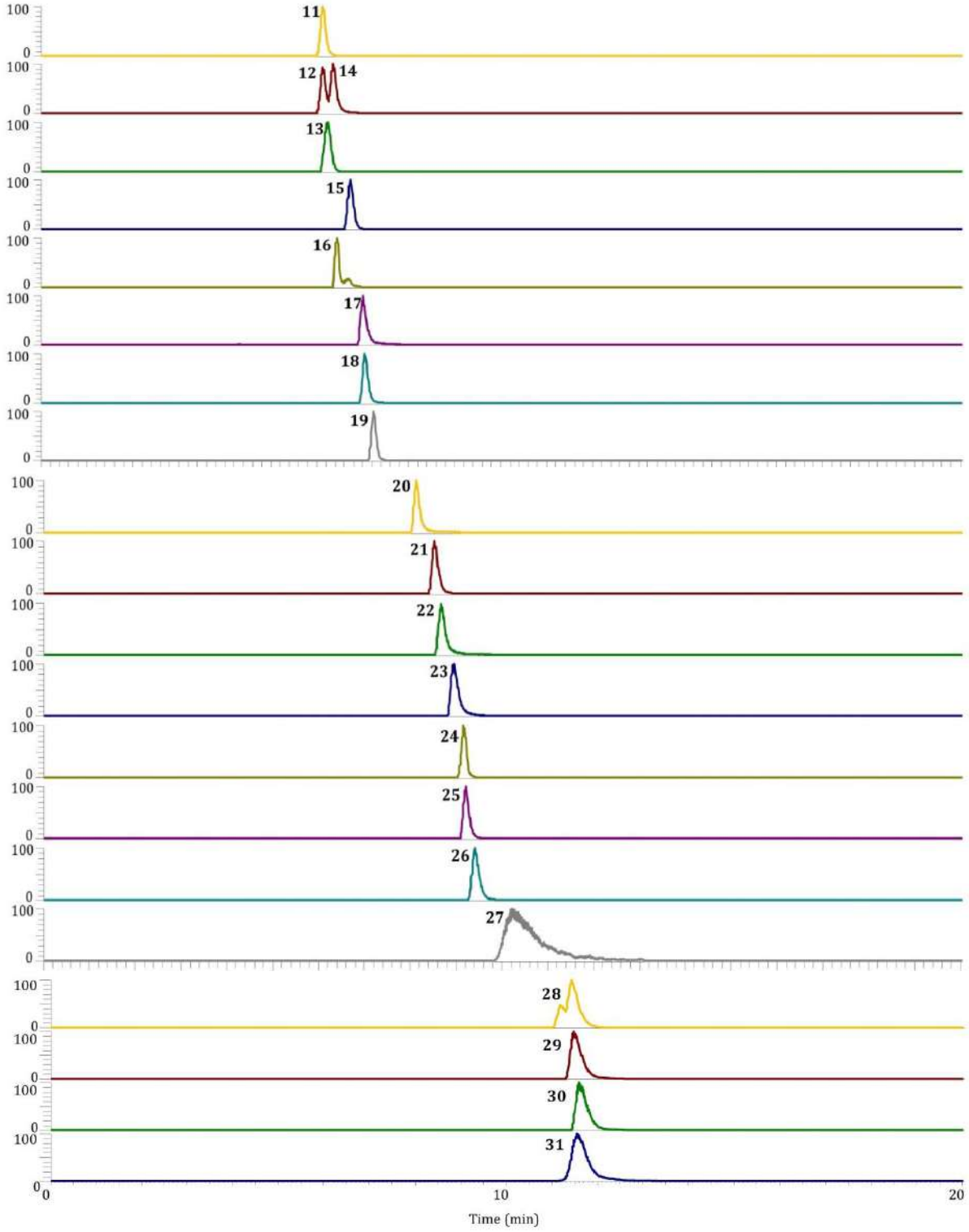

Figure S2. HPLC-HRMS chromatograms of the metabolites 11 to 31. For peak identification see Table S2.

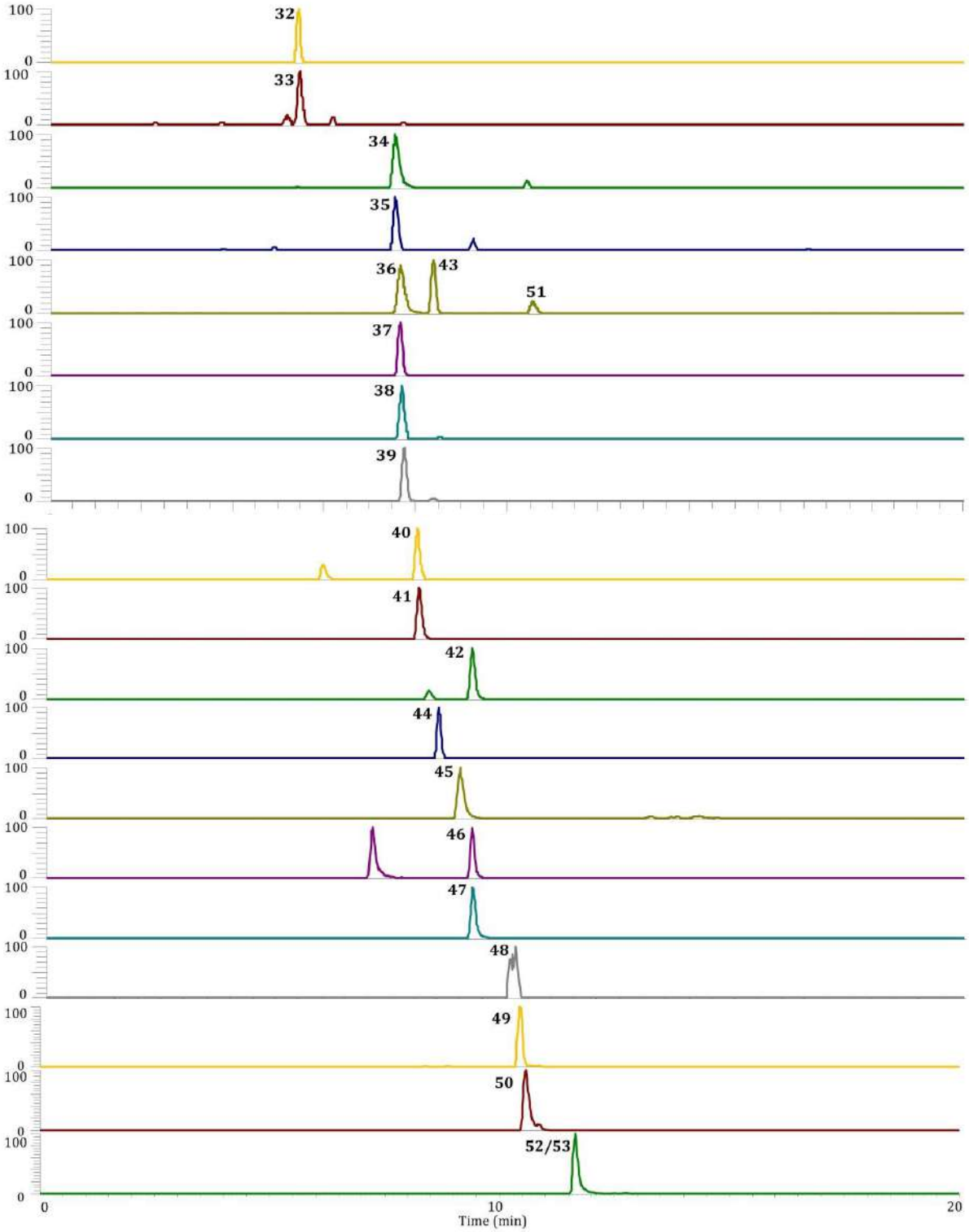

Figure S3. HPLC-HRMS chromatograms of the metabolites 32 to 53. For peak identification see Table S3.

Artículo 2

Changes on the Antioxidant Activity and Metabolite Profile of Three Onion Varieties during the Elaboration of 'Black Onion'

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Changes in the antioxidant activity and metabolite profile of three onion varieties during the elaboration of 'black onion'

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ABSTRACT

This study aims to investigate whether the heat treatment applied during the production of black onion, a novel derived product made from fresh onion, produces changes in the content of flavonoids, organosulfur compounds, organic acids, water soluble sugars and amino acids in three onion varieties ('Shallot', 'Chata' and 'Echalion'). The total flavonoid content decreased up to 12-fold in black onions compared with fresh onions while the quantities of isoalliin, the main organosulfur compound in black onions, drastically increased during the process. Moreover, the levels of fructose and glucose significantly increased during the elaboration process, contributing to the sweetness of black onions. The influence of heating on their antioxidant capacity showed a decreasing trend of the ORAC antioxidant activity of onion, while ABTS and DPPH did not show a clear tendency. These results present a comprehensive phytochemical characterization of black onions, highlighting the significant influence of the heating process on their phytochemical composition.

1. Introduction

The *Allium* genus belongs to the Liliaceae family, which includes garlic (*Allium sativum* L.), onion (*Allium cepa* L.), leeks (*Allium porrum* L.), chives (*Allium fistolosum* L.) and Shallots (*Allium ascalonicum* L.). All of these vegetables have been used historically for medicinal purposes for over 4000 years to treat a large number of conditions. Epidemiological evidence suggests that diets rich in *Allium* vegetables such as onion and garlic may decrease the risk of cancers (Nicastro, Ross, & Milner, 2015; Pourzand et al., 2016) and cardiovascular diseases (Bahadoran, Mirmiran, Momenan, & Azizi, 2017), in addition to protecting against the development of metabolic diseases such as diabetes (Akash, Rehman, & Chen, 2014). Moreover, there is substantial *in vitro* evidence showing that bioactive compounds present in onion or garlic have anti-inflammatory, anti-diabetic and anti-atherogenic properties (Santhosha, Jamuna, & Prabhavathi, 2013).

Onion in particular is a rich source of bioactive constituents, including flavonoids and organosulfur compounds, whose main components are thiosulfinates (aka alkane(ene) thial-S-oxide) and sulfur volatiles and which most of the biological health-promoting properties are attributed to. These compounds are formed upon damage or crushing of onions. After that, the enzyme alliinase is release from the vacuoles of cells and catalysed the cleavage of S-alk(en)yl-L-cysteine derivatives to sulfenic acid intermediates. These intermediates are highly reactive and rapidly produce thiosulfinate compounds via condensation reactions. The major onion thiosulfinate is isoalliin together with other precursor compounds such as S-methylcysteine sulfoxide (methiin) being precursors of a wide range of sensory-active and health-promoting compounds (Tapiero, Townsend, & Tew, 2004) including anti-inflammatory (Arreola et al., 2015), antimicrobial (Barba, Esteve, & Frígola, 2014) and anti-obesity (Souza et al., 2011; Yoshinary et al., 2012) effects. The subsequence condensation of sulfenic acid intermediates results into the formation of lachrymatory factor (thiopropanal S-oxide), thiosulfonates, bissulfines, sulphines including diallyl sulphide, diallyl disulphide and diallyl trisulfide, zwejebelanes, and cepaenes, all of which contribute to the flavour of onion (Nicastro et al.,

Abbreviations: UHPLC-HRMS, ultra-high-performance liquid chromatography coupled to high resolution mass spectrometry; FA, formic acid; RH, Relative Humidity; DW, Dry Weight; GABA, Gamma-AminoButyric Acid

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2015) and have anti-cancer activity (Seki et al., 2008; Yang et al., 2006). Among flavonoids, high levels of quercetin and isorhamnetin glycosides have been found in onion (Lanzotti, 2006; Liguori et al., 2017).

Onions are commonly consumed in prepared foods either fresh or after being subjected to a wide variety of cooking methods (Juániz et al., 2016). Among them, boiling, frying and griddling have been shown to induce significant changes in onion composition, bioactive compounds either being gained or lost, depending on the process (Kim, Lee, Shin, & Yoo, 2016). Moreover, one of the main advantages of cooking is to make onions more acceptable for consumers, eliminating their pungency and increasing their palatability and sweetness. In this sense, a novel commercially available product derived from onion, known as "black onion", has been developed by processing (aging) raw onion in a temperature- and humidity-controlled room without using any artificial additives. This aging process is a spontaneous fermentation for 30–60 days at 60–80 °C and high humidity (90% RH) (Chung, Kwon, Chung, & Chun, 2011). During the manufacturing process, a series of modifications to the compositional and sensory characteristics of the fresh product are produced. It has been shown that black onion differs significantly in terms of odour and aromas from the fresh onion, avoiding the pungency and burning characteristics of fresh onion mainly due to the decrease of different chemical families like sulfur and aromatic compounds, sulfur organic compounds and nitrogen oxides as stated by Wang et al. (Wang, Liu, Ma, Wang, & Zhao, 2015), with new aromas (like toffee and liquiorice) that are more palatable to many consumers. The final product has a more pleasing mouthfeel with fruit-like sweetness and improved organoleptic properties with less or non-stimulating smell compared with fresh onion (Ríos-Ríos, Montilla, Olano, & Villamiel, 2019). Black onion is considered a gourmet product that is consumed either directly as an aperitif or used in the haute cuisine for the preparation of sauces and seasoning, especially meats, salads and desserts. Moreover, due to the treatment, black onion increases its shelf-life avoiding spoilage during storage, a common issue in fresh vegetables. Likewise, previous studies have shown that black garlic, an analogous aged food product, presents higher nutritional characteristics than the fresh product (Jung et al., 2014). However, up to date there is no information about the nutritional and phytochemical composition of the aged black onion, available in the markets for the consumers. To the best of our knowledge there is only one study dealing with discrimination between onion samples at different processing times based on the content of volatile sulfur compounds (Wang et al., 2015).

The aim of this study was to determine the impact of the heat treatment on antioxidant activity and on flavonoids, organosulfur compounds, amino acids, organic acids and sugars profiles during the production of black onion from three different varieties of raw onion: 'Shallot', 'Chata' and 'Echalion'.

2. Material and methods

2.1. Chemicals

The reference flavonoid compounds rutin, luteolin, (−)-epicatechin, quercetin-3-*O*-glucoside, apigenin, quercetin, kaempferol-3-*O*-rutinoside, isorhamnetin, and the amino acids glycine, glutamic acid, leucine, isoleucine, proline, methionine, alanine, tyrosine, asparagine, lysine, histidine, ornithine, glutamine, serine, threonine, phenylalanine, tyrosine, aspartic acid, gamma-aminobutyric acid (GABA), arginine, valine and tryptophan were purchased from Sigma-Aldrich (Madrid, Spain). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), pyruvic, citric, quinic, succinic, fumaric, salicylic, oxalic, shikimic, malic, tartaric and ascorbic acids were obtained from Sigma-Aldrich (Madrid, Spain). Alliin, s-allyl-L-cysteine (SAC) together with sucrose, maltose, glucose and fructose, and formic acid were acquired from Sigma-Aldrich (Madrid, Spain). Ammonium formate, ammonium acetate and ethanol were obtained from Sigma-Aldrich. Acetronitrile and methanol were of LC-MS grade. Potassium phosphate monobasic was purchased from Panreac Química (Barcelona, Spain).

2.2. Materials and sample preparation

Ten kg of authenticated 'Shallot', 'Chata' and 'Echalion' onion varieties were obtained from a local supplier. The manufacturing process of black onion was carried out at IFAPA's facilities (Palma del Río, Córdoba, Spain). These three onion varieties were selected based on the surface-volume ratio of the onion bulbs and their similar relative sizes, which makes the elaboration process of black onion more efficient. The heat equipment consisted of a food warming and heated holding cabinet (Edesa Industrial S.L., Mondragón, Spain) and the dryer equipment consisted of an ECO EVO drier (Tred Technology S.R.L., Ripalimosani, Italy). The manufacturing process involved the following stages: i) the onions were checked for defects such as injuries, scars or rots. Once the onions were deemed suitable, preparatory conditioning was carried out consisting of cleaning the outer layers and cutting the basal plate of the onion. ii) Heating process: in this phase, the bulbs were subjected to relative humidity conditions close to saturation (90% RH) and at a temperature between 65 and 70 °C in the presence of oxygen (20.9%) for 28 days. To study the evolution during the heating process, 0.5 kg of onion were taken at T0 (initial stage) and every 7 days until day 28: T1 (after 7 days), T2 (after 14 days), T3 (after 21 days) and T4 (after 28 days). A final phase involved applying a drying process to the T3 onion samples. The drying was performed with dehumidified hot air. To this end, the drier equipment first cooled the air to condense its humidity (final relative humidity of ~15%) and then the air was heated to 50 °C. This hot air was sent to the onion samples at high speed thought a turbine. The onion bulbs are dried after 24 h under these conditions. The samples from this stage are named T4S samples. Both the heating and the drying process of the three onion varieties were run in triplicate. All the onion samples were grinded to a final particle size of 10 μm using a cryogenic grinder with liquid nitrogen mill equipment (Freezer Mill model 6870, Fisher Scientific, Waltham, MA USA) and stored at −80 °C until analysis.

2.3. Analysis of flavonoids, amino acids and organosulfur compounds

Onion samples were extracted following the previously optimized procedure reported (Moreno-Rojas et al., 2018). Changes in (poly) phenols, amino acids and organosulfur compounds during the heating process were monitored using an UHPLC-PDA-MS system (Thermo Scientific, San José, CA, USA). The analysis of flavonoids, amino acids and organosulfur compound was carried out following a previously validated methodology (Moreno-Rojas et al., 2018). Figs. S1–S5 and S8, in Supported Information showed typical UHPLC-HRMS chromatograms of flavonoids, amino acids and organosulfur compounds in onion samples.

2.4. Extraction of organic acids

The extraction method followed the one previously described by Cuevas et al. (2015) with some modifications. 0.5 g of onion sample was mixed with 1 mL of deionized water with 3% phosphoric acid for 2 min at room temperature. The mixture was sonicated for 10 min and then centrifuged at 15,000 rpm for 15 min. The supernatant was collected and residues were re-extracted twice using 1 mL of the same solvent following the same protocol described previously. All the supernatants were pooled together and frozen at −80 °C until UHPLC-DAD analysis.

2.5. Analysis of organic acids

Organic acids were analysed by using an UHPLC-DAD (Thermo

Scientific, San José, CA, USA) consisting of a HPLC pump, a DAD detector and an autosampler operating at 4 °C. The separation of organic acids was performed on a 250 × 4.6 mm i.d. Synergi 4 μm Hydro-RP column (Phenomenex) and maintained at 22 °C. The mobile phase, 20 mM potassium phosphate, was pumped for 20 min at a flow rate of 0.7 mL/min using an isocratic method. The organic acids were identified by comparing the retention time and the maximum wavelength with pure organic acid standards at 254 nm for ascorbic acid and 220 nm for the rest of organic acids. Quantification was performed by reference to 0.1–100.0 mg/L calibration curves of their respective standards. The citric and fumaric acids co-eluted under this chromatographic condition and were quantified together. Fig. S6 in Supported Information showed a typical UHPLC-DAD chromatogram of organic acids in raw and black onion samples.

2.6. Extraction of simple sugars

Onion samples were extracted following the method previously described by Shanmugavelan et al. (2013) adapted to our samples: 0.5 g of sample was mixed with 1 mL of deionized water:ethanol (20:80, v/v) for 2 min at room temperature and the mixture was sonicated for 15 min and then centrifuged at 15,000 rpm for 15 min. The supernatant was collected and the residues were re-extracted twice using 1 mL of the same solvent following the same protocol previously described. All the supernatants were pooled together and frozen at −80 °C until HPLC-RID analysis.

2.7. Analysis of simple sugars

The identification and quantification of glucose, fructose and sucrose in onion samples were carried out using an HPLC-RID system (Perkin Elmer, Waltham, MA, USA) consisting of an HPLC pump, a RID detector, and an autosampler operating at 4 °C. The separation of the simple sugars was performed on a 250 × 4.6 mm i.d. Luna 5 μm NH_2 column (Phenomenex) and maintained at 40 °C. The mobile phases, A: deionized water and B: acetonitrile, (20% phase A–80% phase B) were pumped at a flow rate of 1.5 mL/min using an isocratic method for 15 min. The sugars were identified by comparing the retention times with pure reference standards. Quantification was performed by reference to the 0.3–50.0 mg/mL calibration curves of fructose and 0.3–10.0 mg/mL of glucose and sucrose. Fig. S7 in Supported Information showed a typical HPLC-RID chromatogram of simple sugars in onion samples.

2.8. Antioxidant activity by ABTS assay

The free radical scavenging activity was measured using the ABTS decolouration method (Re et al., 1999) with some modifications (Madrona, Pereira-Caro, Bravo, Mateos, & Espartero, 2011). The antioxidant activity was expressed as μM of Trolox equivalents per gram of dry sample (μM TE/g DW). Each value is the average of three determinations.

2.9. Antioxidant activity by DPPH assay

Free radical DPPH (1,1-diphenyl-2-picryl-hydrazyl) scavenging capacity was determined by using the previously described methods Sánchez-Moreno, Larrauri, and Saura-Calixto (1998). The antioxidant activity was expressed as μM of Trolox equivalents per gram of dry sample (μM TE/g DW). Each value is the average of three measurements.

2.10. Antioxidant activity by ORAC assay

The oxygen radical scavenging activity was measured by the ORAC assay according to the method previously published by Huang, Ou, Hampsch-Woodill, Flanagan, and Prior (2002) and modified by Madrona et al. (2011). The final results were calculated according to Madrona et al. (2011). ORAC values are expressed as mM Trolox equivalents per gram of dry sample (mM TE/g DW).

2.11. Statistical analysis

Statistical analyses were performed on the basis of three analytical replicates measured on each sample. A one-way ANOVA was carried out to assess for significant differences (significant model was accepted for a p-value < 0.05) using the SPSS Statistic Program (v. 22). Next, Fisher's LSD pairwise comparison was performed on the data. A two-way ANOVA was used to compare the mean differences in the antioxidant activity during the heating process and to understand if there is an interaction between the heating process and the onion varieties using R software (v.3.5.0). Principal Component Analysis (PCA) was carried out as an unsupervised method using SIMCA software (v.15.0.2) to evaluate whether the changes on the profiles of flavonoids, organosulfur compounds, amino acids, organic acids and sugars were different enough to distinguish among process stages and onion varieties.

3. Results and discussion

3.1. Changes in flavonoids Profile.

A total of seven flavonoids were identified and quantified in the onion samples. Quercetin and quercetin derivatives including quercetin-3-*O*-glucuronide and two isomers of quercetin-diglucuronide were the main flavonoids in raw onion varieties (T0), accounting for 95.7, 97.2 and 97.6% of the total flavonoids in 'Shallot', 'Chata' and 'Echalion' varieties, respectively (Table 1). These results are in line with those reported by Juániz et al. (2016), who demonstrated that 90% of total flavonoids in raw onion were quercetin and quercetin derivatives. Other minor compounds such as isorhamnetin, luteolin and isorhamnetin glucoside were detected (Table 1). It is noteworthy that the 'Shallot', 'Chata' and 'Echalion' onion varieties showed similar concentrations of flavonoids. During the production of black onion (T4), the initial flavonoid content of the three onion varieties decreased up to 6-fold, 12-fold and 6-fold for 'Shallot', 'Chata' and 'Echalion' varieties, respectively. In general, the biggest differences were observed when comparing samples from T0 and T1, and further T1 and T2 intervals (Table 1). Of note is that isorhamnetin content increased in the T1 stage (7 days), arguably due to the hydrolysis of isorhamnetin glucosides during the heat treatment, and then decreased again in T2 (14 days) in all varieties. In stage T4S, quercetin was the major flavonoid present in the black onion samples, accounting for 93.7, 98.8 and 99.2% of the total flavonoid content in the 'Shallot', 'Chata' and 'Echalion' onion varieties, respectively. As Table 1 shows, 'Echalion' black onion showed a higher concentration of flavonoids with 41.10 mg/100 g DW, followed by the 'Shallot' variety with 36.80 mg/100 g DW. 'Chata' black onion had the lowest concentration (19.71 mg/100 g DW).

The significant decrease in flavonoid content during the production of black onion can be explained considering the heat conditions and incubation time. Cooking or heat treatments of onion samples has been shown to produce a significant increase in the concentrations of flavonoids in the final product since these treatments were performed for short periods of time. For instance, Sharma et al. (2015) evaluated the effect of different heating treatments on the flavonoid content of six onion varieties. They observed a positive effect of heat treatment on the total flavonoid content in five onion varieties when the maximum temperature was 120 °C. When heat treatment reached 150 °C, the total flavonoid content decreased drastically. Although the temperature used during black onion production was less aggressive than in other studies, the heat treatment was prolonged over time, thus affecting the final content of flavonoids of the black onion. Moreover, the losses in the phenolic content during the production of black onion are argue

Table 1
Concentration (mg/100 g DW) of flavonoids presented in onion samples at different stages during the production of black onion for three varieties (Shallot, Chata and Echalion). Data is expressed as mean values (n = 6).

Compounds	T0	T1	T2	T3	T4	T4S
	Shallot' variety					
Quercetin	91.08^{a}	61.01^{b}	29.17^{d}	34.41^{d}	42.64^{c}	34.46^{d}
Isorhamnetin	3.31^{c}	9.51^{a}	4.17^{b}	2.53^{d}	1.61^{e}	1.81^{de}
Quercetin-3-O-glucoside	19.29^{a}	1.84^{b}	1.81^{b}	0.80^{c}	0.66^{c}	0.52^{c}
Luteolin	1.83^{a}	nq	nq	nq	nq	nq
Quercetin diglucoside	46.27^{a}	nq	nq	nq	nq	nq
Quercetin-4-O-glucoside	65.93^{a}	59.62^{b}	3.17^{c}	0.48^{c}	0.54^{c}	nq
Isorhamnetin-4-O-glucoside	4.91^{a}	5.42^{a}	nq	nq	nq	nq
Total	**232.62^{a}**	**137.40^{b}**	**38.32^{c}**	**38.21^{c}**	**45.46^{c}**	**36.80^{c}**
	Chata' variety					
Quercetin	75.33^{a}	42.12^{b}	27.71^{c}	14.62^{d}	20.50 ^{cd}	19.47^{d}
Isorhamnetin	0.31^{d}	6.14^{b}	2.34^{c}	6.71^{a}	1.96^{c}	0.24^{d}
Quercetin-3-O-glucoside	8.56^{a}	0.72^{b}	0.44^{b}	0.20^{b}	nq	nq
Luteolin	1.96^{a}	0.26^{b}	nq	nq	nq	nq
Quercetin diglucoside	57.28^{a}	nq	nq	nq	nq	nq
Quercetin-4-O-glucoside	95.07^{a}	11.07^{b}	nq	nq	nq	nq
Isorhamnetin-4-O-glucoside	4.64^{a}	nq	nq	nq	nq	nq
Total	**243.15^{a}**	**60.31^{b}**	**30.49^{c}**	**21.54^{c}**	**22.46^{c}**	**19.71^{c}**
	Echalion' variety					
Quercetin	119.70^{a}	93.82^{b}	54.53^{c}	19.10^{e}	30.54^{de}	40.76 ^{cd}
Isorhamnetin	2.26^{c}	11.46^{a}	4.01^{b}	1.62^{c}	nq	nq
Quercetin-3-O-glucoside	13.19^{a}	0.85^{b}	1.51^{b}	0.34^{b}	0.28^{b}	0.34^{b}
Luteolin	0.88^{a}	nq	nq	nq	nq	nq
Quercetin diglucoside	37.48^{a}	nq	nq	nq	nq	nq
Quercetin-4-O-glucoside	74.64^{a}	19.32^{b}	3.40^{c}	nq	nq	nq
Isorhamnetin-4-O-glucoside	3.00^{a}	nq	nq	nq	nq	nq
Total	**251.14^{a}**	**125.45^{b}**	**63.45^{c}**	**21.06^{d}**	**30.82^{d}**	**41.10^{cd}**

Different letters (one-way ANOVA) denote significant differences ($p < 0.05$) among the five stages for the same compound.

attributed to the oxidation of flavonoids to semiquinoid intermediates and the respective quinones, which normally react further with other quinones to produce dark melanin pigments (Friedman, 1996) or with proteins to produce dark polymers (Kroll, Rawel, & Rohn, 2003).

3.2. Changes in the amino acid profile

A total of 21 amino acids were identified and quantified in the onion samples. Arginine, glutamine, asparagine and glutamic acid were the main amino acids in the raw onion varieties (T0), accounting for the 80.5, 80.1 and 72.0% of the total amino acids in 'Shallot', 'Chata' and 'Echalion' varieties, respectively (Table S1, Supporting Information). These results are in line with those reported by Hansen (2001), who reported that glutamine, arginine, asparagine, glutamic acid and lysine are the main amino acids present in different parts of onions bulbs. The results from our study showed that arginine was the major amino acid in black onion for the three varieties, accounting for the 41.6, 40.1 and 31.1% of the total amino acid content, respectively. Black onions made with the 'Shallot' and 'Echalion' varieties showed lower concentrations of total amino acids compared with the 'Chata' variety (Table S1, Supporting Information).

Some specific compounds comprising phenylalanine, leucine, isoleucine, GABA, valine, proline, alanine, glycine, serine, aspartic acid and ornithine showed an increase during the production process of black onion (Table S1, Supporting Information). A similar behaviour was observed during the production of black garlic (Molina-Calle, de Medina, Priego-Capote, & de Castro, 2017). In agreement with those results, Kimura et al. (2017) showed that during the production of black garlic the amino acids leucine, isoleucine and phenylalanine increased 1.06-fold, 1.67-fold and 2.43-fold, respectively. On the contrary, there are some other amino acids, such as tryptophan, methionine, glutamic acid and glutamine, whose content decreased during the black onion production (Table S1, Supporting Information). Our results are in line with those found by Molina-Calle et al. (2017), who observed an important decrease in the levels of tryptophan during black garlic production. The three varieties showed a significant increase in GABA concentration during the heating process, 2-fold, 4-fold and 4-fold for the 'Shallot', 'Chata' and 'Echalion' varieties, respectively. GABA is a non-protein amino acid with health benefits that has a role in decreasing hypertension levels and cancer cell proliferation (Yoshimura et al., 2010; Oh & Oh, 2004). In addition, the important decrease in the glutamine content (more than 99% for the three varieties) observed in our study could be explained taking into account that free glutamine, together with asparagine and glutamic acid, are the major precursors of brown products in the Maillard reaction and are responsible for the dark appearance of black onion (Niquet & Tessier, 2007).

3.3. Changes in the organosulfur compound profile

A total of 27 organosulfur compounds were identified and quantified in onion samples. γ-Glutamyl-S-(1-propenyl) cysteine sulfoxide, γ-Glutamyl-S-(2-propenyl) cysteine sulfoxide, Isoalliin and γ–Glutamyl-S-(1-propenyl) cysteine are the main organosulfur compounds in raw onion varieties (T0), accounting for the 66.3, 53.8 and 53.7% of the total organosulfur compounds in 'Shallot', 'Chata' and 'Echalion' varieties, respectively (Table S2, in Supporting Information). These results are in line with those reported by Wiczkowski (2011), who reported that isoallin is the predominant flavour precursor in onion. Isoallin was also found to be the major organosulfur compound in black onion for the three varieties studied, accounting for 83.6, 82.4 and 83.8% of the total organosulfur compound content, respectively (Table S2, in Supporting Information).

A significant increase was observed in the concentration of organosulfur compounds between T0 and T1 for all varieties (24.4, 41.4 and 48.4% increase for 'Shallot', 'Chata' and 'Echalion' varieties respectively). Subsequently, a gradual decrease in the total concentration of these compounds was observed up to T4, this being more noticeable in the case of the 'Echalion' variety (Table S2, Supporting Information). During the heat treatment, the total content of organosulfur compounds showed a decrease of 31.2% for 'Shallot' variety, while the 'Chata' and 'Echalion' varieties showed an increase of 24.8 and 25.7% in the total content of organosulfur compounds, respectively. At T4S in particular, isoallin was the organosulfur compound with the highest concentration for all varieties, accounting for more than 80% of the total content of organosulfur compounds (703.55 mg/100 g DW, 1360.12 mg/100 g DW, and 1242.68 mg/100 g DW in 'Shallot', 'Chata' and 'Echalion' respectively). Furthermore, the process has an important impact on γ-glutamil-S-(1-propenyl)-L-cysteine sulfoxide (Fig. 1). This compound decreased 10-fold during the whole heating process for 'Shallot', 4-fold for 'Echalion' and 3-fold for the 'Chata' samples. Similar data were found for cycloalliin and γ-glutamil-S-(1-propenyl)-L-cysteine by Molina-Calle et al. (2017) during the production of black garlic. These authors suggested that the decrease in the organosulfur compounds during the heat treatment could be due to the formation of intermediate compounds such as thiosulfinates and the subsequent transformation to organosulfur volatiles (diallyl disulfides and diphenyl disulfides)

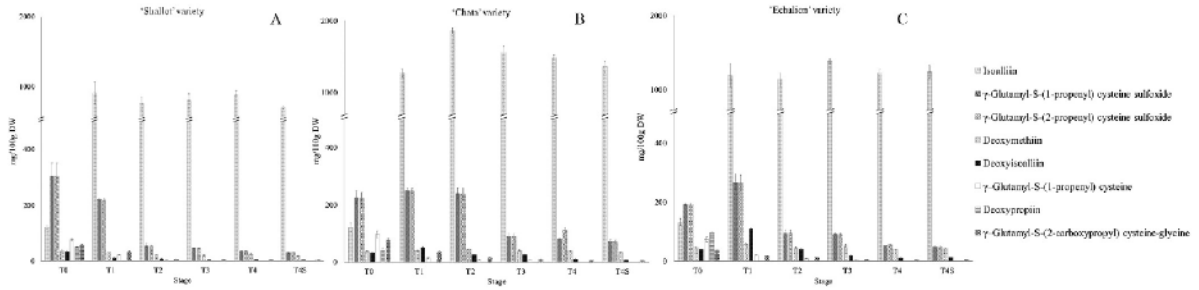

Fig. 1. Changes in organosulfur compound content during the production process of black onion for (A) raw 'Shallot' onion, (B) raw 'Chata' onion and (C) raw 'Echalion' onion. Data are expressed as mg/100 g DW as mean values (n = 3). Different letters (one-way ANOVA) denote statistically significant differences between the five stages.

(Molina-Calle, Priego-Capote, & Luque de Castro, 2017; Yu, Wu, Rosen, Hartman, & Ho, 1994).

3.4. Changes in the organic acid profile

The most abundant organic acids found in the raw onion samples were malic, tartaric and oxalic acids, which were present in the largest amounts in the T0 samples. The concentration of these organic acids increased significantly from T0 to T4S samples (Table 2), while the remaining organic acids did not present a clear trend during black onion production. In the last stage of the process (T4S), tartaric acid was the major organic acid quantified in the black onions, followed by malic acid (Table 2). The two organic acids accounted for 93.3% of the total content of organic acids in 'Shallot', 87.5% of the total content in 'Chata' and 95.3% of the total content in 'Echalion'. These results are in line with those obtained by Colina-Coca, de Ancos, and Sánchez-Moreno (2014) in the first stage of the heat process. In addition, regarding the pyruvic acid content, our three varieties of black onion samples are classified as low pungency, according to Dhumal, Datir, and Pandey (2007).

3.5. Changes in the sugar profile

The total sugars content in the black onions of the three varieties studied increased significantly with regard to the fresh onions. More specifically, a significantly increase in fructose levels was found in the black onions compared with raw onions in all the varieties, representing up to 10-fold, 2-fold and 5-fold increases for the 'Shallot', 'Chata' and 'Echalion' varieties, respectively (Fig. 2). In general, the fructose content increased greatly from T0 to T2, with a concomitant decrease in sucrose levels, and from then on the amount of fructose was constant until the end of the production process. Similar behaviour was observed for the glucose content in all the onion varieties (Fig. 2). These results are in line with those obtained by Martínez-Casas, Lage-Yusty, and López-Hernández (2017), who showed a 10-fold increase in fructose content during black garlic production. The substantially increased

Table 2
Concentration (mg/100 g DW) of organic acids presented in onion samples at different stages during the production of black onion for three varieties (Shallot, Chata and Echalion). Data is expressed as mean values (n = 6).

Compounds	T0	T1	T2	T3	T4	T4S
	Shallot' variety					
Oxalic Acid	131.00^{ab}	142.23^{a}	100.25^{c}	98.40^{c}	116.08^{b}	92.25^{c}
Tartaric Acid	236.21^{e}	2151.24^{a}	1645.54^{cd}	1549.31^{d}	1980.22^{b}	1717.82^{c}
Pyruvic Acid	19.94^{a}	15.54^{b}	12.28^{c}	9.97^{c}	19.83^{a}	16.32^{b}
Malic Acid	994.91^{c}	1685.51^{a}	1229.77^{b}	1213.97^{b}	1327.48^{b}	1012.86^{c}
Citric + Fumaric Acids	60.23^{a}	31.17^{b}	15.28^{e}	20.36^{d}	26.27^{c}	21.93^{d}
Succinic Acid	64.30^{a}	63.23^{a}	50.67^{b}	48.66^{b}	52.95^{b}	66.73^{a}
Total	$\mathbf{1506.59^{d}}$	$\mathbf{4088.91^{a}}$	$\mathbf{3053.79^{c}}$	$\mathbf{2940.67^{c}}$	$\mathbf{3522.83^{b}}$	$\mathbf{2927.92^{c}}$
	Chata' variety					
Oxalic Acid	364.46^{a}	219.58^{d}	282.25^{b}	240.92^{c}	238.43^{c}	240.04^{c}
Tartaric Acid	438.32^{c}	1933.63^{b}	2905.86^{a}	2936.68^{a}	2651.35^{a}	2869.62^{a}
Pyruvic Acid	52.39^{b}	53.31^{b}	62.90^{a}	31.21^{d}	43.35^{c}	27.60^{d}
Malic Acid	622.11^{a}	508.79^{a}	612.63^{a}	523.03^{a}	566.00^{a}	538.79^{a}
Citric + Fumaric Acids	52.05^{b}	42.01^{c}	32.69^{d}	34.75^{d}	72.41^{a}	28.91^{d}
Succinic Acid	124.78^{d}	117.97^{d}	225.08^{a}	227.97^{a}	167.19^{c}	189.57^{b}
Total	$\mathbf{1654.11^{c}}$	$\mathbf{2875.30^{b}}$	$\mathbf{4121.41^{a}}$	$\mathbf{3994.56^{a}}$	$\mathbf{3738.72^{a}}$	$\mathbf{3894.53^{a}}$
	Echalion' variety					
Oxalic Acid	101.44^{d}	171.66^{a}	118.40^{b}	112.91^{bc}	102.78^{cd}	115.47^{b}
Tartaric Acid	278.28^{c}	2708.32^{a}	2336.01^{b}	2826.94^{a}	2493.95^{b}	2827.04^{a}
Pyruvic Acid	1.33^{e}	4.74^{a}	4.42^{b}	3.61^{c}	3.22^{d}	4.77^{a}
Malic Acid	1781.34^{a}	1388.81^{c}	1042.31^{d}	1535.16^{bc}	1359.43^{c}	1574.68^{b}
Citric + Fumaric Acids	32.53^{b}	22.01^{d}	19.47^{e}	19.77^{e}	45.55^{a}	30.12^{c}
Succinic Acid	84.78^{a}	9.38^{e}	36.50^{d}	55.41^{c}	52.42^{c}	66.07^{b}
Total	$\mathbf{2279.70^{d}}$	$\mathbf{4304.91^{ab}}$	$\mathbf{3557.10^{c}}$	$\mathbf{4553.80^{a}}$	$\mathbf{4057.34^{b}}$	$\mathbf{4618.15^{a}}$

Different letters (one-way ANOVA) denote significant differences ($p < 0.05$) among the five stages for the same compound.

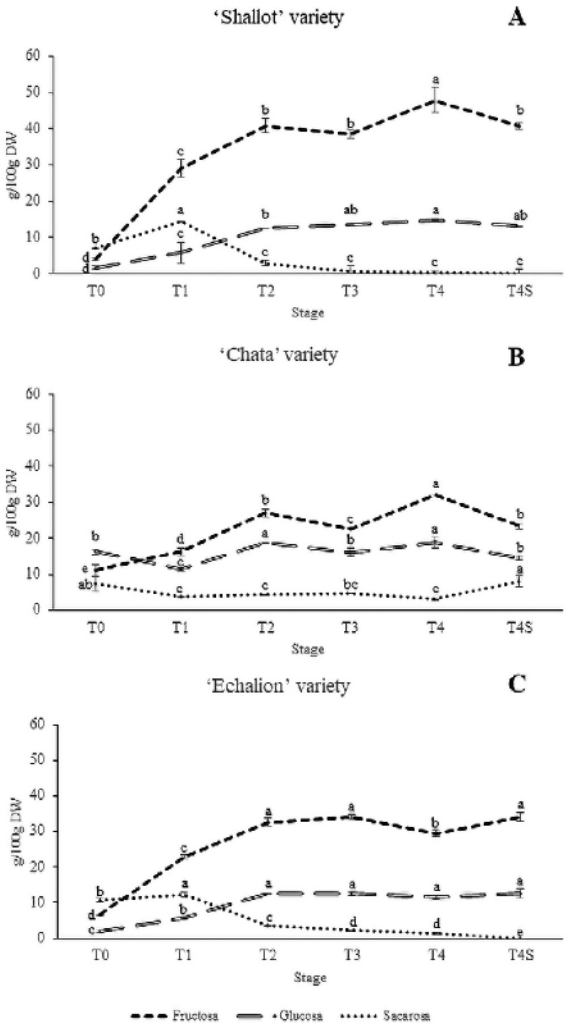

Fig. 2. Evolution of simple sugars during production process of black onion for (A) raw 'Shallot' onion, (B) raw 'Chata' onion and (C) raw 'Echalion' onion. Data are expressed as g/100 g DW as mean values (n = 3). Different letters (one-way ANOVA) denote statistically significant differences between the five stages.

levels of fructose and glucose in black onions are arguably due to the potential hydrolysis of fructans during de thermal process of black onion production (Yuan, Sun, Chen, & Wang, 2016). In addition, the sweetness of fructose is more than twice that of glucose and 1.7 times that of sucrose. These results are further evidence that the sweetness of black onion is mainly due to the increase in the level of fructose during the heat treatment (Yuan, Sun, Chen, & Wang, 2018; Zhang et al., 2015). The increase in glucose content during the process may also contribute to a greater or lesser extent to the characteristic sweetness of black onion (Yuan et al., 2018; Zhang et al., 2015; Edwards, Rossi, Corpe, Butterworth, & Ellis, 2016).

It is worth noting that at the beginning of the process, the 'Chata' variety presented much higher glucose and fructose contents than the 'Shallot' and 'Echalion' varieties. During thermal processing, the increase in these simple sugars was more marked in the 'Shallot' and 'Echalion' varieties, which could lead to an effect on their final taste that conditions the final sensorial acceptance of the product.

3.6. Changes in the total antioxidant capacity

In order to evaluate the changes in the antioxidant capacity of black onion during different stages of the production process, three different antioxidant activity measurements were performed: ABTS, DPPH and ORAC assays. At time T0, the 'Shallot' and 'Echalion' onions showed higher antioxidant activity measured by ABTS and ORAC assays than the 'Chata' variety. Meanwhile, the DPPH assay showed that the three varieties presented similar antioxidant activity (Fig. 3A, 3B and 3C). With regard to the heating process, the three varieties showed different trends with regard to antioxidant activity measured by ABTS. The 'shallot' variety showed a decrease in the antioxidant activity from T0 to T1, reaching the initial value at T2 and then remaining stable to T4S. Meanwhile, the 'Chata' variety showed a significant increase in the ABTS values from T0 to T2, and then these values decreased up to T4S. In general, the antioxidant activity measured by the ABTS assay did not show significant differences between the T0 and T4S stages for the 'Shallot' and 'Chata' varieties. In the case of the 'Echalion' variety, the antioxidant activity gradually increased from T0 to T4S, showing a significant difference if the T0 stage is compared with T4S.

DPPH assays showed a marked decrease in the antioxidant activity values from the initial stage (T0) up to T1 for the 'Shallot' and 'Chata' varieties. From T1 to T2, the DPPH values showed an increase and remained stable until T4 and T3 for the 'Shallot' and 'Chata' varieties, respectively. From these stages, the DPPH values significantly decreased up to the final stage. In general, the antioxidant activity measured by DPPH assay decreased from T0 to T4S for the 'Shallot' and 'Chata' varieties. It is noteworthy that the 'Echalion' variety did not show important variations in the DPPH values during the heating treatment.

The ORAC assays showed an appreciably decrease in the antioxidant activity from the initial stage (T0) up to T1 for the three varieties. From T1 to T4, although there were slight oscillations in the antioxidant activity values, they all remained under the values of the initial stage (T0). In general, the antioxidant activity by ORAC assay greatly decreased from T0 to T4S in all the varieties.

It is worth mentioning that the differences in the results from the three antioxidant activity assays (ORAC, ABTS and DPPH) could be explained taking into account the chemistry principles upon which the antioxidant assays are based (Ou, Huang, Hampsch-Woodill, Flanagan, & Deemer, 2002). For example, the ORAC assay typically measures peroxyl-radical scavenging with a more relevant significance, while ABTS and DPPH involve organic radicals who presented large compound with steric issues (Craft, Kerrihard, Amarowicz, & Pegg, 2012). Our findings are in agreement with those of Dudonne, Vitrac, Couriere, Woillez, and Mérillon (2009), who reported that 30 plant extracts showed different antioxidant activities with various reaction mechanisms. Moreover, the different trends regarding antioxidant activity could also be attributed to the increase in or the formation of specific compounds with a different number of hydrogen atoms. In this regard, the thermal treatment to produce black onion broke the glucosides of flavonoids to form aglycones, which possess different antioxidant properties. Additionally, the Maillard reaction products formed by the non-enzymatic browning, including a wide variety of intermediates products formed during the Maillard reaction and, ultimately, melanoidins, could be other factors in the different antioxidant activity trends. Thus, different classes of compounds during the stages of the heating process may have different contribution to ORAC, DPPH and ABTS values (Bernaert, De Loose, Van Bockstaele, & Van Droogenbroeck, 2013). Therefore, antioxidant profiles must be based on a variety of types of antioxidant activities because reactive oxygen species are formed by a number of different mechanisms and can be determined by a variety of techniques.

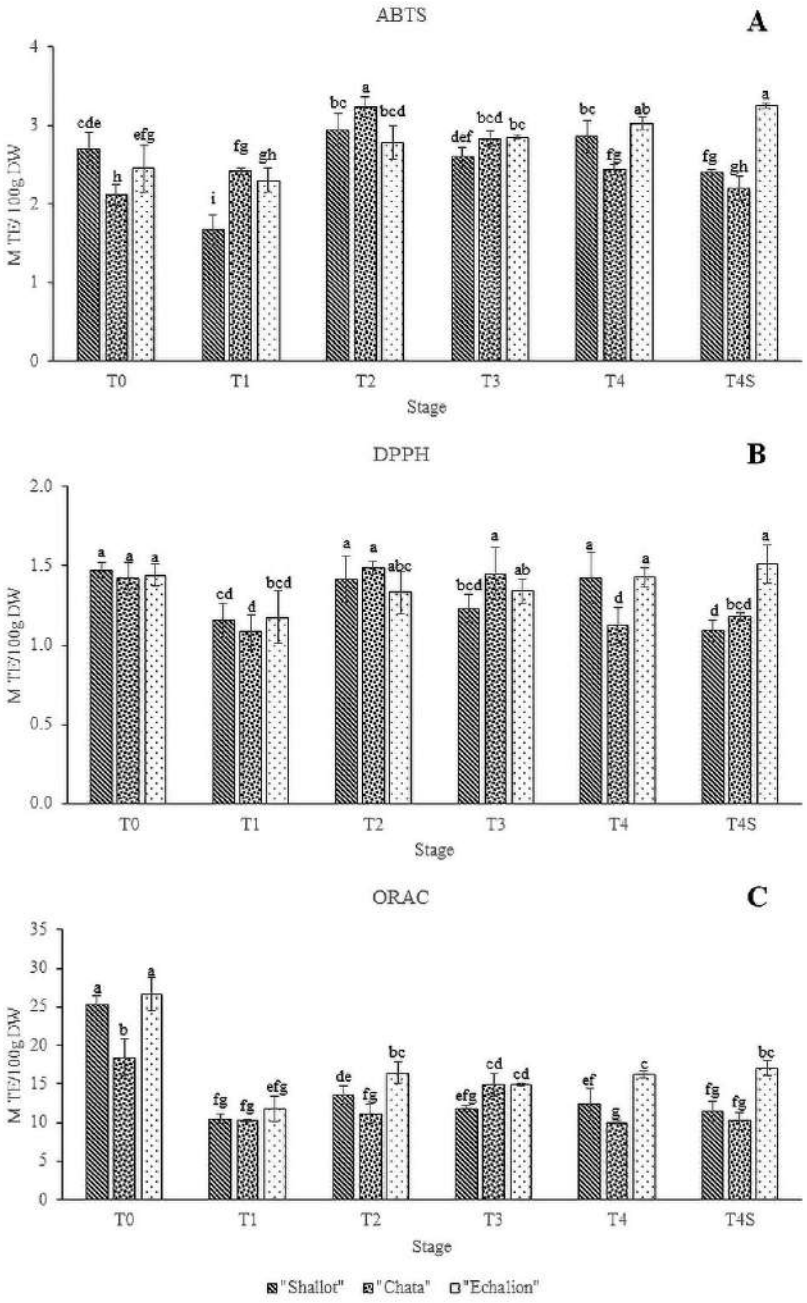

Fig. 3. Antioxidant activity for 'Shallot', 'Chata' and 'Echalion' varieties during production process of black onion measured by (A) ABTS, (B) DPPH and (C) ORAC assays. Data are expressed M TE/100 g DW as mean values (n = 9). Different letters (Two-Ways ANOVA) denote statistically significant differences between the five stages.

3.7. Overall changes and remarks

With the aim of determining at a glance the influence of the heating process during the production of different varieties of black onion on their levels of flavonoids, organosulfur compounds, amino acids, organic acids and sugar, a multivariate treatment of the data was used applying PCA (principal component analysis) (Fig. 4). Fig. 4A shows the score plot according to the two principal components selected (PC). PC1, accounting for 46% of the total variance, shows a clear discrimination among samples based on the different stages of the heating process, separating between T0 samples (initial stage) and the rest (T1 to T4S). Fewer differences were found between T1 to T4S. Thus, these results highlight that the major changes in the phytochemical composition took place during the first stage of the black onion process. In addition, the most significant variables that contribute to sample discrimination were glucose, fructose, isoallin and tartaric acid (Fig. 4B),

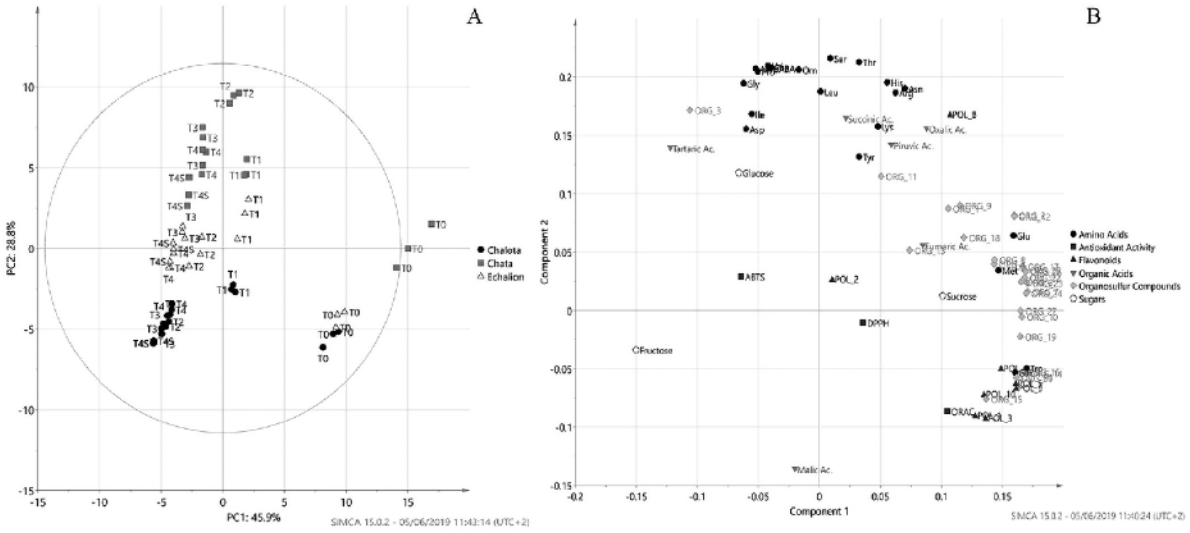

Fig. 4. Scores (A) and loadings (B) of PCA comparing data from samples of three different onion varieties during black onion production.

linked to the dramatic increase in their concentration from the T0 to T1 stages. Some amino acids, including asparagine, glycine, GABA, proline, alanine and valine, also contributed to the differentiation between onion samples during the black onion production. PC2, which explained 29% of the total variance, showed a clear discrimination of black onion samples at the last stage of the heating process based on onion varieties. For instance, black onion from the 'Shallot' variety is different to that from the 'Chata' and 'Echalion' varieties. The loading plot reveals that fructose, glucose and malic acid contributed largely to this separation.

4. Conclusions

The heating process applied to black onion production, consisting of controlling the humidity and temperature conditions (90–95% RH and 65–70 °C) for 28 days, induces several phytochemical modifications in three onion varieties, which results in a loss of total flavonoids and increases in organosulfur compounds, predominantly isoalliin, but also fructose, glucose and tartaric acid. These changes are influenced by the sensitivity of the individual compound to modification or degradation under the heating process, although others parameters such as pH, the presence of oxygen during the process along with the presence of other phytochemicals in the matrix also impact on their alterations.

Black onion from the 'Shallot' variety presented a different phytochemical profile compared with those obtained from the 'Chata' and 'Echalion' varieties. Indeed, during thermal processing, the increase in simple sugars is more marked in the 'Shallot' variety, which could affect the final taste that conditions the final sensorial acceptance of the product. It is important to highlight that black onion from all the varieties presented isoalliin as the main bioactive compound. Future research should focus on understanding the bioavailability and biological effects of the isoalliin present in black onion as there is no data on the bioavailability and bioactivity of this organosulfur compound in the literature.

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CRediT authorship contribution statement

Alicia Moreno-Ortega: Investigation, Writing - original draft. **Gema Pereira-Caro:** Investigation, Writing - review & editing. **José Luis Ordóñez:** Investigation. **José Manuel Muñoz-Redondo:** Formal analysis. **Rafael Moreno-Rojas:** Writing - review & editing. **Jesús Pérez-Aparicio:** Resources. **José Manuel Moreno-Rojas:** Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.125958.

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SUPPLEMENTARY INFORMATION

Changes on the Antioxidant Activity and Metabolite Profile of Three Onion Varieties during the Elaboration of 'Black Onion'

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Table S1. Concentration (mg/100g DW) of amino acids presented in onion samples at different stages during the production of black onion for three varieties (Shallot, Chata and Echalion). Data is expressed as mean values (n=6).

Compounds	***T0***	***T1***	***T2***	***T3***	***T4***	***T4S***
	Shallot' variety					
Phenylalanine	11.95 ^{c}	24.92 ^{a}	19.68 ^{b}	19.12 ^{b}	20.61 ^{b}	14.01 ^{c}
Leucine	22.42 ^{b}	41.60 ^{a}	27.56 ^{b}	23.54 ^{b}	27.65 ^{b}	16.20 ^{c}
Isoleucine	9.59 ^{c}	17.59 ^{a}	18.50 ^{a}	16.69 ^{a}	18.32 ^{a}	13.54 ^{b}
Tryptophan	53.68 ^{a}	18.65 ^{b}	3.87 ^{c}	3.60 ^{c}	4.36 ^{c}	3.04 ^{c}
GABA	8.84 ^{c}	27.70 ^{a}	24.30 ^{ab}	24.29 ^{ab}	26.05 ^{ab}	18.91 ^{b}
Methionine	7.57 ^{a}	6.41 ^{b}	3.69 ^{c}	2.93 ^{cd}	3.34 ^{c}	2.38 ^{d}
Valine	24.79 ^{c}	44.17 ^{a}	43.58 ^{a}	39.16 ^{ab}	42.65 ^{a}	34.01 ^{b}
Proline	5.99 ^{c}	24.11 ^{a}	20.46 ^{ab}	19.91 ^{b}	20.93 ^{ab}	17.15 ^{b}
Tyrosine	102.94 ^{a}	111.84 ^{a}	74.36 ^{bc}	72.13 ^{bc}	79.99 ^{b}	56.70 ^{c}
Alanine	22.34 ^{d}	73.31 ^{ab}	70.64 ^{abc}	68.27 ^{bc}	80.98 ^{a}	62.37 ^{c}
Treonine	41.89 ^{ab}	45.41 ^{a}	31.46 ^{cd}	27.96 ^{cd}	33.36 ^{bc}	23.26 ^{d}
Glycin	9.24 ^{d}	15.97 ^{c}	19.15 ^{b}	21.26 ^{b}	24.79 ^{a}	20.31 ^{b}
Glutamic Acid	198.67 ^{a}	159.73 ^{b}	73.78 ^{c}	53.83 ^{c}	69.78 ^{c}	46.28 ^{c}
Serine	30.17 ^{a}	29.94 ^{a}	26.39 ^{ab}	22.67 ^{b}	26.82 ^{ab}	19.57 ^{b}
Glutamine	871.79 ^{a}	8.03 ^{b}	2.20 ^{b}	1.98 ^{b}	2.39 ^{b}	1.58 ^{b}
Asparagine	255.04 ^{a}	226.73 ^{a}	134.47 ^{b}	105.85 ^{b}	125.20 ^{b}	81.19 ^{b}
Aspartic Acid	58.22 ^{c}	49.15 ^{c}	72.40 ^{b}	79.24 ^{ab}	86.29 ^{a}	81.49 ^{ab}
Arginine	1055.18 ^{a}	1134.90 ^{a}	587.58 ^{b}	453.22 ^{b}	564.08 ^{b}	426.84 ^{b}
Lisine	98.43 ^{a}	123.86 ^{a}	65.15 ^{b}	55.40 ^{b}	70.47 ^{b}	48.56 ^{b}
Histidine	61.57 ^{a}	56.29 ^{a}	38.44 ^{b}	35.65 ^{b}	43.13 ^{b}	31.92 ^{b}
Ornithine	7.68 ^{b}	9.90 ^{a}	10.40 ^{a}	8.72 ^{ab}	9.33 ^{ab}	7.57 ^{b}
Total	**2957.99** ^{a}	**2250.22** ^{b}	**1368.06** ^{c}	**1155.40** ^{c}	**1380.52** ^{c}	**1026.88** ^{c}
	Chata' variety					
Phenylalanine	23.30 ^{e}	40.42 ^{d}	59.52 ^{ab}	62.16 ^{a}	52.38 ^{bc}	49.20 ^{c}
Leucine	32.23 ^{c}	51.26 ^{ab}	57.99 ^{a}	51.76 ^{ab}	52.32 ^{ab}	42.91 ^{bc}
Isoleucine	16.52 ^{c}	32.98 ^{b}	42.97 ^{b}	43.52 ^{a}	39.31 ^{ab}	32.19 ^{b}
Tryptophan	74.08 ^{a}	17.24 ^{b}	8.90 ^{c}	6.09 ^{c}	6.27 ^{c}	5.37 ^{c}
GABA	22.93 ^{c}	113.76 ^{b}	153.48 ^{a}	117.86 ^{ab}	122.96 ^{ab}	87.69 ^{b}
Methionine	10.83 ^{a}	7.51 ^{b}	6.19 ^{bc}	5.48 ^{cd}	4.78 ^{cd}	4.12 ^{d}
Valine	47.51 ^{c}	74.81 ^{b}	106.29 ^{a}	94.88 ^{ab}	90.86 ^{ab}	75.63 ^{b}
Proline	20.03 ^{b}	58.96 ^{a}	79.38 ^{a}	69.32 ^{a}	57.83 ^{a}	54.93 ^{a}
Tyrosine	102.00 ^{b}	130.16 ^{ab}	153.28 ^{a}	131.95 ^{ab}	125.91 ^{ab}	109.92 ^{b}
Alanine	64.31 ^{d}	154.22 ^{c}	243.28 ^{a}	211.10 ^{ab}	199.05 ^{b}	177.73 ^{bc}
Treonine	88.75 ^{b}	115.55 ^{ab}	145.84 ^{a}	117.63 ^{ab}	114.17 ^{ab}	102.59 ^{b}

Glycin	18.18	c	37.29	b	57.35	a	54.44	a	51.46	a	51.17	a
Glutamic Acid	229.49	a	160.12	bc	186.57	ab	119.36	bc	127.61	bc	97.65	c
Serine	68.85	c	94.97	bc	140.34	a	118.06	ab	104.25	b	109.75	ab
Glutamine	1355.62	a	5.34	b	6.30	b	4.61	b	4.27	b	3.52	b
Asparagine	588.13	ab	547.25	b	731.59	a	545.87	b	570.70	ab	464.81	b
Aspartic Acid	67.39	d	90.83	d	172.83	c	253.53	a	200.54	bc	233.76	ab
Arginine	1234.16	c	1626.01	b	2014.81	a	1523.22	bc	1549.92	bc	1282.29	c
Lisine	108.48	c	168.77	ab	182.54	a	156.10	ab	130.87	bc	109.91	c
Histidine	67.88	d	92.39	ab	104.96	a	87.46	bc	80.77	bcd	75.35	cd
Ornithine	11.62	d	25.88	c	34.33	b	40.06	a	26.89	c	25.31	c
Total	**4252.31**	**ab**	**3645.73**	**ab**	**4688.73**	**a**	**3814.46**	**ab**	**3713.12**	**ab**	**3195.81**	**b**
	Echalion' variety											
Phenylalanine	15.23	c	28.52	b	36.14	ab	36.52	ab	33.32	ab	39.65	a
Leucine	39.70	d	58.77	a	47.08	bc	50.81	b	40.58	cd	45.16	bcd
Isoleucine	21.35	b	38.20	a	39.40	a	45.90	a	38.97	a	46.37	a
Tryptophan	67.75	a	18.38	b	5.97	c	4.81	c	4.37	c	4.54	c
GABA	16.43	c	92.31	ab	84.48	ab	101.60	a	72.84	b	72.09	b
Methionine	9.32	a	7.30	ab	9.22	a	4.26	bc	3.74	c	4.06	bc
Valine	32.70	b	65.03	a	62.24	a	76.66	a	63.55	a	76.55	a
Proline	13.28	c	45.10	b	45.88	b	58.33	a	45.22	b	48.12	b
Tyrosine	147.38	a	174.36	a	146.86	a	155.03	a	133.26	a	149.91	a
Alanine	19.50	c	97.02	b	93.19	b	121.07	a	105.86	ab	108.43	ab
Treonine	39.04	b	64.33	a	54.13	a	58.46	a	50.41	ab	53.69	a
Glycin	9.59	c	21.02	b	23.82	b	33.85	a	33.54	a	33.55	a
Glutamic Acid	194.72	a	153.39	b	93.03	c	94.47	c	64.54	d	71.75	cd
Serine	33.37	c	61.59	ab	52.72	b	65.84	a	52.81	b	59.95	ab
Glutamine	783.98	a	4.34	b	3.06	b	3.17	b	2.36	b	2.56	b
Asparagine	202.49	b	266.24	a	211.42	ab	202.58	b	150.11	b	163.98	b
Aspartic Acid	54.36	b	52.87	b	80.17	b	122.33	a	150.28	a	142.47	a
Arginine	752.27	b	1053.41	a	641.74	b	732.61	b	591.18	b	602.45	b
Lisine	149.00	ab	211.87	a	140.88	b	152.68	ab	115.62	b	126.53	b
Histidine	70.29	b	90.60	a	68.04	b	71.27	ab	60.85	b	65.53	b
Ornithine	15.46	a	20.75	a	16.94	a	19.95	a	20.30	a	18.46	a
Total	**2687.22**	**a**	**2625.40**	**a**	**1956.42**	**b**	**2212.21**	**ab**	**1833.72**	**b**	**1935.81**	**b**

Different letters (one-way ANOVA) denote significant differences ($p<0.05$) among the five stages for the same compound

Table S2. Concentration (mg/100g DW) of organosulfur compounds presented in onion samples at different stages during the production of black onion for three varieties (Shallot, Chata and Echalion). Data are expressed as mean values (n=6).

Compounds	***T0***	***T1***	***T2***	***T3***	***T4***	***T4S***
	Shallot' variety					
S-methyl-cysteine sulfoxide (Methiin)	11.77 ^{b}	38.91 ^{a}	9.28 ^{bc}	6.84 ^{cd}	7.33 ^{cd}	4.67 ^{d}
γ-Glutamyl-*S*-methyl cysteine sulfoxide	10.58 ^{a}	6.78 ^{b}	5.03 ^{d}	4.80 ^{d}	5.53 ^{c}	3.92 ^{e}
(*S*-(E)-(1-propenyl) cysteine sulfoxide (Isoalliin)	121.39 ^{b}	913.25 ^{a}	766.52 ^{a}	804.30 ^{a}	877.53 ^{a}	703.55 ^{a}
γ-Glutamyl-*S*-(1-propenyl) cysteine sulfoxide	306.02 ^{a}	222.06 ^{b}	56.37 ^{c}	47.92 ^{c}	35.49 ^{c}	30.18 ^{c}
S-[(E)-1-Propenyl]cysteine *S*-oxide	9.73 ^{a}	0.00 ^{b}	0.00 ^{b}	0.00 ^{b}	0.00 ^{b}	0.00 ^{b}
3-Methyl-1,4-thiazane-5-carboxylic acid sulfoxide (Cycloalliin)	29.10 ^{a}	17.78 ^{b}	6.08 ^{c}	5.04 ^{c}	6.37 ^{c}	4.18 ^{c}
S-(2-propenyl) cysteine sulfoxide (Alliin)	10.13 ^{a}	5.16 ^{b}	3.83 ^{d}	3.53 ^{e}	4.14 ^{c}	2.98 ^{f}
γ-Glutamyl-*S*-(2-propenyl) cysteine sulfoxide	305.74 ^{a}	219.14 ^{b}	53.80 ^{c}	47.45 ^{c}	35.16 ^{c}	30.23 ^{c}
S-propyl-cysteine sulfoxide (Propiin)	9.81 ^{b}	16.68 ^{a}	6.38 ^{c}	4.91 ^{cd}	6.63 ^{c}	3.55 ^{d}
γ–Glutamyl-*S*-propyl cysteine sulfoxide	14.35 ^{a}	8.08 ^{b}	4.87 ^{cd}	4.42 ^{d}	5.73 ^{c}	3.65 ^{d}
S-methyl cysteine (Deoxymethiin)	35.14 ^{a}	30.05 ^{ab}	21.76 ^{c}	21.07 ^{c}	24.95 ^{bc}	18.75 ^{c}
γ-Glutamyl-*S*-methyl cysteine	10.56 ^{a}	7.13 ^{b}	5.03 ^{c}	4.73 ^{cd}	6.21 ^{b}	3.93 ^{d}
1-Propenyl-cysteine (Deoxyisoalliin)	34.01 ^{a}	10.53 ^{b}	8.24 ^{bc}	4.51 ^{c}	5.28 ^{bc}	4.22 ^{c}
γ–Glutamyl-*S*-(1-propenyl) cysteine	77.32 ^{a}	22.39 ^{b}	3.86 ^{c}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}
S-Propyl-L-cysteine (Deoxypropiin)	51.35 ^{a}	0.00 ^{b}	0.00 ^{b}	0.00 ^{b}	0.00 ^{b}	0.00 ^{b}
γ–Glutamyl-*S*-(propyl) cysteine	10.42 ^{a}	4.57 ^{b}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}
S-(2-carboxypropyl)cysteine	9.97 ^{a}	5.79 ^{b}	4.90 ^{c}	4.19 ^{d}	4.52 ^{cd}	3.32 ^{e}
S-(2-carboxypropyl) cysteine-glycine	22.36 ^{b}	25.30 ^{a}	10.37 ^{c}	6.36 ^{d}	6.58 ^{d}	4.86 ^{e}
γ–Glutamyl-*S*-(2-carboxypropyl) cysteine-glycine	58.39 ^{a}	31.92 ^{b}	7.20 ^{c}	4.71 ^{c}	4.90 ^{c}	3.88 ^{c}
γ–Glutamyl-*S*-(2-carboxypropyl) cysteine glycine hexoside	9.73 ^{a}	0.00 ^{b}	0.00 ^{b}	0.00 ^{b}	0.00 ^{b}	0.00 ^{b}
γ–Glutamyl-(2-carboxyethyl) cysteine-glycine	9.73 ^{a}	0.00 ^{b}	0.00 ^{b}	0.00 ^{b}	0.00 ^{b}	0.00 ^{b}
γ–Glutamyl-*S*-(*S*-methyl) cysteine-glycine	9.94 ^{a}	4.87 ^{b}	3.79 ^{d}	3.66 ^{d}	4.43 ^{c}	3.10 ^{e}
S-(*S*-propyl) cysteine	12.90 ^{a}	7.94 ^{b}	4.11 ^{cd}	3.96 ^{cd}	5.54 ^{c}	3.21 ^{d}
γ–Glutamyl-*S*-(*S*-1-propenyl) cysteine	9.79 ^{a}	4.88 ^{b}	3.59 ^{d}	3.59 ^{d}	4.30 ^{c}	2.93 ^{e}
γ–Glutamyl-*S*-(*S*-1-propenyl) cysteine-glycine	11.30 ^{a}	4.48 ^{b}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}
S-(*S*-1-propenyl) cysteine	10.68 ^{a}	6.20 ^{b}	4.09 ^{d}	4.02 ^{d}	5.05 ^{c}	3.21 ^{e}
γ–Glutamyl-*S*-(*S*-propyl) cysteine-glycine	11.01 ^{a}	4.45 ^{b}	3.51 ^{c}	3.41 ^{c}	4.11 ^{b}	2.87 ^{d}
Total	**1223.26** ^{b}	**1618.32** ^{a}	**992.63** ^{c}	**993.42** ^{c}	**1059.79** ^{bc}	**841.17** ^{c}
	Chata' variety					

S-methyl-cysteine sulfoxide (Methiin)	37.45	a	43.39	a	42.15	a	18.07	b	23.38	b	14.33	b
γ-Glutamyl-*S*-methyl cysteine sulfoxide	17.67	a	10.15	c	12.15	b	8.32	de	8.83	d	7.29	e
(*S*-(E)-(1-propenyl) cysteine sulfoxide (Isoalliin)	120.82	e	1271.60	d	1864.07	a	1557.54	b	1485.09	bc	1360.12	cd
γ-Glutamyl-*S*-(1-propenyl) cysteine sulfoxide	226.18	a	250.67	a	239.43	a	89.52	b	80.73	b	72.03	b
S-[(E)-1-Propenyl]cysteine *S*-oxide	16.81	a	0.00	b	0.00	b	0.00	b	0.00	b	0.00	b
3-Methyl-1,4-thiazane-5-carboxylic acid sulfoxide (Cycloalliin)	31.14	a	20.57	b	18.24	b	9.68	c	10.46	c	8.15	c
S-(2-propenyl) cysteine sulfoxide (Alliin)	17.34	a	6.84	b	7.21	b	6.18	c	6.01	c	5.32	d
γ-Glutamyl-*S*-(2-propenyl) cysteine sulfoxide	224.13	a	249.02	a	237.74	a	89.53	b	112.33	b	70.89	b
S-propyl-cysteine sulfoxide (Propiin)	19.31	a	16.60	ab	16.03	ab	9.81	bc	11.20	bc	7.09	c
γ–Glutamyl-*S*-propyl cysteine sulfoxide	19.19	a	8.36	b	8.91	b	6.77	c	7.00	c	5.89	d
S-methyl cysteine (Deoxymethiin)	37.06	bc	38.98	b	45.40	a	40.61	ab	37.55	bc	33.86	c
γ-Glutamyl-*S*-methyl cysteine	17.67	a	10.19	c	12.16	b	8.32	de	8.83	d	7.29	e
1-Propenyl-cysteine (Deoxyisoalliin)	30.88	b	52.13	a	26.10	b	26.11	b	10.16	c	7.10	c
γ–Glutamyl-*S*-(1-propenyl) cysteine	96.73	a	14.10	b	7.69	bc	6.02	bc	0.00	c	0.00	c
S-Propyl-L-cysteine (Deoxypropiin)	39.99	a	0.00	b	0.00	b	0.00	b	0.00	b	0.00	b
γ–Glutamyl-*S*-(propyl) cysteine	17.22	a	0.00	b	0.00	b	0.00	b	0.00	b	0.00	b
S-(2-carboxypropyl)cysteine	17.31	a	8.81	b	8.54	b	8.45	b	6.08	c	5.43	c
S-(2-carboxypropyl) cysteine-glycine	29.90	b	40.41	a	22.66	bc	17.64	c	7.82	d	6.94	d
γ–Glutamyl-*S*-(2-carboxypropyl) cysteine-glycine	77.97	a	35.06	b	14.63	c	10.06	cd	6.23	d	5.70	d
γ–Glutamyl-*S*-(2-carboxypropyl) cysteine glycine hexoside	16.81	a	0.00	b	0.00	b	0.00	b	0.00	b	0.00	b
γ–Glutamyl-(2-carboxyethyl) cysteine-glycine	16.81	a	0.00	b	0.00	b	0.00	b	0.00	b	0.00	b
γ–Glutamyl-*S*-(*S*-methyl) cysteine-glycine	16.84	a	6.95	c	7.90	b	6.57	c	6.65	c	5.63	d
S-(*S*-propyl) cysteine	23.76	a	9.21	b	10.27	b	6.72	b	8.54	b	6.18	b
γ–Glutamyl-*S*-(*S*-1-propenyl) cysteine	16.85	a	6.34	c	7.17	b	5.90	c	6.12	c	5.17	d
γ–Glutamyl-*S*-(*S*-1-propenyl) cysteine-glycine	18.29	a	6.01	bc	6.85	b	5.79	c	5.96	bc	5.14	c
S-(*S*-1-propenyl) cysteine	18.26	a	7.43	c	8.98	b	6.85	cd	7.26	c	5.69	d
γ–Glutamyl-*S*-(*S*-propyl) cysteine-glycine	18.31	a	5.96	bc	6.72	b	5.72	cd	5.92	bcd	5.07	d
Total	**1240.68**	**e**	**2118.79**	**b**	**2630.97**	**a**	**1950.17**	**bc**	**1862.16**	**c**	**1650.29**	**d**
	Echalion' variety											
S-methyl-cysteine sulfoxide (Methiin)	50.45	b	56.16	a	21.54	c	18.10	c	9.18	d	9.10	d
γ-Glutamyl-*S*-methyl cysteine sulfoxide	11.64	a	9.11	b	6.54	d	7.66	c	5.50	e	6.08	de
(*S*-(E)-(1-propenyl) cysteine sulfoxide (Isoalliin)	131.63	c	1195.10	ab	1142.33	b	1385.21	a	1230.47	ab	1242.68	ab
γ-Glutamyl-*S*-(1-propenyl) cysteine sulfoxide	192.83	b	266.02	a	95.43	c	90.62	c	54.61	d	48.37	d

S-[(E)-1-Propenyl]cysteine *S*-oxide	11.30 a	0.00 b	0.00 b	0.00 b	0.00 b	0.00 b
3-Methyl-1,4-thiazane-5-carboxylic acid sulfoxide (Cycloalliin)	20.97 a	20.34 a	9.15 b	8.83 b	5.90 b	6.34 b
S-(2-propenyl) cysteine sulfoxide (Alliin)	11.53 a	6.19 b	5.24 c	4.90 cd	4.45 d	4.57 d
γ-Glutamyl-*S*-(2-propenyl) cysteine sulfoxide	192.93 b	264.06 a	95.14 c	90.33 cd	56.20 de	48.21 e
S-propyl-cysteine sulfoxide (Propiin)	14.48 b	22.37 a	11.32 bc	9.41 cd	5.94 d	6.84 d
γ–Glutamyl-*S*-propyl cysteine sulfoxide	18.81 a	13.76 b	8.68 c	8.00 cd	5.79 d	6.70 cd
S-methyl cysteine (Deoxymethiin)	47.23 bc	57.59 a	47.02 bc	53.19 ab	39.37 d	44.72 cd
γ-Glutamyl-*S*-methyl cysteine	11.64 a	9.10 b	6.55 d	7.59 c	6.05 d	6.23 d
1-Propenyl-cysteine (Deoxyisoalliin)	39.92 b	109.03 a	40.51 b	18.50 c	9.99 d	12.43 cd
γ–Glutamyl-*S*-(1-propenyl) cysteine	74.03 a	20.64 b	8.45 c	4.69 c	0.00 c	0.00 c
S-Propyl-L-cysteine (Deoxypropiin)	97.79 a	0.00 b	0.00 b	0.00 b	0.00 b	0.00 b
γ–Glutamyl-*S*-(propyl) cysteine	12.41 a	5.51 b	0.00 c	0.00 c	0.00 c	0.00 c
S-(2-carboxypropyl)cysteine	11.74 a	6.54 b	6.17 b	5.21 c	4.81 c	5.14 c
S-(2-carboxypropyl) cysteine-glycine	16.54 b	21.83 a	17.14 b	7.65 c	5.99 c	6.89 c
γ–Glutamyl-*S*-(2-carboxypropyl) cysteine-glycine	36.63 a	17.39 b	11.72 c	5.85 d	4.79 e	5.42 de
γ–Glutamyl-*S*-(2-carboxypropyl) cysteine glycine hexoside	11.30 a	0.00 b	0.00 b	0.00 b	0.00 b	0.00 b
γ–Glutamyl-(2-carboxyethyl) cysteine-glycine	11.30 a	0.00 b	0.00 b	0.00 b	0.00 b	0.00 b
γ–Glutamyl-*S*-(*S*-methyl) cysteine-glycine	11.33 a	5.93 b	4.92 cd	5.09 c	4.55 e	4.69 de
S-(*S*-propyl) cysteine	14.97 a	8.30 b	5.05 d	5.63 c	4.58 d	4.75 d
γ–Glutamyl-*S*-(*S*-1-propenyl) cysteine	11.34 a	5.61 b	4.50 cd	4.67 c	4.17 d	4.36 cd
γ–Glutamyl-*S*-(*S*-1-propenyl) cysteine-glycine	12.93 a	4.36 b	4.35 b	4.53 b	0.00 c	0.00 c
S-(*S*-1-propenyl) cysteine	11.87 a	6.99 b	5.51 c	5.66 c	4.83 d	5.15 cd
γ–Glutamyl-*S*-(*S*-propyl) cysteine-glycine	11.98 c	2.62 a	4.42 b	4.61 b	4.14 b	4.34 b
Total	**1101.53 c**	**2134.53 a**	**1561.68 b**	**1755.92 b**	**1471.31 b**	**1483.02 b**

Different letters (one-way ANOVA) denote significant differences ($p<0.05$) among the five stages for the same compound

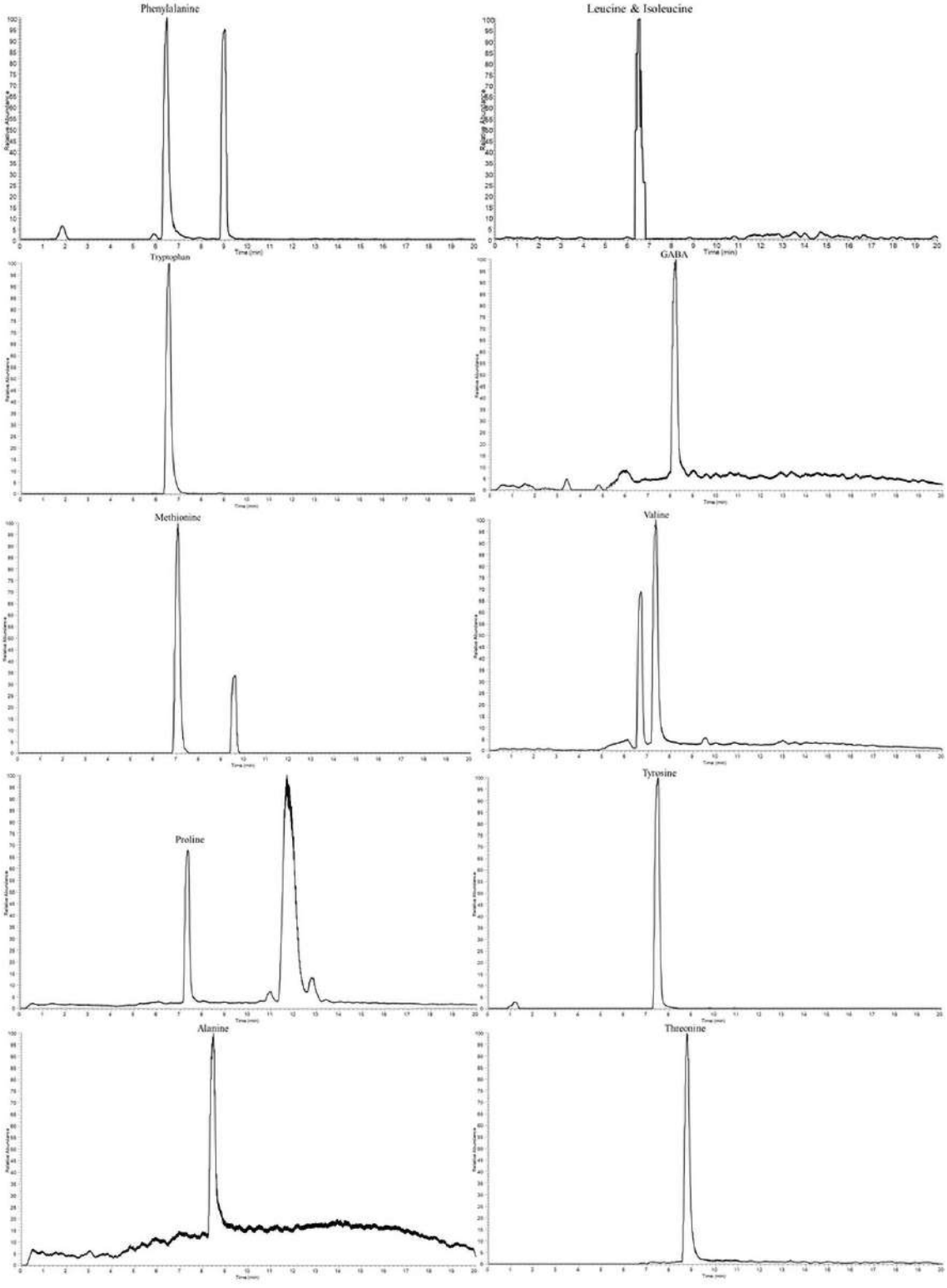

Figure S1. UHPLC-HRMS chromatograms of phenylalanine, leucine, isoleucine, tryptophan, GABA, methionine, valine, proline, tyrosine, alanine and threonine in onion samples.

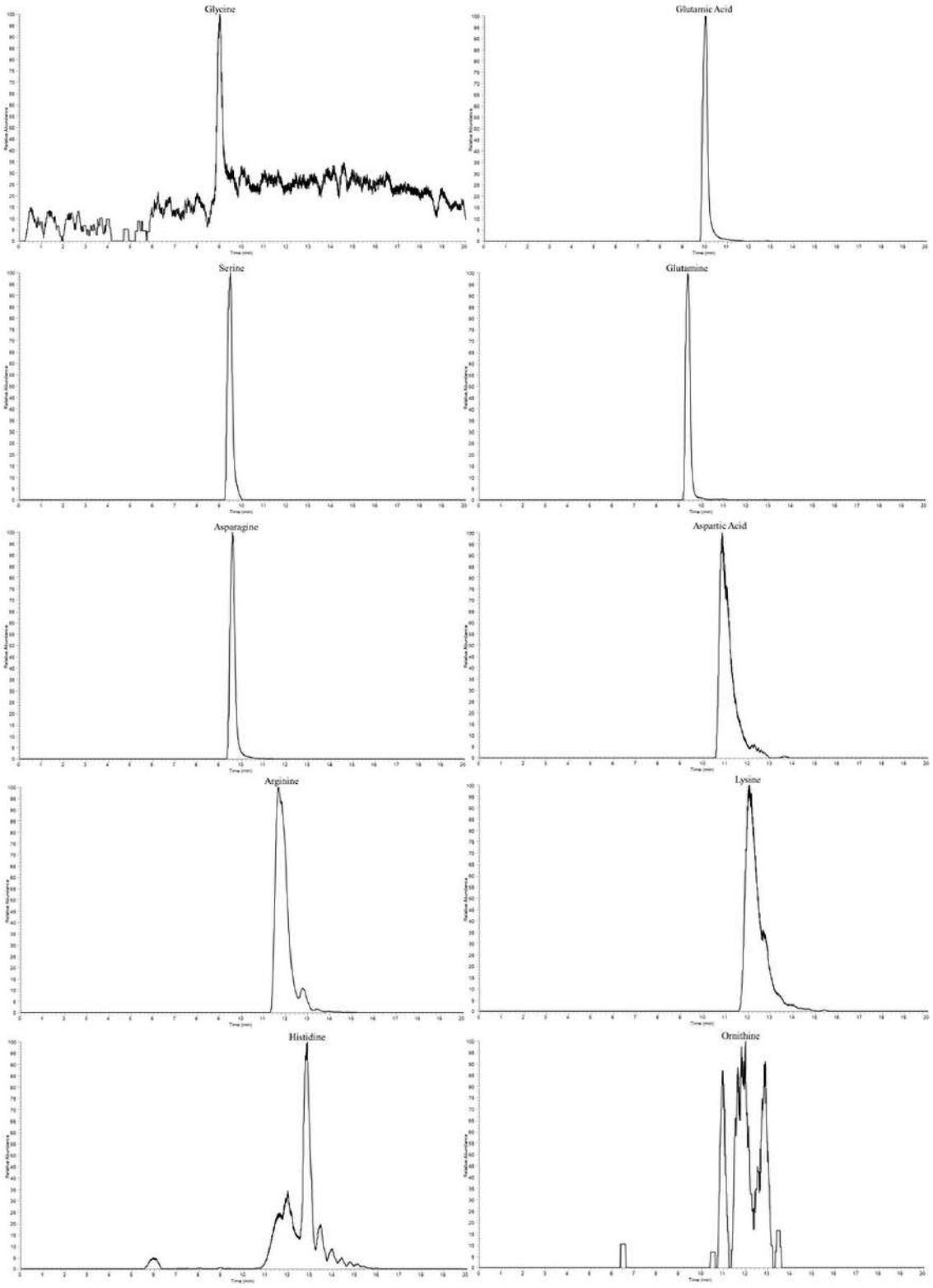

Figure S2. UHPLC-HRMS chromatograms of glycine, glutamic acid, serine, glutamine, asparagine, aspartic acid, arginine, lysine, histidine and ornithine in onion samples.

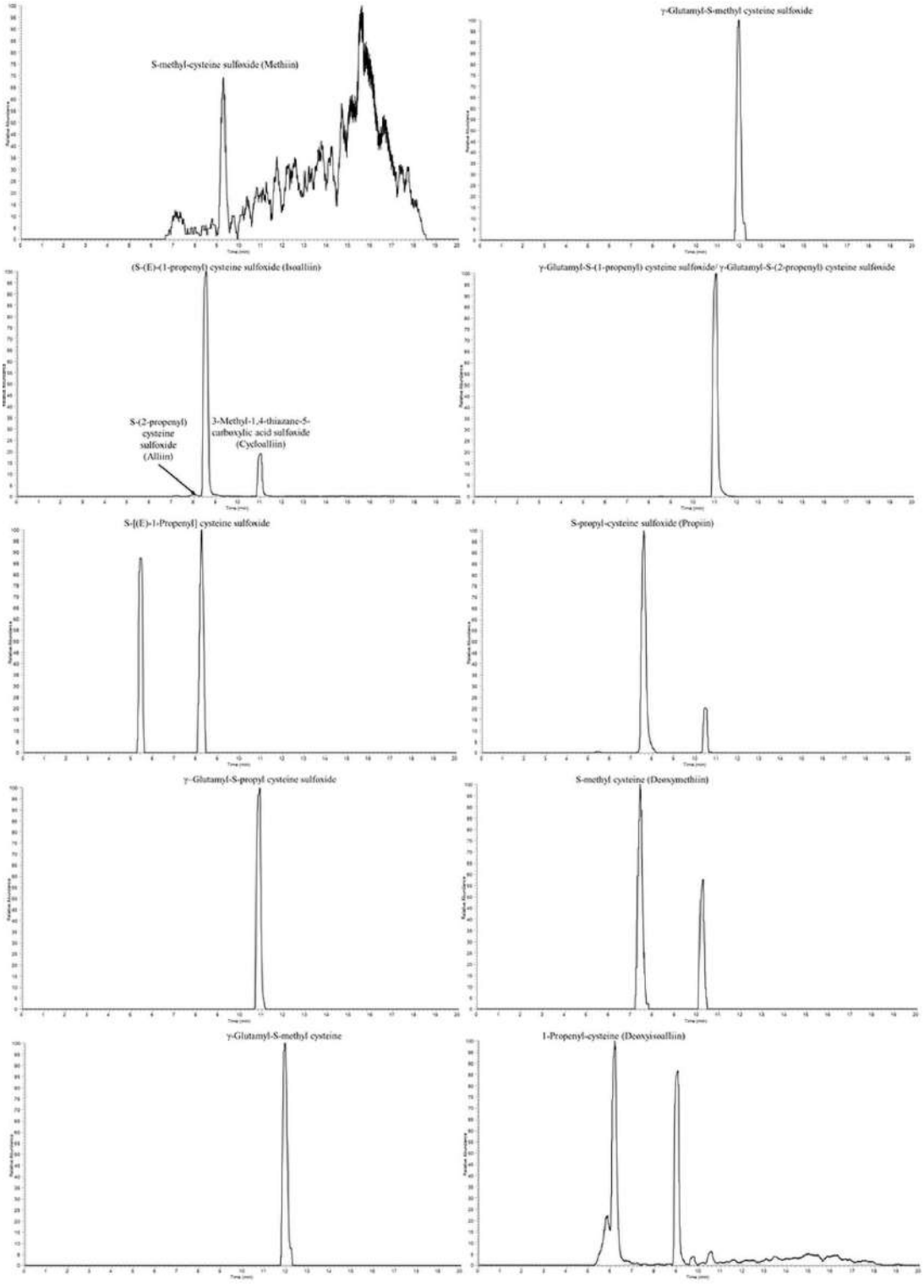

Figure S3. UHPLC-HRMS chromatograms of S-methyl-cysteine sulfoxide (Methiin), γ-Glutamyl-S-methyl cysteine sulfoxide, (S-(E)-(1-propenyl) cysteine sulfoxide (Isoalliin), 3-Methyl-1,4-thiazane-5-carboxylic acid sulfoxide (Cycloalliin), S-(2-propenyl) cysteine sulfoxide (Alliin), γ-Glutamyl-S-(1-propenyl) cysteine sulfoxide, γ-Glutamyl-S-(2-propenyl) cysteine sulfoxide, S-[(E)-1-Propenyl] cysteine sulfoxide, S-propyl-cysteine sulfoxide (Propiin), γ–Glutamyl-S-propyl cysteine sulfoxide, S-methyl cysteine (Deoxymethiin), γ-Glutamyl-S-methyl cysteine and 1-Propenyl-cysteine (Deoxyisoalliin) in onion samples.

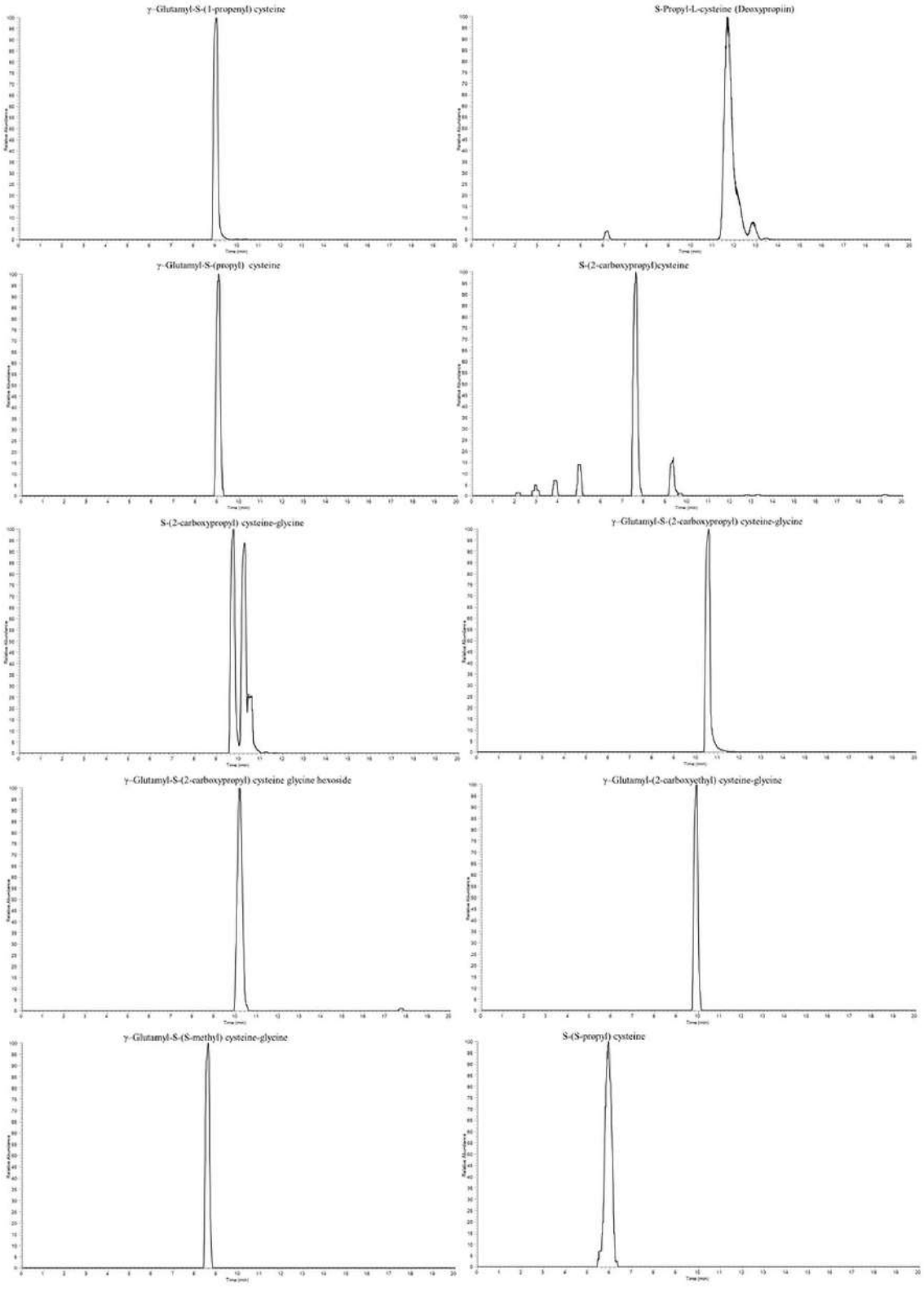

Figure S4. UHPLC-HRMS chromatograms of γ–Glutamyl-S-(1-propenyl) cysteine, S-Propyl-L-cysteine (Deoxypropiin), γ–Glutamyl-S-(propyl) cysteine, S-(2-carboxypropyl) cysteine, S-(2-carboxypropyl) cysteine-glycine, γ–Glutamyl-S-(2-carboxypropyl) cysteine-glycine, γ–Glutamyl-S-(2-carboxypropyl) cysteine-glycine hexoside, γ–Glutamyl-(2-carboxyethyl) cysteine-glycine, γ–Glutamyl-S-(S-methyl) cysteine-glycine and S-(S-propyl) cysteine in onion samples.

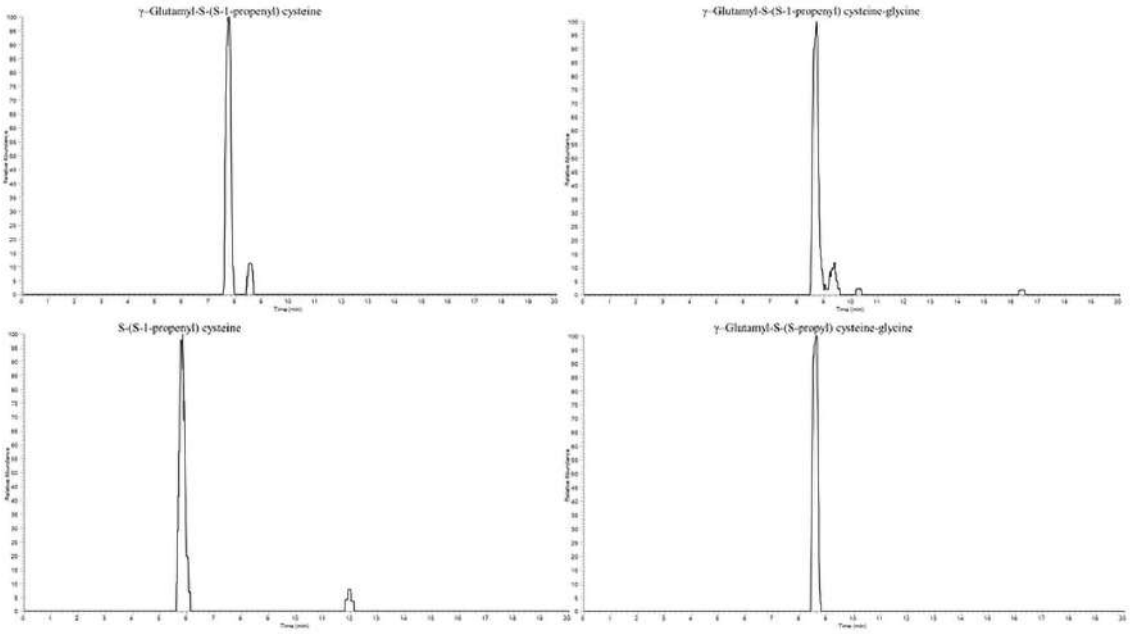

Figure S5. UHPLC-HRMS chromatograms of γ–Glutamyl-S-(S-1-propenyl) cysteine, γ–Glutamyl-S-(S-1-propenyl) cysteine-glycine, S-(S-1-propenyl) cysteine and γ–Glutamyl-S-(S-propyl) cysteine-glycine in onion samples.

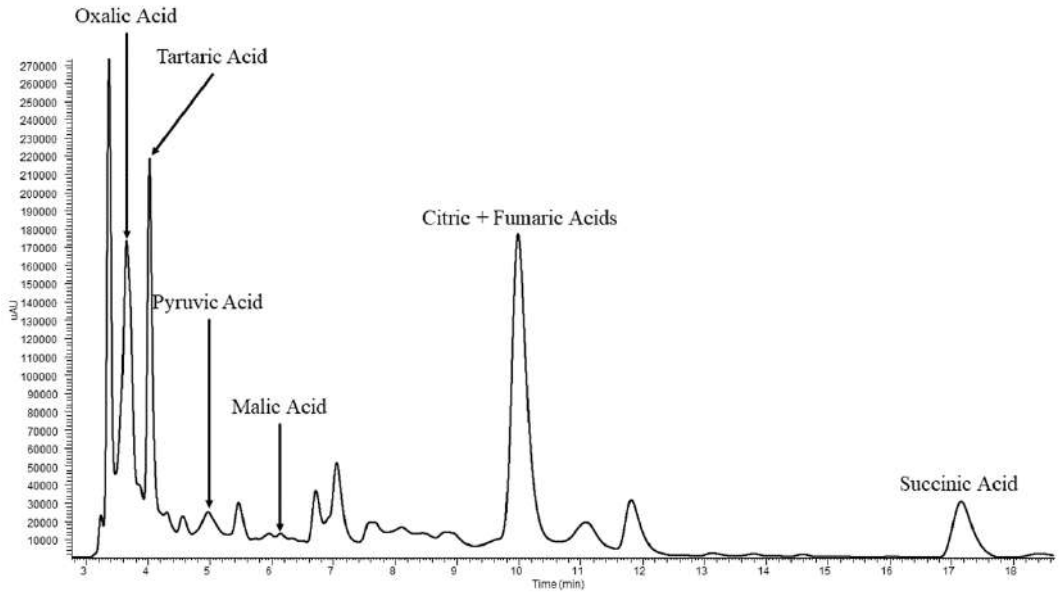

Figure S6. UHPLC-DAD chromatograms of oxalic acid, tartaric acid, pyruvic acid, malic acid, citric acid, fumaric acid and succinic acid in onion samples.

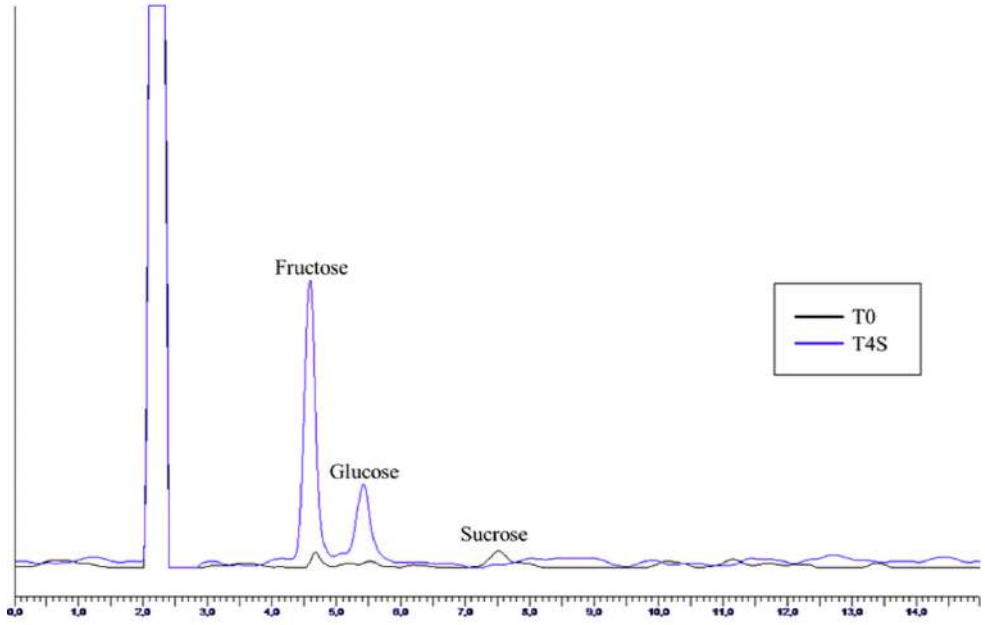

Figure S7. HPLC-RID chromatograms of fructose, glucose and sucrose in T0 and T4S onion samples.

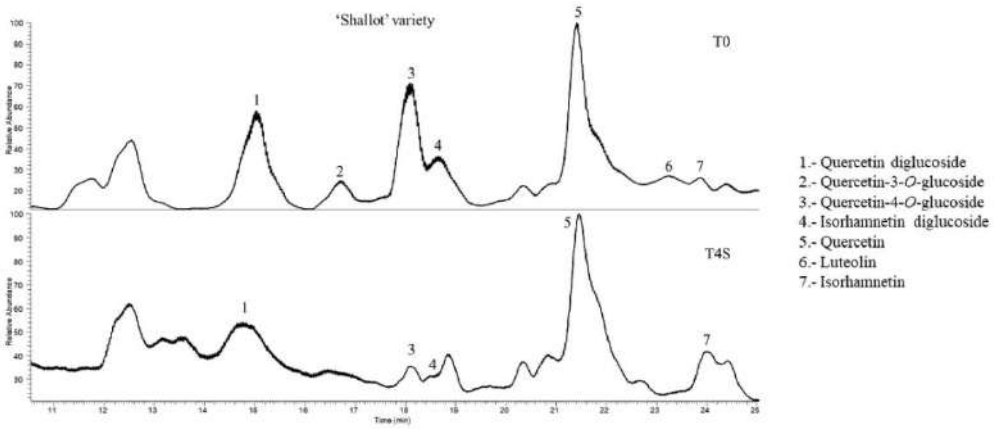

Figure S8. UHPLC-MS chromatograms of quercetin, isorhamnetin, luteolin, quercetin diglucoside, quercetin-3-*O*-glucoside, quercetin-4-*O*-glucoside and isorhamnetin diglucoside in T0 and T4S onion samples.

CAPÍTULO 4

CAPÍTULO 4: RESULTADOS OBJETIVOS 2 Y 3

Artículo 3

Bioaccessibility of Bioactive Compounds of 'Fresh Garlic' and 'Black Garlic' Through In-Vitro Gastrointestinal Digestion

Alicia Moreno-Ortega, Gema Pereira-Caro, José Luis Ordóñez, Rafael Moreno-Rojas, Víctor Ortíz-Somovilla, José Manuel Moreno-Rojas

Foods

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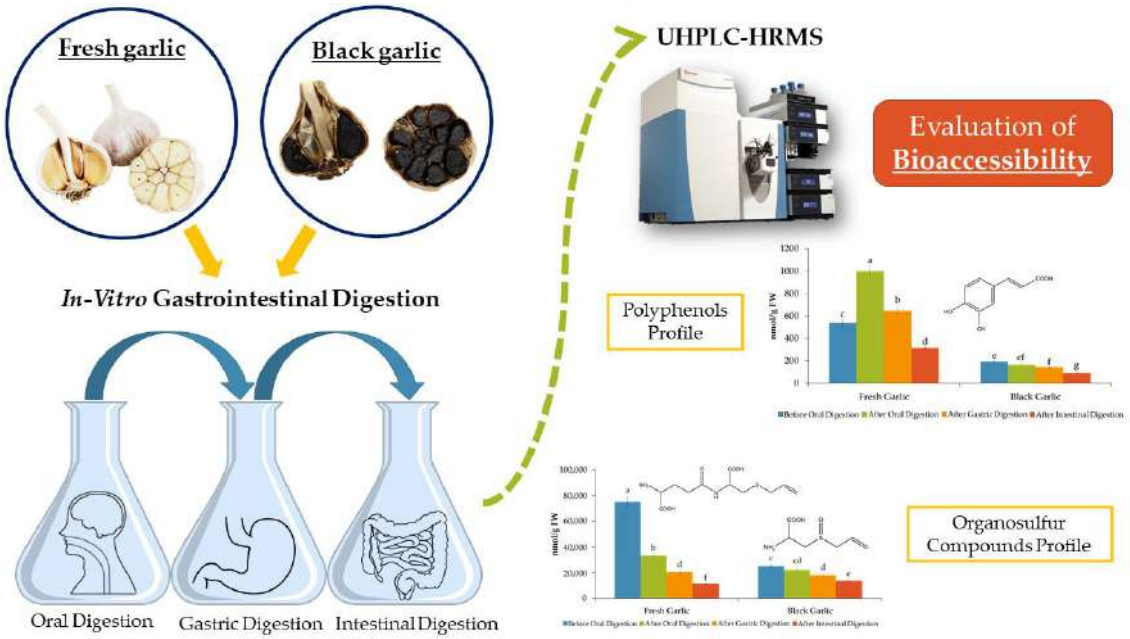

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Article

Bioaccessibility of Bioactive Compounds of 'Fresh Garlic' and 'Black Garlic' through In Vitro Gastrointestinal Digestion

Alicia Moreno-Ortega [1,2], Gema Pereira-Caro [1], José Luis Ordóñez [1], Rafael Moreno-Rojas [2], Víctor Ortíz-Somovilla [1] and José Manuel Moreno-Rojas [1,*]

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Abstract: Numerous studies have reported health benefits associated with the consumption of fresh and black garlic, which are characterized by the presence of polyphenols and organosulfur compounds (OS). This study aims to analyze the bioaccessibility of the bioactive compounds in fresh and black garlic after in vitro gastrointestinal digestion by monitoring the individual profile of these compounds by ultra-high-performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-HRMS). Polyphenols decreased from the beginning of the digestive process, is mainly affected during intestinal digestion. Regarding the OS, the S-alk(en)yl-L-cysteine (SACs) derivatives were more influenced by the acidic conditions of the gastric digestion, while the γ-glutamyl-S-alk(en)yl-L-cysteine (GSAk) derivatives were more susceptible to intestinal digestion conditions in both the fresh and black garlic samples. In conclusion, after in vitro gastrointestinal digestion, the compounds with the highest bioaccessibility were vanillic acid (69%), caffeic acid (52%), γ−glutamyl-S-methyl-L-cysteine sulfoxide (GSMCS) (77%), and S-allylmercapto-L-cysteine (SAMC) (329%) in fresh garlic. Meanwhile, in black garlic, the main bioaccessible compounds were caffeic acid (65%), GSMCS (89%), methionine sulfoxide (262%), trans-S-(1-propenyl)-L-cysteine (151%), and SAMC (106%). The treatment (heating + humidity) to obtain black garlic exerted a positive effect on the bioaccessibility of OS compounds, 55.3% of them remaining available in black garlic, but only 15% in fresh garlic. Polyphenols showed different behavior regarding bioaccessibility.

Keywords: fresh garlic; black garlic; polyphenols; organosulfur compounds; simulated in vitro digestion; bioaccessibility

1. Introduction

Garlic (*Allium sativum* L.) belongs to the *Allium* genus, which is one of the main bulbs consumed either fresh or cooked, and traditionally used as a medicinal plant around the world, or as a basic ingredient in the Mediterranean diet [1,2]. The health benefits of garlic consumption include the prevention of certain non-communicable diseases comprising some types of cancer, cardiovascular diseases, and diabetes, among others [3–5]. These benefits have been associated mostly with the presence of bioactive compounds, such as phenolic and organosulfur compounds [6]. For instance, the regular consumption of garlic has been linked with a reduction in the risk of some types of cancer (gastric, colorectal, lung, or breast cancer) and with beneficial effects on metabolic syndrome,

by delaying lipid absorption and inhibiting cholesterol synthesis [7], and on cardiovascular diseases and hypertension, by decreasing blood pressure and oxidative stress [8]. Preventive effects on obesity by improving insulin sensitivity and decreasing adiposity have also been reported in animal models [9]. The last decade has seen an increase in the popularity of black garlic, a derived product obtained from fresh garlic under specific temperature and humidity conditions [10,11]. Although obtained from fresh garlic, its composition is quite different. It presents higher contents than fresh garlic of reducing sugars, organic acids, and bioactive compounds, particularly S-allyl-cysteine (SAC) and coumaric acid [12,13]. This results in a product with different organoleptic characteristics, such as a darker color, sweeter taste, softer texture and less pungent flavor, making it much more attractive to consumers. Despite the differences between both of them, the consumption of black garlic has been shown to improve human health [14–17], although the mechanism of how phenolic compounds or organosulfur compounds from garlic achieve this is not fully understood.

To better understand the physiological response of black garlic consumption and to compare it with fresh garlic, it is of paramount importance to evaluate the effect of the digestive processes on the stability and availability for the absorption of black garlic bioactives within the human gastrointestinal tract [18]. Simulated gastrointestinal digestion is an in vitro model that aims to simulate the physiological conditions of the upper gastrointestinal tract, namely, the oral, gastric and small intestine phases, and it is frequently used to evaluate the bioaccessibility of a wide variety of bioactive compounds in foods [19,20]. For instance, numerous studies have analyzed the bioaccessibility of phenolic compounds in different food matrices, including berries [21], plant-based beverages [22], tomato [23], and coffee [20]. However, there is limited data on the bioaccessibility of organosulfur compounds, some of the main bioactive compounds of the *Allium* genus. A recent study by Torres-Palazzolo et al. [24] reported the bioaccessibility of different organosulfur compounds in raw versus cooked garlic, showing a 100% bioaccessibility of allicin in raw garlic, while in cooked garlic the bioaccessibility presented 52, 57, 66, and 87% for ajoene, 2-vinyl-4H-1,3-dithiin (2VD), diallyl disulfide (DADS) and diallyl trisulfide (DATS), respectively. To our knowledge, there are no more studies into the effect of gastrointestinal digestion on the individual profile of the phenolic and organosulfur compounds in fresh and black garlic. Therefore, the aim of this study was to determine the impact of simulated gastrointestinal digestion on both fresh and black garlic by determining the bioaccessibility of the (poly)phenols and organosulfur compounds by ultra-high-performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-HRMS) and to evaluate the effect of the treatment used in black garlic elaboration on the bioaccessibility of bioactive compounds.

2. Materials and Methods

2.1. Chemicals

For simulated salivary, gastric, and intestinal fluids (SSF, SGF, and SIF), sodium chloride and magnesium chloride hexahydrate were purchased from Fisher Scientific (Madrid, Spain); sodium bicarbonate and ammonium carbonate were supplied by Sigma-Aldrich (Madrid, Spain); and potassium dihydrogen phosphate was obtained from VWR International Eurolab (Barcelona, Spain). The following were used for digestion: α-amylase from human saliva (300–1500 U/mg protein), pepsin (3.2–4.5 U/mg protein), and pancreatin from porcine pancreas (4 × UPS). Bile salts and calcium chloride were purchased from Sigma-Aldrich (Madrid, Spain), HCl was obtained from Merck (Darmstadt, Germany), and NaOH was acquired from Fisher Scientific (Madrid, Spain). Reference flavonoid compounds, including gallic, caffeic, coumaric, and ferulic acids, catechin, and (−)-epicatechin were purchased from Sigma-Aldrich (Madrid, Spain). Alliin, s-allyl-L-cysteine (SAC), and formic acid (FA) were acquired from Sigma-Aldrich (Madrid, Spain). Ammonium acetate, ammonium formate, and ethanol were obtained from Sigma-Aldrich. Acetonitrile and methanol were of LC-MS grade.

2.2. *Materials and Sample Preparation*

Fresh and black garlic (Spring White Garlic) were supplied by a local supplier (La Abuela Carmen®, Córdoba, Spain). The black garlic samples were obtained from fresh garlic using the protocol previously described [25]. The fresh and black garlic were peeled, and their cloves were ground to a final particle size of 10 µm using a cryogenic grinder with liquid nitrogen mill equipment (Freezer Mill model 6870, Fisher Scientific, Waltham, MA USA) and stored at −80 °C until the in vitro gastrointestinal digestion process.

2.3. *In Vitro Gastrointestinal Digestion*

The in vitro gastrointestinal digestion protocol previously applied by Juániz et al. [26] was adapted to our laboratory. Briefly, the whole process took place in a stirred water bath (Unitronic Reciprocating Shaking Bath model 6,032,011, J.P. Selecta, Barcelona, Spain) at 37 °C with 100 mL amber glass bottles containing 2 g of each sample in triplicate. For the development of the oral phase, 14 mL of SSF solution (Table S1, Supporting Information) was added to the bottles with the samples, together with 250 µL of α-amylase solution (1.3 mg/mL), 0.1 mL of 0.3 M CaCl2, and 5.65 mL of distilled water. The mixture was shaken at 37 °C for 30 min. Then, the gastric phase was started by adjusting the mixture to pH 3 using a 1 M HCl solution. The following step involved adding 15 mL of SGF solution (Table S1, Supporting Information) to the samples, together with 1.19 mL of a pepsin solution (100 mg of pepsin/1 mL of 0.1 M HCl solution), 0.01 mL of 0.3 M CaCl2, and 3.8 mL of distilled water. The mixture was incubated again at 37 °C for 120 min. Next, for the intestinal phase, 22 mL of SIF solution was added to the samples (Table S1, Supporting Information) together with 10 mL of pancreatin solution (8 mg/mL), 5 mL of bile salts (25 mg/mL), 0.08 mL of 0.3 M CaCl2, and 9.92 mL distilled water. Then, 1 M NaOH solution was employed to adjust the pH to 7. Finally, the mixture was incubated for 120 min at 37 °C.

Samples were taken before before oral digestion (BOD) and after oral digestion (AOD) and after gastric (AGD) and intestinal digestion (AID). These samples were lyophilized and stored at −80 °C until extraction and analysis.

2.4. *Phenolic and Organosulfur Compounds Extraction and Analysis*

A previous optimized and validated methodology was used for the extraction and analysis of the organosulfur and phenolic compounds from the digested fresh and black garlic samples [27]. Briefly, 0.5 of fresh or black garlic lyophilized and ground was mixed with 5 mL of deionized water-methanol (50:50, *v/v*) for 2 min at room temperature, and the mixture was sonicated for 15 min and then centrifuged at 4900 rpm for 15 min. The supernatant was collected, and residues were re-extracted twice using 5 mL of the same solvent by following the same protocol described previously. All the supernatants were pooled and frozen at −80 °C until UHPLC-HRMS analysis.

The analysis of polyphenols and organosulfur compounds in fresh and black garlic extracts were carried out using a UHPLC-PDA-MS mass spectrometer system (Thermo Scientific, San José, CA, USA) comprising of a UHPLC pump, a PDA detector scanning from 200 to 600 nm, and an autosampler operating at 4 °C (Dionex Ultimate 3000 RS, Thermo Corporation). Separation of flavonoids was performed on a 100 × 2.1 mm i.d. 1.8 µm Zorbax SB-C18 RRHD column (Agilent, Santa Clara, CA, USA) preceded by a guard pre-column of the same stationary phase and maintained at 40 °C. The mobile phases, A—acidified water 1% formic acid, and B—acetonitrile, were pumped at a flow rate of 0.15 mL min^{-1} with a 33 min gradient starting in 3% B and maintained during 1 min, then rising 60% B in 24 min, maintained during 3 min and then rising 70% B in 5 min. After that, the column was equilibrated to the previous conditions within 5 min. The separation of organosulfur compounds in fresh and black onion extracts were based on a 2.1 × 150 mm ACQUITY UPLC 1.7 µm BEH amide column (equipped with an ACQUITY UPLC BEH amide 1.7 µm van-guard pre-column) (Waters, Spain), which was maintained at 35 °C and eluted using two mobile phases: A—deionized water with 5 mM of ammonium acetate, 5 mM ammonium formate, and 1% formic acid, and B—acetonitrile,

over the course of 20 min at 0.4 mL min^{-1}. The gradient started with 5% of A rising 10% A in 0.5 min, then rising 30% A in 8 min following 46% of A after 4.5 min and finally return to 5% A in 3 min and maintained during 4 min to equilibrate the column to the initial conditions.

After passing through the flow cell of the PDA detector, the column eluate went directly to an Exactive Orbitrap mass spectrometer (Thermo Scientific, San José, CA, USA) fitted with a Heated Electrospray Ionization Probe (HESI) operating in positive ionization mode for the determination of OS compounds and in negative ionization mode for the determination of polyphenols [27].

The identification of polyphenols and OS compounds were achieved as follows: (i) By comparing the exact mass and the retention time with available standards, (ii) in the absence of standards, compounds were tentatively identified by comparing the exact theoretical mass of the molecular ion with the measured accurate mass of the molecular ion and searched against metabolite databases, including Metlin, Phenol Explorer, and more general chemical databases, such as PubChem and ChemSpider. Compounds having molecular masses within the pre-specified tolerance (≤5 ppm) of the query masses are retrieved from these databases. The quantification of phenolic compounds and OS compounds was carried out by selecting the exact theoretical mass of the molecular ion by reference to standard curves prepared in diluted fresh and black garlic extracts, obtaining a linear regression analysis with R^2 values of >0.998 (n = 6). In the absence of reference compounds, they were quantified by reference to the calibration curve of a closely related parent compound. The detection and quantification limits varied from 0.0004 to 0.007 ng μL^{-1} and from 0.012 to 0.024 ng μL^{-1} for polyphenols and were 0.03 and 0.1 ng μL^{-1}, for OS compounds, respectively.

2.5. *Bioaccessibility of (Poly)Phenols and Organosulfur Compounds*

The bioaccessibility index was used for calculating the percentage of bioaccessibility of (poly)phenols and organosulfur compounds after simulated gastrointestinal digestion [28,29].

$$\text{Bioaccessibility} = \text{FC/IC} * 100$$

where FC is the Final Concentration (compound concentration after simulated gastrointestinal digestion), and IC is the Initial Concentration (compound concentration before simulated gastrointestinal digestion).

2.6. *Statistical Analysis*

Statistical analyses were performed based on six replicate measures of each sample. A one-way ANOVA was carried out using R software (v. 3.6.3, R Core Team, Vienna, Austria) to determine significant differences between the stages of the in vitro gastrointestinal digestion, the significance being accepted for a p-value < 0.05. Next, Fisher's LSD pairwise comparison was performed on the data.

3. Results and Discussion

3.1. *Bioaccessibility of Phenolic Compounds after Simulated Gastrointestinal Digestion*

Before oral digestion, a total of five (poly)phenols were identified in the fresh garlic and four in the black garlic (Table 1, Figure 1). Details of their identification and quantification are presented in the Supporting Information, Table S2. Among phenolic acids, caffeic acid and gallic acid were the main compounds in the fresh garlic, accounting for 95.3% of the total phenolic content, while these compounds plus coumaric acid were the main ones found in the black garlic, representing 99.4% of the total polyphenol content. These results are in line with those reported by Kim et al. [30], who demonstrated that caffeic acid was the major phenolic compound in fresh garlic, while in black garlic, it was coumaric acid.

Table 1. Concentration (nmol/g FW) of (poly)phenols presented in fresh and black garlic samples at different stages during the simulated gastrointestinal digestion. Data are expressed as mean values (n = 6).

Compounds	BOD	AOD	% Recovery	AGD	% Recovery	AID	% Recovery-Bioaccessibility	p-Value
				Fresh Garlic				
Benzoic Acid	nq	162 ab	-	141 b	-	173 a	-	***
Vanillic acid	18.3 a	15.5 a	84.7	11.7 b	63.9	12.7 b	69.4	***
Gallic acid	58.7 b	105 a	180	119 a	203	14.8 c	25.2	***
Caffeic acid	455 b	712 a	156	370 b	81.3	112 c	24.6	***
Ferulic Acid	6.8 a	5.6 b	82.4	3.8 c	55.9	3.5 c	51.5	***
(+)-Catechin	nq	nq	-	0.01 b	-	0.11 a	-	***
(−)-Epicatechin	nq	0.092 a	-	0.04 b	-	0.03 c	-	***
Epigallocatechin	0.19 c	0.39 b	203	0.44 a	230.0	0.07 d	35.3	***
Chlorogenic acid	nq	0.054 a	-	0.04 b	-	0.02 c	-	***
Total	539 c	1001 a	185.9	646 b	120.0	316 d	58.6	***
				Black Garlic				
Gallic Acid	14.7 a	11.2 b	76.7	nq	-	nq	-	***
Caffeic Acid	142 a	131 ab	92.3	113 b	79.6	92 c	64.8	***
Coumaric Acid	37.1 a	25.3 c	68.2	33.1 b	89.4	nq	-	***
Epigallocatechin gallate	1.2 a	0.95 b	79.4	nq	-	nq	-	***
Total	195 a	168 b	86.2	146 c	74.9	92 d	47.2	***

Different letters (one-way ANOVA) denote significant differences ($p < 0.05$) among the five stages for the same compound. Ns, non-significant; *** p-value < 0.001. nq, No-quantified. BOD, Before Oral Digestion; AOD, After Oral Digestion; AGD, After Gastric Digestion; AID, After Intestinal Digestion.

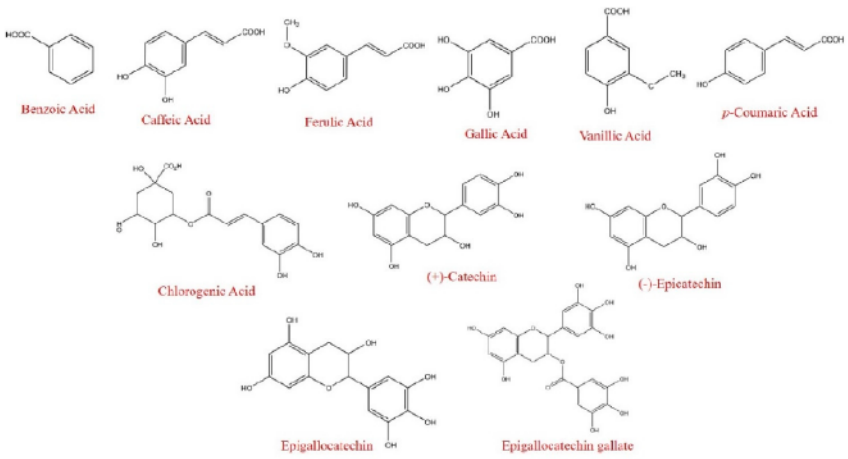

Figure 1. Chemical structures of fresh and black garlic polyphenols.

After in vitro oral digestion, the total polyphenol content of the fresh garlic significantly increased (1001 nmol/g FW, Fresh Weight), accounting for a mean recovery of 185.9%. This increase was mainly attributed to the 1.5 and 1.8-fold increase in the levels of caffeic acid and gallic acid, respectively, along with the appearance of a substantial amount of benzoic acid (162 nmol/g FW) (Table 1). During the oral digestion, epicatechin, and chlorogenic acid also appeared in the fresh garlic at low concentrations (0.09 and 0.05 nmol/g FW, respectively). The increase in hydroxycinnamic acids, including gallic, coumaric, and ferulic acid, during the oral stage of the simulated digestion of soymilk [31], and flours from persimmon fruit [32] has been reported by other authors. It is considered to be mainly due to pH and enzymatic activity, which could induce the breakdown between these compounds and other food components.

After in vitro gastric digestion, the content of most of the (poly)phenols in the fresh garlic showed a notable decrease, with recoveries ranging from 55.9% for ferulic acid to 81.3% for caffeic acid (Table 1); while the concentration of gallic acid (203% recovery with regard to the initial content in the fresh garlic) remained stable compared with its concentration after oral digestion. The flavonoid epigallocatechin also showed a significant increase in its concentration after gastric digestion with a recovery of 230%, although it remained a minor component. These results agree with those reported by Lucas-González et al. [32], who found an increase in the gallic acid content of 160 and 176.7% in two types of flours obtained from persimmon fruit after oral and gastric digestion. In addition, catechin appeared for the first time during this stage, although it was a marginal compound. Despite the significant decrease in some polyphenols after gastric digestion, the total phenolic content of the fresh garlic after in vitro gastric digestion was 646 nmol/g FW, representing a mean recovery of 120% at the end of this stage (Table 1). During intestinal digestion, the last step of the digestion process, there was a significant reduction in the total content of (poly)phenols, 58.6% being the mean bioaccessibility index of (poly)phenols in the fresh garlic (Table 1). Individually, most of the polyphenols presented initially in the fresh garlic significantly decreased their concentration after in vitro digestion, vanillic acid (69.4%), and ferulic acid (51.5%) being the phenolics that presented the highest bioaccessibility, followed by epigallocatechin (35.3%). Meanwhile, gallic acid and caffeic acid were more likely to be affected by the in vitro digestion, with bioaccessibility indexes of 25.2 and 24.6%, respectively. It is worth mentioning that benzoic acid, catechin, epicatechin, and chlorogenic acid appeared after in vitro digestion, but were not identified in fresh garlic. These compounds arguably come from the breakdown of supramolecular structures that store phenolic compounds as the apparently weak bond of these phenolic acids with dietary fiber allows them to be released more easily during simulated gastrointestinal digestion [33]. Nevertheless, except for benzoic acid, these compounds were found in low amounts, accounting for 0.05% of the total phenolic content after the complete digestion.

Regarding black garlic, the content of almost all the (poly)phenols significantly decreased after oral digestion, with a mean recovery of 86.2%. Phenolics, such as gallic acid, coumaric acid, and epigallocatechin gallate, presented recoveries of 76.7, 68.2, and 79.4% respectively, while caffeic acid seemed not to be seriously affected by oral digestion, with a recovery of 92.3%.The stability of caffeic acid after oral digestion in black garlic could be a consequence of a more complex black garlic matrix, which may affect its bioaccessibility. Indeed, it has been shown that the matrix components, such as the proteins, carbohydrates, and fiber, could affect phytochemical bioaccessibility, and therefore, their release from the food matrix during gastrointestinal digestion [31,34].

After gastric digestion, the total content of phenolic compounds in black garlic significantly decreased, with a recovery of 74.9%. Two phenolic compounds, gallic acid, and epigallocatechin gallate, disappeared after this step. Likewise, the content of caffeic acid, the main phenolic compound in black garlic, slightly decreased, with a recovery of 79.6% with regard to its initial amount. However, the content of coumaric acid increased by 31.0% during this stage. Moreover, during the last in vitro digestion step, the in vitro intestinal digestion, there was a significant loss in the quantity of almost all the (poly)phenols, caffeic acid being the only (poly)phenol present after the in vitro digestion process of the black garlic, with a bioaccessibility index of 64.8% (Table 1). Overall, the total phenolic content of the black garlic during the whole in vitro digestion process decreased, the mean bioaccessibility index of (poly)phenols in black garlic being 47.2% (Table 1).

In summary, the total amount of (poly)phenols in the fresh garlic increased significantly after oral digestion, mainly due to the notable emergence of benzoic acid and the significant increase in caffeic and ferulic acid (Figure 2). Subsequently, the (poly)phenolic content decreased during the rest of the gastrointestinal digestion, this being more pronounced during the intestinal digestion. This trend could be related to the fact that the phenolic content in garlic is mainly represented by phenolic acids, which are more unstable under the conditions of intestinal digestion (basic pH), while at acid pH, these compounds are easily released from the matrix [35]. In contrast, in the black garlic, although there was a significant decrease in the content of polyphenols during the gastrointestinal digestion (Figure 2), with only caffeic acid remaining bioaccessible, the effect of the gastric and intestinal conditions was not so marked when compared with fresh garlic. Moreover, it is noteworthy that the gastrointestinal digestion had a negative effect on the bioaccessibility of the polyphenols in both matrices, 58.6% and 42.7% remaining bioaccessible in fresh garlic and black garlic, respectively.

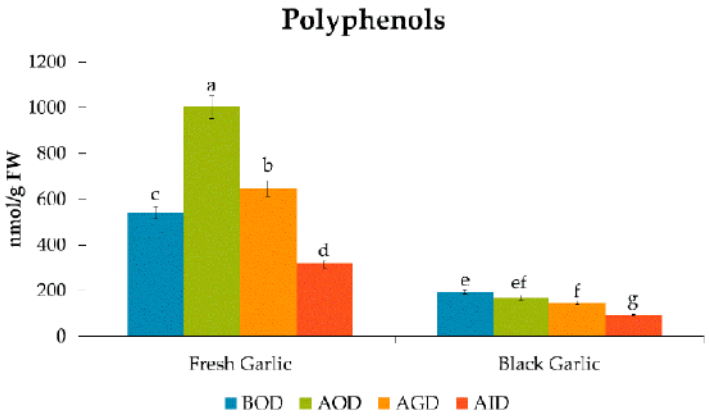

Figure 2. Quantities of total polyphenols during the in vitro gastrointestinal digestion. Data are expressed as nmol/g FW as mean values ($n = 6$). Different letters (one-way ANOVA) denote statistically significant differences between the stages of simulated gastrointestinal digestion (p-value < 0.05). (BOD, Before Oral Digestion; AOD, After Oral Digestion; AGD, After Gastric Digestion; AID, After intestinal digestion).

3.2. Bioaccessibility of Organosulfur Compounds after Simulated Gastrointestinal Digestion

A total of 23 organosulfur compounds (OS) were identified and quantified in the fresh garlic before the simulated gastrointestinal digestion, including 13 S-alk(en)yl-L-cysteine derivatives (SACs) and 10 γ-glutamyl-S-alk(en)yl-L-cysteine derivatives (GSAks), which accounted for 76.8% and 23.1% of the total OS in the samples, respectively (Table 2, Figure 3). Details of their identification and quantification are presented in Table S3 (Supporting Information). Among them, alliin (41706 nmol/g FW), cycloalliin (5251 nmol/g FW), methiin (4425 nmol/g FW), and trans-S-(1-propenyl)-L-cysteine (S1PC) (2909 nmol/g FW) were the main S-alk(en)yl-L-cysteine (SACs) derivatives in the fresh garlic, while γ−glutamyl-S-allyl-L-cysteine (GSAC) (13,714 nmol/g FW), γ-glutamyl-S-methyl-L-cysteine (GSMC) (1580 nmol/g FW) and γ-glutamyl-S-(1-propenyl) cysteine sulfoxide (G1PCS) (1311 nmol/g FW) were the main GSAk derivatives. These results are in line with those reported by Goncharov et al. [36], who found that alliin is the major organosulfur compound in raw garlic along with other organosulfur derivatives, including S1PC.

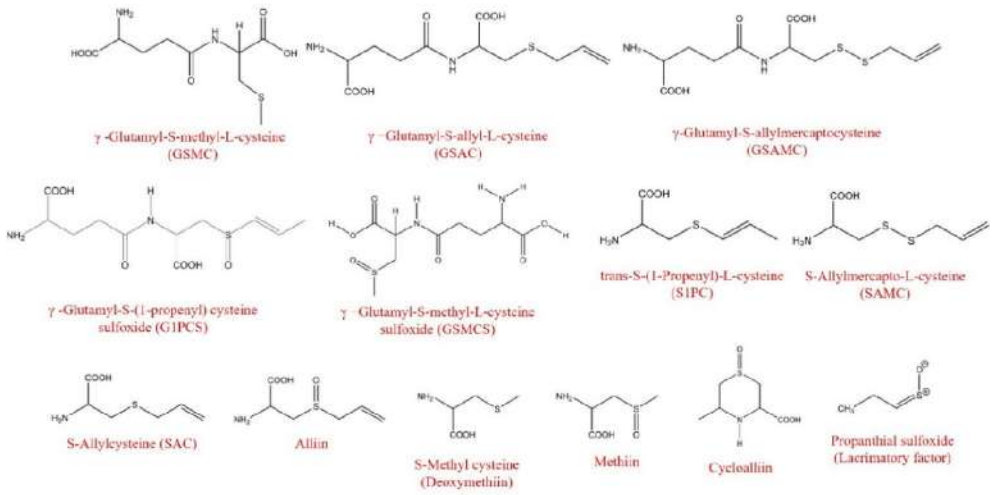

Figure 3. Chemical structures of fresh and black garlic organosulfur compounds.

After oral digestion, the total content of organosulfur compounds in the fresh garlic decreased notably, showing a mean recovery of total OS of 44.4% (Table 2). The compounds mostly affected during the oral digestion were the SACs derivatives, with a mean recovery of 37.1%, while the GSAk derivatives were less affected, with a mean recovery of 68.6%. Among them, the levels of γ–glutamyl-S-(S-1-propenyl)cysteine-glycine, γ–glutamyl-S-(S-1-methyl)cysteine-glycine, γ–glutamyl- S-(S-1-propyl) cysteine (GS1PC), γ–glutamyl-S-allyl-L-cysteine (GSAC), γ–glutamyl-S-allylmercaptocysteine (GSAMC) and γ–glutamyl-cysteine decreased, which is probably, at least in part, a consequence of the enzyme hydrolysis during the oral phase [37] resulting in the loss of the γ–glutamyl group and in the formation of other OS compounds. For instance, S-allylmercapto-L-cysteine (SAMC), with a recovery of 156% after oral digestion, likely originates from the hydrolysis of GSAMC, as has been indicated in Figure 4.

Table 2. Concentration (nmol/g FW) of organosulfur compounds presented in fresh and black garlic samples at different stages during the simulated gastrointestinal digestion. Data are expressed as mean values ($n = 6$).

Organosulfur Compounds.	BOD	AOD	% Recovery	AGD	% Recovery	AID	% Recovery-Bioaccessibility	*p*-Value
				Fresh Garlic				
γ-Glutamyl-S-Alk(en)yl-L-Cysteine Derivatives (GSAk)								
γ-Glutamyl-S-(2-carboxypropyl) cysteine-glycine	287 a	253 a	88.2	163 b	56.9	71 c	24.6	***
γ-Glutamyl-S-(S-1-propenyl) cysteine-glycine	159.0 a	65.6 b	41.3	45.5 c	28.6	11.3 d	7.1	***
γ-Glutamyl-S-(S-methyl) cysteine-glycine	10.3 a	4.0 b	39.0	2.2 c	21.3	0.8 d	8.0	***
γ-Glutamyl-S-methyl-L-cysteine (GSMC)	1580 a	1490 a	94.3	899 b	56.9	598 c	37.9	***
γ-Glutamyl-S-(propenyl) cysteine (GS1PC)	6.7 a	3.5 b	52.3	2.7 b	40.3	1.5 c	22.8	***
γ-Glutamyl-S-allyl-L-cysteine (GSAC)	13,714 a	8837 b	64.4	5849 c	42.7	3328 d	24.3	***
γ-Glutamyl-S-allylmercaptocysteine (GSAMC)	343 a	158 b	46.0	97 c	28.1	35 d	10.2	***
γ-Glutamyl-cysteine	36.1 a	19.3 b	53.5	10.6 c	29.3	4.4 d	12.2	***
γ-Glutamyl-S-methyl-L-cysteine sulfoxide (GSMCS)	17.0 a	14.5 b	85.3	13.0 b	76.1	13.0 b	76.6	***
γ-Glutamyl-S-(1-propenyl) cysteine sulfoxide (G1PCS)	1311 a	1142 a	87.1	706 b	53.8	526 b	40.1	***
Total GSAk Derivatives	17,464 a	11,987 b	68.6	7788 c	44.6	4589 d	26.3	***
S-Alk(en)yl-L-Cysteine Derivatives (SACs)								
S-Methylcysteine (Deoxymethiin)	534 a	270 b	50.7	118 c	22.1	52 d	9.8	***
S-Allylcysteine (SAC)	1144 a	1017 a	88.9	384 c	33.6	696 b	60.8	***
S-(2-Carboxypropyl) cysteine	88 a	50 b	57.0	34 c	38.4	21 d	24.1	***
S-allylglutathione (SAG)	2.7 a	1.4 b	52.9	0.8 c	29.2	0.4 d	14.2	***
trans-S-(1-Propenyl)-L-cysteine (S1PC)	2909 a	1868 b	64.2	1216 c	41.8	662 d	22.8	***
S-Allylmercapto-L-cysteine (SAMC)	164 c	256 b	156.4	296 b	181.1	539 a	329.3	***
S-Allylsulfenic acid (Lacrimatory factor)	1517 a	887 b	58.5	624 c	41.2	291 d	19.2	***
Alliin	41,706 a	11,621 b	27.9	5937 c	14.2	2207 c	5.3	***
S-Methyl-l-cysteine sulfoxide (Methiin)	4425 a	2651 b	59.9	2176 c	49.2	1668 d	37.7	***
S-Propyl-L-cysteine sulfoxide (Propiin)	26.89 a	3.7 b	13.7	1.9 bc	7.2	0.9 c	3.2	***
Cycloalliin	5251 a	2741 b	52.2	1855 c	35.3	771 d	14.7	***
Methionine sulfoxide	14.5 a	8.4 c	57.9	8.7 c	60.0	10.5 b	72.4	***

Table 2. *Cont.*

Organosulfur Compounds.	BOD	AOD	% Recovery	AGD	% Recovery	AID	% Recovery-Bioaccessibility	*p*-Value
				Fresh Garlic				
N-Acetyl-S-allyl-L-cysteine (NASAC)	60.3 b	69.1 a	114.7	37.5 c	62.2	10.4 d	17.2	***
Total SACs Derivatives	57,842 a	21,444	37.1	12,689 c	22.0	6929 d	12.0	***
Total OS Compounds	75,306 a	33,431 b	44.4	20,477 c	27.2	11,518 d	15.3	***
				Black Garlic				
γ-Glutamyl-S-Alk(en)yl-L-Cysteine (GSAk)								
γ-Glutamyl-S-(S-1-propenyl) cysteine-glycine	119 a	120 a	100.9	120 a	101.1	nd	-	***
γ-Glutamyl-S-methyl-L-cysteine (GSMC)	199 a	206 a	103.7	200 a	100.7	nd	-	***
γ-Glutamyl-S-allyl-L-cysteine (GSAC)	4393 a	3900 ab	88.8	3597 b	81.9	934 c	21.3	***
γ-Glutamyl-S-allylmercaptocysteine (GSAMC)	504 a	510 a	101.2	560 a	111.3	287 b	57.1	***
γ-Glutamyl-S-methyl-L-cysteine sulfoxide (GSMCS)	380	384	101.1	340	89.4	338	88.9	ns
γ-Glutamyl-S-(1-propenyl) cysteine sulfoxide (G1PCS)	3452 a	3346 a	96.9	2645 b	76.6	2305 b	66.8	***
Total GSAk Derivatives	9046 a	8466 a	93.6	7463 b	82.5	3864 c	42.7	***
S-Alk(en)yl-L-Cysteine (SACs)								
S-methyl cysteine (Deoxymethiin)	168 b	166 b	98.7	218 a	129.8	nd	-	***
S-Allyl-L-cysteine (SAC)	7683 a	6189 b	80.5	4066 c	52.9	2803 d	36.5	***
S-(2-carboxypropyl) cysteine	115 b	116 b	100.7	149 a	130.3	nd	-	***
S-allylmercapto-L-cysteine (SAMC)	408 ab	358 b	87.7	224 c	54.9	434 a	106.4	***
S-allylglutathione	46.6 a	43.6 a	93.6	nd	-	nd	-	
trans-S-(1-Propenyl)-L-cysteine (S1PC)	205 c	198 c	96.6	243 b	118.5	310 a	151.2	***
S-allylsulfenic acid (Lacrimatory factor)	935 a	818 ab	87.5	648 b	69.4	895 a	95.7	**
S-(S-propyl) cysteine	229 b	355 a	155.3	nd	-	nd	-	***
Alliin	5611 a	4735 ab	84.4	4423 b	78.8	4302 b	76.7	**
S-methyl-cysteine sulfoxide (Methiin)	243 a	255 a	104.9	235 a	96.6	nd	-	***
Methionine sulfoxide	502 b	535 b	106.6	411 c	81.9	1314 a	262.0	***
Total SACs Derivatives	16,145 a	13,768 b	85.3	10,617 c	65.8	10,059 c	62.3	***
Total OS Compounds	25,191 a	22,234 b	88.3	18,080 c	71.8	13,923 d	55.3	***

Different letters (one-way ANOVA) denote significant differences ($p < 0.05$) among the five stages for the same compound. Ns, non-significant; ** *p*-value < 0.01; *** *p*-value < 0.001. nd, No-detected. BOD, Before Oral Digestion; AOD, After Oral Digestion; AGD, After Gastric Digestion; AID, After Intestinal Digestion.

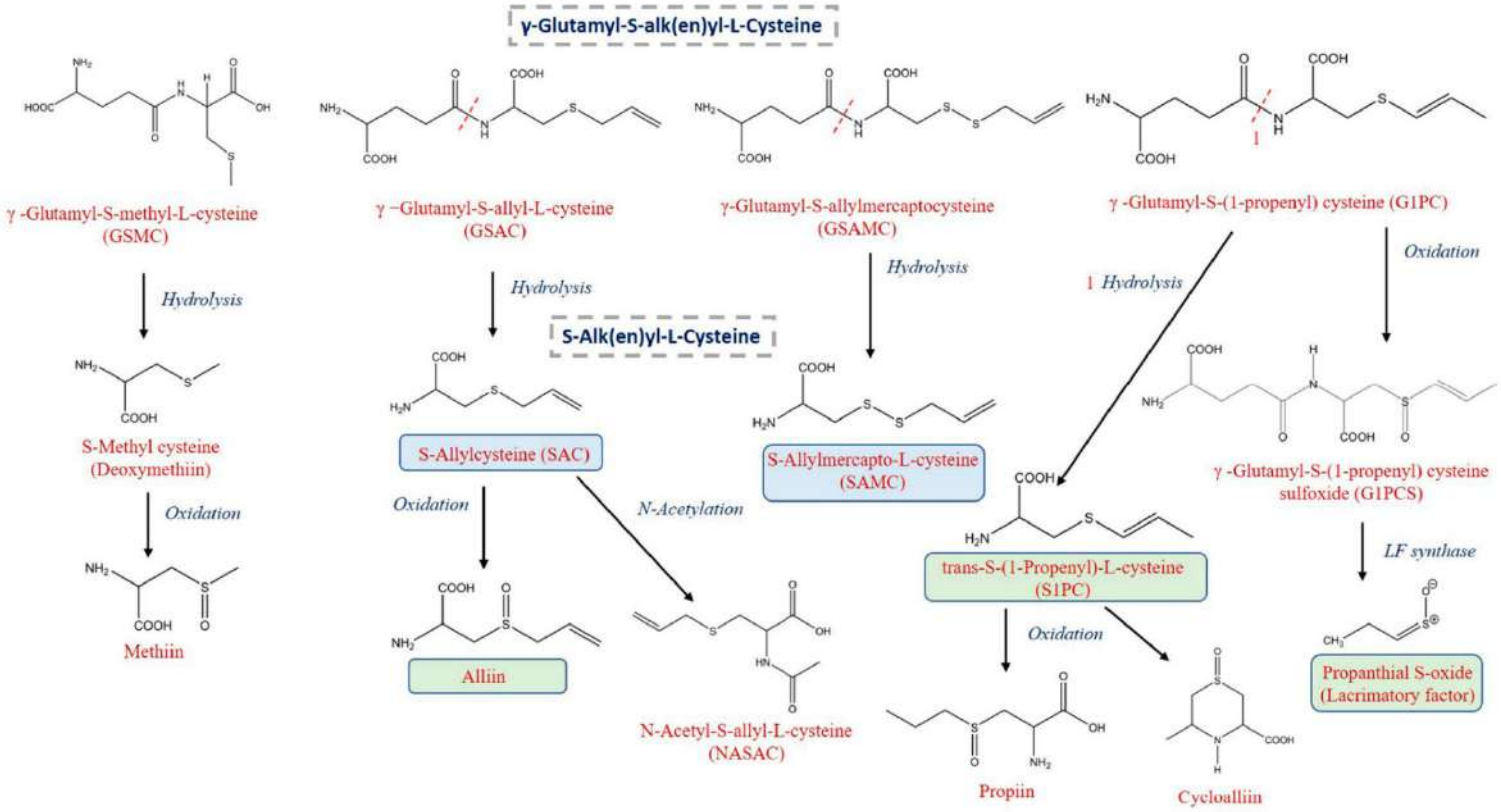

Figure 4. Proposed breakdown pathway of organosulfur compounds during in vitro gastrointestinal digestion.

After the gastric digestion of the fresh garlic samples, a reduction was observed in the concentration of almost all the OS compounds, with a mean recovery of 27.2%, highlighting the susceptibility of these compounds to simulated gastric conditions. The mean recovery of GSAk derivatives was 44.6%, ranging from 21.3% for γ–glutamyl-S-(S-methyl) cysteine-glycine to 76.1% for GSMCS; while for SACs derivatives, the recovery ranged from 7.2% for propiin to 62.2% for NASAC, with a mean recovery of 22.0%. It is of note that the concentration of SAMC continued to increase after gastric digestion, accounting for 181.1% of recovery (Table 2). This could be explained by the fact that SAMC is a metabolite from alliin produced when it reacts with cysteine after ingestion of fresh garlic [38]. This is arguably due to the hydrolysis of its precursor GSAMC, whose concentration decreased by almost 90% during the in vitro digestion (Figure 4) [39].

At the end of the digestion process, the organosulfur compounds from the fresh garlic showed a total mean bioaccessibility of 15.3%, GSAK derivatives (26.3%) showing a higher bioaccessibility index than SACs derivatives (12.0%). The organosulfur compound GSAC, followed by GSMC and G1PCS, was the main GSAk derivative observed after the gastrointestinal digestion of the fresh garlic (38.7%), while alliin, methiin, cycloalliin, SAC, SAMC, and S1PC were the main SACs derivatives, accounting for 56.8% of the total OS content. The OS compounds with the highest bioaccessibility indexes were SAC, methionine sulfoxide, GSMCS, and SAMC, with 60.8, 72.4, 76.6, and 329.3%, respectively. To the best of our knowledge, this is the first time that the in vitro gastrointestinal stability of fresh garlic OS has been studied.

Regarding the black garlic, a total of 17 organosulfur compounds were identified and quantified (Table 2, Figure 3), of which 64.1% were SACs derivatives, SAC (30.5%), and alliin (22.3%) being the main ones. The main GSAk derivatives were GSAC (17.4%) and G1PCS (13.7%), accounting for 31.1% of the total OS in the black garlic (Table 2). These data are in line with Molina-Calle et al. [40], who also tentatively identified SAC, alliin, and GSAC in the extracts from black garlic.

After the oral digestion of the black garlic, OS compounds showed better stability than in the fresh garlic, with an average of 88.3%, the recovery rates ranging from 80.5 to 106.6% (Table 2). The significant 1.5-fold increase in S-(S-propyl) cysteine after the oral digestion of the black garlic is noteworthy (Table 2). After gastric digestion, significant changes were observed in the concentration of GSAC and G1PCS, which lead to a significant decrease in the concentration of GSAk derivatives after this digestion step with a mean recovery of 82.5%. Moreover, some SACs derivatives, including S-allylglutathione and S-(S-propyl) cysteine disappeared, and others, such as SAC, SAMC, the lacrimatory factor, alliin, and methionine sulfoxide were strongly affected by the gastric conditions, their concentrations decreasing significantly; other SACs derivatives, such as deoxymethiin, S-(2-carboxypropyl)cysteine, S1PC, and methiin, were stable or their concentrations even significantly increased (Table 2).

After intestinal digestion, the total OS content in the black garlic decreased significantly, with a mean recovery of 55.3%. Particularly, GSAk derivatives were seriously affected, showing a mean recovery of 42.7%. This marked a decrease in their concentration is likely due to the great instability of these compounds under alkaline pH conditions (intestinal digestion), consistent with earlier reports [41]. Moreover, compounds, such as methiin, deoxymethiin, S-(2-carboxypropyl) cysteine, GSMC, and γ–Glutamyl-S-(S-1-propenyl) cysteine-glycine, were not detected after the intestinal phase. Conversely, three organosulfur compounds, namely, SAMC, S1PC, and methionine sulfoxide, showed an increase in their concentration during the simulated intestinal digestion, with respective recovery rates of 106.4, 151.2, and 262.0%. The 3-fold increase in methionine sulfoxide after gastrointestinal digestion is probably due to the oxidation of methionine during the in vitro digestion [42].

Overall, the bioaccessibility index of total organosulfur compounds was higher for black than fresh garlic, 55.3 and 15.3%, respectively (Figure 5). Indeed, 7 of the 13 organosulfur compounds found in both matrices—GSMCS, G1PCS, GSAMC, alliin, methionine sulfoxide, trans-S-(1-propenyl)-L-cysteine and lacrimatory factor— showed higher bioaccessibility indexes in black garlic (Table 2). Regarding the individual OS compounds, alliin was the main SACs derivative present in both matrices, presenting a bioaccessibility of 5.3 and 76.6% in fresh and black garlic, respectively. This means that even though

the content of alliin in fresh garlic was 7-fold higher than in black garlic before oral digestion, after this process, its content was almost 2-fold higher in black garlic, suggesting a higher bioaccessibility for this compound in the black garlic. In contrast, SAMC showed a significantly higher bioaccessibility index in fresh (329.3%) compared to black garlic (106.4%), despite presenting 164 nmol/g FW and 408 nmol/g FW, respectively, before digestion. Related to this, Xiao et al. [43] and Yi et al. [44] reported that SAMC presented activity that inhibited tumor growth in in vivo models. Furthermore, as discussed above, SAMC is a metabolite derived from the metabolic pathways of GSAMC [38,39], and the decrease in this compound was evidenced throughout the digestive process. Conversely, a higher percentage of bioaccessibility of methionine sulfoxide was found in the black garlic, with 262.0%, compared with 72.6% in the fresh garlic.

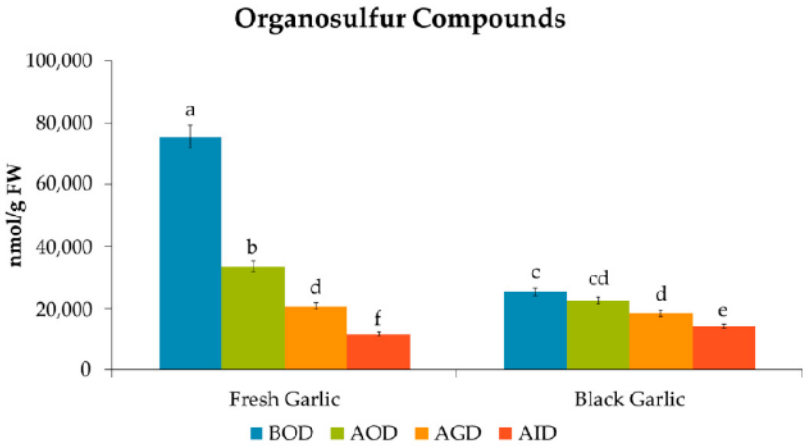

Figure 5. Quantities of total organosulfur compounds during the in vitro gastrointestinal digestion. Data are expressed as nmol/g FW as mean values (n = 6). Different letters (one-way ANOVA) denote statistically significant differences between the stages of simulated gastrointestinal digestion (p-value < 0.05). (BOD, Before Oral Digestion; AOD, After Oral Digestion; AGD, After Gastric Digestion; AID, After intestinal digestion).

Taking the results from this study together, a putative breakdown pathway of OS compounds in fresh and black garlic during in vitro gastrointestinal digestion is proposed (Figure 4). The first step represents the scission mediated by hydrolysis and gamma-glutamyl to yield the corresponding S-alk(en)yl-L-cysteine derivatives, which subsequently can be further converted by oxidation of the S-group to sulfoxide derivatives.

4. Conclusions

Considering the limited information available, the present study was undertaken to investigate the effects of in vitro simulated gastrointestinal digestion on the recovery and bioaccessibility of the individual (poly)phenolic and OS compounds in black garlic, and compare the results with its counterpart (fresh garlic). A notable increase in polyphenol compounds was observed during the oral digestion of fresh garlic, followed by a significant decrease during the subsequent steps of the gastrointestinal digestion, benzoic acid being the main polyphenol remaining at the end of the digestion process. Meanwhile, in black garlic, the polyphenol content decreased from the beginning of the digestive process, caffeic acid the only polyphenol still remaining. Regarding OS content, SACs derivatives were more influenced by the gastric digestion, while GSAk derivatives were more sensitive to intestinal digestion conditions in both the fresh and black garlic. Conversely, OS compounds in the black garlic presented greater stability throughout the digestive process than those in the fresh garlic. The bioaccessibility indexes of OS compounds in the fresh garlic ranged from 3.2%

for propiin to 329.3% for SAMC, while in the black garlic, they ranged from 21.3 to 262% for GSAC and methionine sulfoxide, respectively. The heat treatment to obtain black garlic has a positive effect on OS bioaccessibility from garlic, but not on polyphenols. A plausible route for the breakdown of OS compounds during gastrointestinal digestion has been proposed.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/9/11/1582/s1, Table S1: Concentrations of electrolytes Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF); Table S2: HPLC-HRMS-Based Identifications of (poly)phenols in fresh and black garlic; Table S3: HPLC-HRMS-based identification of organosulfur compounds in fresh and black garlic.

Author Contributions: The authors' responsibilities were as follows: Conceptualization: J.M.M.-R., G.P.-C. and R.M.-R.; Methodology, Software, Validation and Form Analysis: A.M.-O., J.L.O. and G.P.-C.; Writing—Original Draft Preparation: A.M.-O. and G.P.-C.; Writing—Review & Editing: G.P.-C. and J.M.M.-R.; Visualization: J.L.O.; Supervision: J.M.M.-R.; Project Administration: G.P.-C., V.O.-S.; Funding Acquisition: J.M.M.-R., V.O.-S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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SUPPLEMENTARY INFORMATION

Bioaccessibility of Bioactive Compounds of 'Fresh Garlic' and 'Black Garlic' Through *In-Vitro* Gastrointestinal Digestion.

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Table S1. Details of the Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF)

	$MgCl_2(H_2O)_6$ (0.15 M)	**KCl (0.5 M)**	**KH_2PO_4 (0.5 M)**	**$(NH_4)_2CO_3$ (0.5 M)**	**$NaHCO_3$ (1 M)**	**NaCl (2 M)**	**Distilled Water**	**Final Volume**
	mL							
SSF	0.313	9.438	2.313	0.038	4.250	-	233.650	**250.0**
SGF	0.250	4.313	0.563	0.313	7.813	7.375	229.375	**250.0**
SIF	0.688	4.250	0.500	0.688	26.563	6.000	211.313	**250.0**

Table S2. HPLC-HRMS-Based Identifications of (Poly)phenols in Fresh and Black Garlics

Peak	RT	Compound	Chemical Formula $[m/z]^-$	Experimental Mass $[m/z]^-$	δ (ppm)	MSI MI level[a]
1	4.7	Epigallocatechin	C15H13O7	305.066	-0.48	2
2	7.2	Benzoic Acid	C7H5O2	121.028	0.68	1
3	8.0	Chlorogenic acid	C16H17O9	353.087	-1.13	1
4	8.2	Catechin	C15H13O6	289.071	-1.09	1
5	8.3	Caffeic acid	C9H7O4	179.034	0.28	1
6	9.7	Gallic acid	C7H5O5	169.013	-2.15	1
7	11.7	Epicatechin	C15H13O6	289.071	-1.09	1
8	18.9	Vanillic acid	C8H7O4	167.034	0.67	2
9	21.1	Ferulic acid	C10H9O4	193.050	1.79	1
10	24.4	*p*-Coumaric acid	C9H7O3	163.039	-1.96	1

[a]Metabolite standards initiative (MSI) metabolite identification (MI) levels [1]. Reference compounds were available for all compounds identified at MSI MI level 1

Table S3. HPLC-HRMS-Based Identifications of organosulfur compounds in Fresh and Black Garlics

Peak	RT	Compound	Chemical Formula $[m/z]^+$	Experimental Mass $[m/z]^+$	δ (ppm)	MSI MI level[a]
1	4.2	S-Allylmercapto-L-cysteine (SAMC)	C6H12NO2S2	194.030	0.85	2
2	4.6	S-Allylcysteine (SAC)	C6H12NO2S	162.058	-1.9	1
3	5.7	S-Methylcysteine (Deoxymethiin)	C4H10NO2S	136.043	0.11	2
4	5.8	S-Propyl-L-cysteine sulfoxide (Propiin)	C6H14NO3S	180.069	2.45	2
5	5.9	S-(S-propyl) cysteine	C6H14NO2S2	196.046	0.36	2
6	5.9	Cycloalliin	C6H12NO3S	178.053	-1.36	2
7	6.6	γ –Glutamyl-S-(S-1-propenyl) cysteine (GS1PC)	C11H19N2O5S	291.101	1.65	2
8	6.7	γ-Glutamyl-S-allylmercaptocysteine (GSAMC)	C11H19N2O5S2	323.073	-1.9	2
9	6.8	S-Allylsulfenic acid (Lacrimatory factor)	C3H7OS	91.021	3.42	2
10	6.8	N-Acetyl-S-allyl-L-cysteine (NASAC)	C8H14NO3S	204.069	-1.39	2
11	6.8	Alliin	C6H12NO3S	178.053	-2.82	1
12	6.8	γ –Glutamyl-S-(S-1-propenyl)cysteine-glycine	C13H22N3O6S2	380.094	0.92	2
13	7.0	S-(2-carboxypropyl) cysteine	C7H14NO4S	208.064	-2.15	2
14	7.1	Trans-S-(1-propenyl)-L-cysteine (S1PC)	C6H12NO2S	162.058	-0.70	2
15	7.3	γ −Glutamyl-S-allyl-L-cysteine (GSAC)	C11H19N2O5S	291.101	1.86	2
16	7.4	S-Allylglutathione	C13H22N3O6S	348.122	-0.23	2
17	7.6	S-Methyl-l-cysteine sulfoxide (Methiin)	C4H10NO3S	152.038	-1.33	2
18	7.6	γ –Glutamyl-S-(S-methyl)cysteine-glycine	C11H20N3O6S2	354.079	-0.51	2

19	7.7	Methionine sulfoxide	C5H11NO3S	166.053	1.94	2
20	8.5	γ -Glutamyl-cysteine	C8H15N2O5S	251.070	-2.45	2
21	8.6	γ –Glutamyl-S-(2-carboxypropyl)cysteine-glycine	C14H24N3O8S	394.128	0.67	2
22	9.0	γ-Glutamyl-S-(1-propenyl) cysteine sulfoxide (G1PCS)	C11H19N2O6S	307.096	-2.05	2
23	9.2	ɣ-Glutamyl-S-methyl-L-cysteine (GSMC)	C9H17N2O5S	265.085	1.56	2
24	10.0	ɣ −Glutamyl-S-methyl-L-cysteine sulfoxide (GSMCS)	C9H17N2O6S	281.080	2.19	2

[a]Metabolite standards initiative (MSI) metabolite identification (MI) levels [1]. Reference compounds were available for all compounds identified at MSI MI level 1

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Artículo 4

Changes in the Organosulfur and Polyphenol Compound Profiles of Black and Fresh Onion during Simulated Gastrointestinal Digestion

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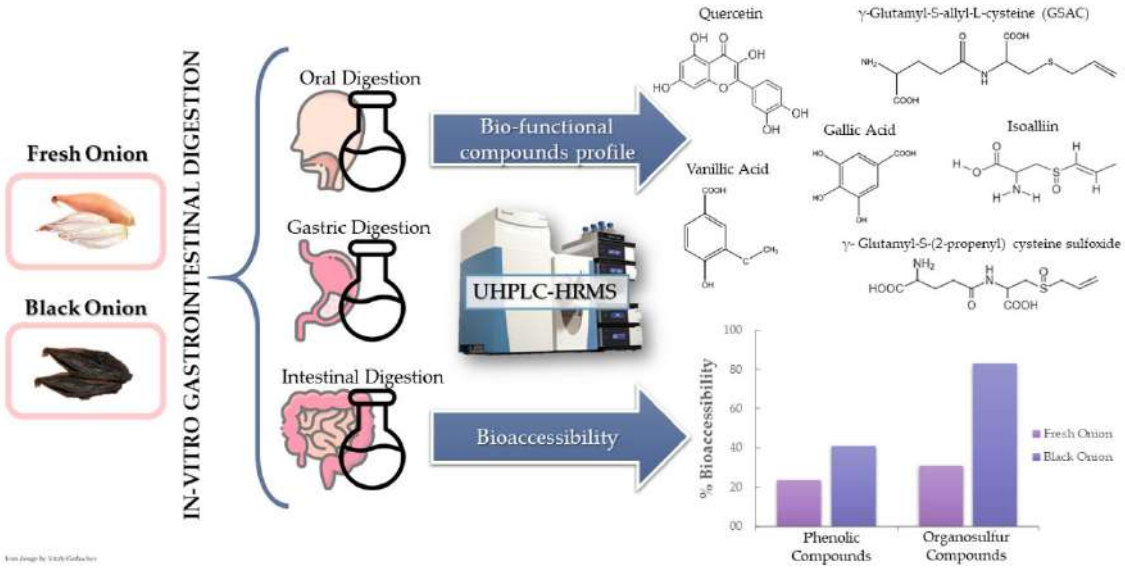

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Changes in the Organosulfur and Polyphenol Compound Profiles of Black and Fresh Onion during Simulated Gastrointestinal Digestion

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Abstract: This study aims to determine the changes in, and bioaccessibility of, polyphenols and organosulfur compounds (OSCs) during the simulated gastrointestinal digestion of black onion, a novel product derived from fresh onion by a combination of heat and humidity treatment, and to compare it with its fresh counterpart. Fresh and black onions were subjected to in-vitro gastrointestinal digestion, and their polyphenol and OSC profiles were determined by ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry (UHPLC-HRMS). Although to a lesser extent than in the fresh onion, the phenolic compounds in the black variety remained stable during the digestion process, presenting a higher bioaccessibility index (BI) with recovery corresponding to 41.1%, compared with that of fresh onion (23.5%). As for OSCs, apart from being more stable after the digestion process, with a BI of 83%, significantly higher quantities (21 times higher) were found in black onion than in fresh onion, suggesting that the black onion production process has a positive effect on the OSC content. Gallic acid, quercetin, isorhamnetin, and γ-glutamyl-S-(1-propenyl)-L-cysteine sulfoxide were the most bioaccessible compounds in fresh onion, while isorhamnetin, quercetin-diglucoside, γ-glutamyl-S-methyl-L-cysteine sulfoxide and methionine sulfoxide were found in black onion. These results indicate that OSCs and polyphenols are more bioaccessible in black onion than in fresh onion, indicating a positive effect of the processing treatment.

Keywords: black onion; fresh onion; polyphenols; organosulfur compounds; simulated digestion; in-vitro digestion; bioaccessibility

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1. Introduction

Onion (*Allium cepa* L.), a member of *Allium* genus, is frequently used in gastronomy around the world and is one of the main ingredients of the Mediterranean diet [1,2]. This product originated in Central Asia, from where its cultivation and consumption spread to the rest of the world [3]. Many medicinal properties have been attributed to members of the *Allium* family throughout history. These properties have made them the focus of many research studies into the relationship between their consumption and the prevention of diseases such as stomach, colorectal, prostate, and breast cancers [4]. Preventing and treating other chronic diseases such as diabetes, cardiovascular diseases, obesity, and metabolic syndromes are some benefits of the regular consumption of *Allium* vegetables, likely due to the presence of biofunctional compounds (polyphenols and OSCs) [5]. Black onion is a novel product that has been developed by the food industry by processing (aging) raw onion under temperature- and humidity-controlled conditions using no artificial additives. The heating treatment (at 60–80 °C) used to produce this

product has been shown to affect its phytochemical composition. Up to 12-fold decreases have been found in flavonoid content, while, in contrast, the OSC content is higher [6]. Black onion shows a series of significant compositional and organoleptic modifications in comparison to the original product, such as a sweeter taste, the lack of a spicy sensation (pungency), and a black-brown colour. The main changes with regard to its composition are a lower phenolic compound content and significant increases in isoalliin, fructose, glucose, and tartaric acid [6]. The elimination of the undesirable characteristics of fresh onion at the sensory and digestive levels enhances consumer acceptance of black onions, increasing the probability of their consumption. To date, there have been no studies on the potential benefits of consuming black onion. However, in-vitro studies have shown that black shallot extract presents higher anticancer and anti-inflammatory activities than fresh onion extract when evaluated in cell lines [7]. Indeed, quercetin, the main polyphenol found in black onion, is related to some beneficial effects, such as an antiproliferative effect on ovarian, breast, and colon cancer cells [8] and a protective effect against certain pathologies related to lipid metabolism, such as atherosclerosis and diabetes [9]. Moreover, isoalliin, the major OSC in both fresh and black onion, and other OSCs have been found to present health-promoting benefits [10]. However, to exert beneficial effects in vivo, polyphenols and OSCs from onion must be bioaccessible, be released from the food matrix, and be ready for absorption [11,12] into the gastrointestinal tract. in-vitro digestion models are a good tool for evaluating the bioaccessibility of biofunctional compounds [13,14], including polyphenols and OSCs. For instance, the literature reports the bioaccessibility of polyphenol compounds in different food matrices, including apples [15], blueberries [16], oranges [17], fresh and black garlic [18], and fresh onion [19]. However, to the best of our knowledge, the effect of in-vitro gastrointestinal digestion on the bioaccessibility of the individual profiles of phenolic compounds and OSCs from black onion remains unknown. Therefore, the aim of this study was to evaluate the bioaccessibility of both polyphenols and OSCs from black and fresh onion during simulated gastrointestinal digestion by monitoring them using ultra-high-performance liquid chromatography coupled with high resolution mass spectrometry (UHPLC-HRMS) and to investigate the possible impact of the transformation process during the production of black onion on bioaccessibility.

2. Materials and Methods

2.1. Chemicals

α-Amylase enzymes from human saliva (937 units/mg protein), pepsin (500 units/mg protein), pancreatin from porcine pancreas (4 × UPS), bile salts, and calcium chloride were purchased from Sigma-Aldrich (Madrid, Spain). HCl was obtained from Merck (Darmstadt, Germany), and NaOH was acquired from Fisher Scientific (Madrid, Spain). Sodium bicarbonate and ammonium carbonate were purchased from Sigma-Aldrich (Madrid, Spain), sodium chloride and magnesium chloride hexahydrate were purchased from Fisher Scientific (Madrid, Spain), and potassium dihydrogen phosphate was obtained from VWR International Eurolab (Barcelona, Spain). Reference flavonoid compounds including isorhamnetin, luteolin, quercetin, quercetin-3-O-glucoside alliin and s-allyl-L-cysteine (SAC) together with formic acid were acquired from Sigma-Aldrich (Madrid, Spain). Ammonium formate, ammonium acetate, and ethanol were obtained from Sigma-Aldrich. Acetonitrile (LC-MS grade) and methanol (LC-MS grade) were obtained from Panreac (Barcelona, Spain).

2.2. Materials and Sample Preparation

Fresh and black "Shallot" onions (*Allium cepa* var. aggregatum) were obtained from a local supplier (La Abuela Carmen®). One batch of fresh onion (5 kg) was divided equally into two groups, one being used to obtain black onion. The black onion samples were obtained by an optimized process that combines heat treatment with controlled humidity conditions, enabling a product with different organoleptic properties to be obtained, as previously described [6]. The fresh and black onions were peeled and ground

using liquid nitrogen with cryogenic grinder mill equipment to obtain a final particle size of 10 µm (Freezer Mill model 6870, Fisher Scientific, Waltham, MA, USA) and stored at −80 °C until the simulated gastrointestinal digestion process.

2.3. Simulated Gastrointestinal Digestion and Evaluation of Bioaccesibility

An in-vitro oral, gastric, and intestinal digestion model, previously reported by Juániz et al. [20], was adapted to obtain a bolus with the right consistency to perform the in-vitro digestion experiments [21]. Briefly, 2 g of each lyophilized onion sample was weighed in a 100 mL amber glass bottle. The whole process was performed in a stirred water bath (Unitronic Reciprocating Shaking Bath model 6032011, J.P. Selecta, Barcelona, Spain) at 37 °C in triplicate. During the oral phase, simulated salivary fluids (SSFs) (Table 1) were used. A total of 14 mL of SSF solution was added to the samples, together with 250 µL of an α-amylase (300–1500 U/mg protein) solution (1.3 mg/mL), 0.1 mL of 0.3 M $CaCl_2$, and 5.65 mL of distilled water. The mixture was shaken at 37 °C for 30 min. For the gastric phase, simulated gastric fluids (SGFs) (Table 1) were used. After the oral phase, it was necessary to adjust the fluids to pH 3 with 1 M HCl solution. Then, 15 mL of SGF solution was added to the samples, together with 1.19 mL of a pepsin (3.2–4.5 U/mg protein) solution, 0.01 mL of 0.3 M $CaCl_2$, and 3.8 mL of distilled water. The pepsin solution was prepared with 1 g of pepsin in 10 mL of 0.1 M HCl. The mixture was incubated at 37 °C for 120 min. For the intestinal phase, simulated intestinal fluids (SIFs) (Table 1) were used. After the gastric phase, 22 mL of SIF solution was added to the samples, together with 10 mL of pancreatin (4 × UPS) solution (8 mg/mL), 5 mL of bile salts (25 mg/mL), 0.08 mL of 0.3 M $CaCl_2$, and 9.92 mL distilled water. Then, 1 M NaOH solution was used to adjust the solution to pH 7. The mixture was incubated for 120 min at 37 °C. Aliquots of the digested samples were taken before oral digestion (BOD) and after every stage of the digestion process: oral, gastric, and intestinal digestion (AOD, AGD, and AID, respectively). These samples were lyophilized and stored at −80 °C until polyphenol and OSC extraction and analysis. The bioaccessibility indices were calculated as percentages of the initial content (before oral digestion, BOD) of the compound (polyphenol or OSC) after simulated gastrointestinal digestion (before oral digestion, BOD) [22,23].

Table 1. Details of the fluids used in the simulated gastrointestinal digestion.

Solution	Concentration	SSFs	SGFs	SIFs
	Molarity	mL	mL	mL
$MagCl_2(H_2O)_6$	0.15	0.313	0.250	0.688
KCl	0.50	9.438	4.313	4.250
KH_2PO_4	0.50	2.313	0.563	0.500
$(NH_4)_2CO_3$	0.50	0.038	0.313	0.688
$NaHCO_3$	1.00	4.250	7.813	26.563
NaCl	2.00	-	7.375	6.000
Distilled Water	-	233.650	229.375	211.313
Final Volume		250	250	250

SSFs: simulated salivary fluids; SGFs: simulated gastric fluids; SIFs: simulated intestinal fluids.

2.4. Polyphenol and Organosulfur Compound Extraction and Analysis

Samples from the in-vitro gastrointestinal digestion were extracted in triplicate following the previously optimized and validated procedure reported by Moreno-Rojas et al. [24]. The polyphenols and OSCs in the fresh and black onion extracts were analysed using an UHPLC-HRMS mass spectrometer system (Thermo Scientific, San José, CA, USA) comprising a UHPLC pump, a PDA detector scanning from 200 to 600 nm, and an autosampler operating at 4 °C (Dionex Ultimate 3000 RS, Thermo Corporation, San José, CA, USA). The chromatographic characteristics of the separation of the polyphenols and OSCs, as well as the details of their identification and quantification, were previously [18,24].

2.5. Statistical Analysis

The results are expressed as the mean of three replicates measured for each sample. Multiple comparisons were performed using a one-way ANOVA using R statistics software (v. 3.6.3) to identify significant differences between the phases of the simulated gastrointestinal digestion, with significance being established at $p < 0.05$. Next, Fisher's LSD pairwise comparison was performed on the data. A principal component analysis (PCA) was performed as an unsupervised method using SIMCA software (v.15.0.2) to determine whether the overall changes in the profiles of polyphenols and OS compounds were different enough to distinguish between the simulated gastrointestinal digestion stages and product types (fresh or black onion).

3. Results and Discussion

3.1. Changes in Polyphenolic Contents of Fresh and Black Onions after Simulated Gastrointestinal Digestion and Bioaccesibility

A total of 17 polyphenols were identified and quantified in the fresh onion samples before simulated gastrointestinal digestion was carried out. Flavonoids were the main ones, accounting for 86.5% of the total content, while phenolic acids represented 13.5%. Overall, quercetin diglucoside (39.7%), quercetin 4-*O*-glucoside (23.6%), vanillic acid (11.6%), and myricetin (9.9%) were the main phenolic compounds in the nondigested fresh onion samples (Table 2). These results are in line with those of Böttcher et al. [25], who reported, among others, quercetin glycosides with glucosyl moieties in 4′-*O* and 3-*O* positions as being the main flavonoids in red and yellow onion cultivars. Regarding black onion, a total of seven polyphenols were identified and quantified, the main type being free quercetin, which accounted for 94% of the total content (Table 2). The remaining polyphenols, representing between 2.6 and 0.06% of the total, were isorhamnetin followed by luteolin, quercetin-diglucoside, quercetin-3-*O*-glucoside, quercetin-4-*O*-glucoside, and isorhamnetin-4-*O*-glucoside (Table 2). This difference in phenolic composition between onion products is mainly attributed to the changes occurring during the black onion elaboration process, as previously reported by Moreno-Ortega et al. [6]. They observed that the heating and humidity conditions used to obtain black onion from fresh onion have an important impact on its physicochemical composition, with free quercetin being the main compound found in black onion from three onion varieties (94% for "Shallot", 99% for "Chata", and 99% for "Echalion"). These decreases in the phenolic content during the production of black onion are arguably attributed to the oxidation of flavonoids to semi-quinoid intermediates and the respective quinones, which normally react further with other quinones to produce dark melanin pigments [26] or with proteins to produce dark polymers [27].

The effect of in-vitro gastrointestinal digestion on the concentrations of individual polyphenols in fresh and black onions is shown in (Table 2). A gradual decrease is observed in the total concentration of polyphenols from the buccal phase (AOD) to the intestinal phase (AID) in both onion products.

Table 2. Concentration (nmol/g FW) of polyphenols found in fresh and black onion samples at different stages of simulated gastrointestinal digestion. Data are expressed as mean values ($n = 3$).

Compounds	BOD	AOD	% Recovery	AGD	% Recovery	AID	% Recovery-Bioaccessibility	*p*-Value
	Fresh Onion							
Phenolic Acids								
p-Coumaric acid	2.48 a	0.93 b	37.5	0.36 c	14.5	0.32 c	12.9	***
Vanillic acid	274 b	365 a	133.2	70 d	25.5	193 c	70.4	***
Gallic acid	3.6 c	4.9 b	136.1	2.6 d	72.2	11.4 a	316.7	***
Caffeic acid	2.05 a	1.01 b	49.3	0.43 c	21.0	0.27 d	13.2	***
Ferulic acid	4.8 b	5.3 a	110.4	2.8 c	58.3	3.0 c	62.5	***
Total Phenolic Acids	287 b	377 a	131.4	76 d	26.5	208 c	72.5	***
Flavonoids								
Morin	56.46 a	2.99 b	5.3	1.69 b	3.0	0.97 b	1.7	***
Quercetin	80 c	81 c	101.3	184 a	230.0	132 b	165.0	***
Epigallocatechin	1.56 a	0.54 c	34.6	0.71 b	45.5	0.20 d	12.8	***
Isorhamnetin	28 c	60 b	214.3	81 a	289.3	59 b	210.7	***
Myricetin	211.4 a	86.1 b	40.7	58.1 c	27.5	11.0 d	5.2	***
Quercitrin	9.1 a	1.3 b	14.7	0.171 c	1.9	0.077 c	0.8	***
Quercetin-4-*O*-glucoside	503 a	247 b	49.1	106 c	21.1	55 d	10.9	***
Isorhamnetin glucoside I	4.06 a	1.81 b	44.6	0.27 c	6.7	0.13 c	3.2	***
Isorhamnetin glucoside II	nd	15.52 a	-	13.09 b	-	4.84 c	-	***
Rutin	12.11 a	0.96 b	7.9	0.53 b	4.4	0.58 b	4.8	***
Quercetin diglucoside	846 a	52 b	6.1	36 b	4.3	23 b	2.7	***
Isorhamnetin diglucoside	90.6 a	16.8 b	18.5	6.5 c	7.2	4.1 c	4.5	***
Total Flavonoids	1842 a	567 b	30.8	488 c	26.5	292 d	15.9	***
Total	2129 a	944 b	44.3	564 c	26.5	500 d	23.5	***

Table 2. *Cont.*

Compounds	BOD	AOD	% Recovery	AGD	% Recovery	AID	% Recovery-Bioaccessibility	*p*-Value
	Fresh Onion							
	Black Onion							
Quercetin	47 a	39 ab	83.3	28 bc	59.6	18c	39.3	***
Isorhamnetin	1.32 a	0.89 b	67.9	0.85 b	64.7	1.08 ab	81.6	**
Luteolin	0.23 a	0.22 a	98.4	0.12 b	51.3	0.14 b	61.1	**
Quercetin diglucoside	0.20 ab	0.21 a	106.5	0.15 b	76.7	0.19 ab	95.4	*
Quercetin-3-*O*-glucoside	0.63 a	0.60 a	94.6	0.57 a	90.6	0.42 b	66.6	**
Quercetin-4-*O*-glucoside	0.81 a	0.57 b	70.2	0.81 a	100.5	0.35 c	43.7	***
Isorhamnetin-4′-*O*-glucoside	0.031 a	0.031 a	98.8	nq	-	nq	-	***
Total	50 a	42 b	83.0	30 c	60.7	21 d	41.1	***

Different letters (one-way ANOVA) denote significant differences ($p < 0.05$) between the four stages for the same compound. Ns: non-significant; * *p*-value < 0.05; ** *p*-value < 0.01; *** *p*-value < 0.001. nq: not quantified; nd: not detected. BOD: before oral digestion; AOD: after oral digestion; AGD: after gastric digestion; AID: after intestinal digestion.

For a more in-depth exploration of the stability of polyphenols from fresh and black onions over the different stages of simulated gastrointestinal digestion and to determine the impact of the elaboration process of black onion on the bioaccessibility of polyphenol compounds, a principal component analysis (PCA) was performed (Figure 1). The first PCA (PC1) described 68% of the total variability (Figure 1A) and showed a clear discrimination between the nondigested and digested black and fresh onion samples. This discrimination was attributed to the presence of specific compounds, including vanillic acid, morin, epigallocatechin, myricetin, quercitrin, rutin, and isorhamnetin diglucoside in fresh onion, while compounds such as luteolin and quercetin-3-*O*-glucoside were characteristic of black onion (Figure 1B). PC2 explained 24% of the total variability and highlighted the significant impact of the gastrointestinal digestion process on the polyphenol profiles of both kinds of onion (Figure 1A). Fresh onions seem to be significantly more greatly affected by the digestive process than black ones, with their polyphenol concentration decreasing from 2129 to 500 nmol/g FW, so that 23% of the total polyphenol content remained; meanwhile 41% of the total polyphenol content in the black onion remained almost intact after the digestion process (from 50 to 21 nmol/g FW) (Table 2). Focusing on fresh onion, the oral phase had the greatest impact on its polyphenol content, followed by gastric digestion, with the intestinal digestion phase being the phase with smallest impact on gastrointestinal stability (Table 2, Figure 2). For instance, oral digestion had a very negative effect on the concentration of flavonoids, but not phenols, the former decreasing by 69% from the initial value. The concentrations of specific compounds, such as morin, quercetin diglucoside, rutin, quercetin-4′-*O*-glucoside, quercitrin and isorhamnetin diglucoside, decreased during the oral digestion of fresh onion, probably as a consequence of the hydrolysis of quercetin and isorhamnetin glucosides. Consequently, there were increases in the free quercetin (1.7-fold) and isorhamnetin (2.1-fold) contents. These results should be considered with caution as the timing of our oral phase process (30 min) did not mimic physiological conditions and, therefore, it is impossible to conclude that the oral phase has a great impact on polyphenol stability. However, the results are somewhat suitable for our purpose, which was to compare the bioaccessibility of polyphenols in two food products: fresh and black onion.

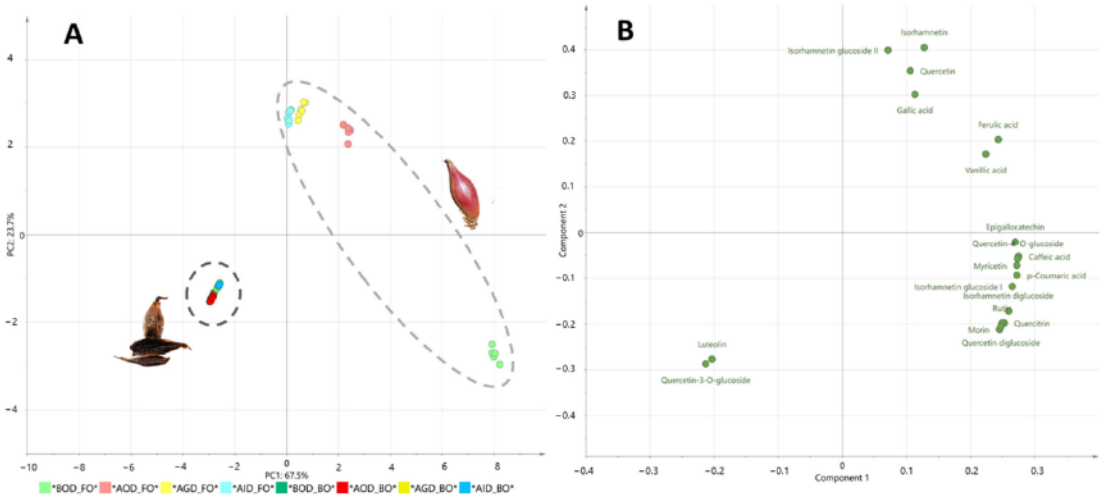

Figure 1. Scores (**A**) and loadings (**B**) obtained in the Principal Component Analysis (PCA) comparing data from polyphenols in fresh and black onion during simulated gastrointestinal digestion.

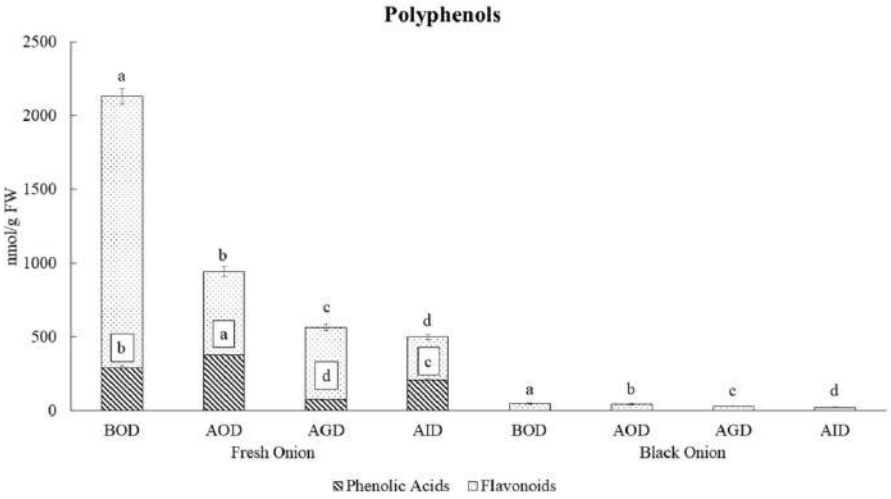

Figure 2. Quantities of phenolic acids and flavonoids during in-vitro simulated gastrointestinal digestion of fresh and black onion. Data are expressed in nmol/g FW as mean values (n = 3). Different letters (one-way ANOVA) denote statistically significant differences between the stages of simulated gastrointestinal digestion (p-value < 0.05).

The increases in free quercetin and isorhamnetin continued during gastric digestion, mainly as a result of their partial hydrolysis by the action of pepsin and the pH. A low pH and gastric enzymes could lead to the hydrolysis of proteins and carbohydrates bound to flavonoids, thus improving their extractability and boosting their hydrolysis, which facilitates the release of aglycones from *O*-glycosides during their passage through the stomach [28,29]. In addition, the pronounced decay in flavonoid content, more so in fresh onion than in black onion, is arguably due to the propensity of flavonoids to interact with other matrix components such as carbohydrates or lipids, as indicated by Gonzales et al. [30], or to the propensity of the digestive enzymes to form complexes, as suggested by De Santiago et al. [31] and Su et al. [32]. The phenolic acids in fresh onion were resistant to the oral phase conditions but were greatly affected during the gastric phase, with only 26.5% remaining (Table 2). Likewise, intestinal digestion resulted in a significant increase (almost 3-fold) in the concentration of most of the phenolic acids in fresh onion compared with those obtained after the gastric phase. This can be explained by considering their release from the food matrix after the activity of enzymes such as pancreatin and pH changes in the duodenum [33].

The polyphenols in black onion were also affected by the digestion process, as Table 2 shows. As the main polyphenol in black onion is quercetin, the total polyphenol content during gastrointestinal digestion is highly influenced by the stability of this compound. Its concentration showed a gradually decrease during the three steps of gastrointestinal digestion, from 47 nmol/g FW before oral digestion to 18 nmol/g FW after the intestinal digestion phase, with 39.3% of its initial content remaining. Overall, the different effects of in-vitro digestion on the polyphenol profiles of fresh and black onion highlight the importance of the food matrix and its interaction with other compounds, particularly in terms of how polyphenols are released during the digestion process, as previously reported by other authors [34–36]. The most bioaccessible compounds in fresh onion were found to be gallic acid (316.7% bioaccessibility index (BI)), quercetin (165% BI), and isorhamnetin (210.7% BI), while in black onion, quercetin-*O*-glucoside (95.4% BI) and isorhamnetin (81.6% BI) were more bioaccessible after in-vitro gastrointestinal digestion (Table 2). These results indicate that the compounds remaining after intestinal digestion—significant quantities of vanillic acid and quercetin-4-*O*-glucoside in fresh onion and free quercetin in black onion—potentially cross the small intestine and reach the colon, where they are subjected to microbiota-mediated metabolism prior to absorption.

3.2. Changes in Organosulfur Compound Profiles of Fresh and Black Onion after Simulated Gastrointestinal Digestion

A total of 24 OSCs were identified and quantified in the fresh onion samples before oral digestion (Table 3), the predominant ones being γ-glutamyl-S-(2-propenyl) cysteine sulfoxide (G2PCS) (2615 nmol/g FW), isoalliin (1348 nmol/g FW), γ-glutamyl-S-(2-carboxypropyl) cysteine–glycine (884 nmol/g FW), γ-glutamyl-S-allyl-L-cysteine (GSAC) (799 nmol/g FW), and propanethial sulfoxide (596 nmol/g FW), accounting for 74.0% of the total initial content (Table 3). The remaining OSCs are listed in Table 3. Variations were found in the OSC profile of black onion samples compared with that of fresh onion, the main differences being found for isoalliin (53,117 nmol/g FW), propanethial sulfoxide (10,663 nmol/g FW), methionine sulfoxide (1073 nmol/g FW), and G2PCS (861 nmol/g FW), representing 98.9% of the total OSC content. The remaining OSCs were γ-glutamyl-S-methyl cysteine sulfoxide (GSMCS), γ-glutamyl-S-propyl cysteine sulfoxide (GSPC), S-(S-propyl) cysteine, methiin, and propiin. These results are in line with those previously reported by Moreno-Rojas et al. [24], who showed that G2PCS, isoalliin, and γ-glutamyl-S-(2-carboxypropyl) cysteine–glycine are the main OSCs in fresh shallot, while isoalliin and G2PCS are the main ones in black onion.

Table 3. Concentrations (nmol/g FW) of organosulfur compounds in fresh and black onion samples at different stages of simulated gastrointestinal digestion. Data are expressed as mean values ($n = 3$).

Compounds	BOD	AOD	% Recovery	AGD	% Recovery	AID	% Recovery-Bioaccessibility	*p*-Value
				Fresh Onion				
γ-Glutamyl-S-alk(en)yl-L-cysteine derivatives (GSAk)								
γ–Glutamyl-S-(2-carboxypropyl) cysteine–glycine	884 a	620 b	70.2	499 c	56.4	253 d	28.6	***
γ–Glutamyl-S-(S-1-propenyl) cysteine–glycine	3.09 a	2.07 b	67.0	1.69 c	54.7	0.94 d	30.5	***
γ–Glutamyl-S-(S-methyl) cysteine–glycine	5.77 a	4.38 b	76.0	2.39 c	41.5	1.35 d	23.4	***
γ–Glutamyl-S-(S-propyl) cysteine–glycine	17.7 a	12.9 b	73.0	7.8 c	43.9	2.8 d	15.9	***
γ-Glutamyl-S-allyl-L-cysteine (GSAC)	799 a	574 b	71.9	437 c	54.7	225 d	28.1	***
γ–Glutamyl-S-(propyl) cysteine (GSPC)	13.78 a	13.09 a	95.0	0.24 b	1.8	nd	0.0	***
γ-Glutamyl-S-methyl cysteine sulfoxide (GSMCS)	15.0 a	9.3 b	62.2	9.1 b	60.8	7.0 c	46.4	***
γ- Glutamyl-S-(2-propenyl) cysteine sulfoxide (G2PCS)	2615 a	1908 b	72.9	1566 c	59.9	1102 d	42.1	***
γ–Glutamyl-S-(propyl) cysteine sulfoxide	323 a	137 b	42.5	94 c	29.1	53 d	16.5	***
γ–Glutamyl-S-(1-propenyl)-L-cysteine sulfoxide (G1PCS)	2.1 c	4.6 a	213.7	2.9 b	136.0	4.4 a	206.3	***
Total GSAk derivatives	4679 a	3286b	70.2	2620 c	56.0	1649 d	35.3	***
S-Alk(en)-yl-L-cysteine derivatives (SACs)								
S-(2-carboxypropyl) cysteine-glycine	333 a	242 b	72.5	198 c	59.3	97 d	29.1	***
S-methyl-cysteine (deoxymethiin)	41.4 a	33.9 b	81.9	20.7 c	50.1	11.1 d	26.8	***
S-Propyl-L-cysteine (deoxypropiin)	359 a	165 b	45.8	128 c	35.6	121 c	33.7	***
S-Allyl-L-cysteine (SAC)	343 a	295 b	85.9	90 c	26.3	68 c	19.9	***
S-allylmercaptoglutathione	0.31 a	0.19 b	60.3	0.17 b	55.6	nd	0.0	***
S-(S-propyl) cysteine	20.9 a	14.3 b	68.3	8.0 c	38.5	13.9 b	66.7	***
S-(2-carboxypropyl) cysteine	11.2 a	9.2 b	82.2	5.6 c	50.4	3.3 d	29.6	***
Alliin	330.7 a	nq	-	nq	-	nd	0.0	***
Isoalliin	1348 a	943 b	70.0	661 c	49.0	379 d	28.1	***
Propanethial sulfoxide (lacrimatory factor)	596a	418 b	70.1	296 c	49.6	175 d	29.3	***
Methyl-L-cysteine sulfoxide (methiin)	160.2 a	14.7 b	9.2	15.8 b	9.9	6.3c	3.9	***

Table 3. *Cont.*

Compounds	BOD	AOD	% Recovery	AGD	% Recovery	AID	% Recovery-Bioaccessibility	*p*-Value
				Fresh Onion				
S-propyl-cysteine sulfoxide (propiin)	31.5 a	1.5 b	4.6	0.6b c	1.9	nd	0.0	***
Cycloalliin	166 a	114 b	68.7	93 c	55.8	62 d	37.6	***
Methionine sulfoxide	12.2 a	6.7 c	54.6	6.3 c	51.2	8.0 b	65.5	***
Total SACs derivatives	3755 a	2256 b	60.1	1523 c	40.6	945 d	25.2	***
Total	8433 a	5543 b	65.8	4142 c	49.2	2594 d	30.8	***
				Black Onion				
γ-Glutamyl-S-alk(en)yl-L-cysteine derivatives (GSAk)								
γ-Glutamyl-S-methyl cysteine sulfoxide (GSMCS)	115 c	104 c	90.4	138 b	119.7	185 a	160.3	***
γ-Glutamyl-S-(2-propenyl) cysteine sulfoxide (G2PCS)	861 a	753 a	87.4	800 a	92.9	603 b	70.0	***
γ–Glutamyl-S-propyl cysteine sulfoxide (GSPCS)	62 b	63 b	101.8	90 a	144.6	nd	0.0	***
Total GSAk derivatives	1039 a	921 ab	88.6	1028 a	99.0	788 b	75.9	***
S-Alk(en)-yl-L-cysteine derivatives (SACs)								
S-(S-propyl) cysteine	178 a	175 a	98.5	90 b	77.9	nd	0.0	***
Isoalliin	53,117 a	45,859 ab	86.3	47,859 ab	90.1	44,199 b	83.2	*
Propanethial sulfoxide (Lacrimatory factor)	10,663 a	9299 ab	87.2	8176 b	76.7	8356 b	78.4	**
S-methyl-cysteine sulfoxide (methiin)	181 a	179 a	99.0	208 a	114.8	nd	0.0	***
S-propyl-cysteine sulfoxide (propiin)	83 b	77 c	92.6	151 a	181.4	nd	0.0	***
Methionine sulfoxide	1073 b	923 b	86.0	1006 b	93.8	1809 a	168.5	***
Total SACs derivatives	65,296 a	56,511 b	86.5	57,489 ab	88.0	54,364 b	83.3	*
Total	66,334 a	57,432 b	86.6	58,517 ab	88.1	55,153 b	83.0	*

Different letters (one-way ANOVA) denote significant differences ($p < 0.05$) between the four stages for the same compound. Ns: non-significant; * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001. nq: not quantified; nd: not detected. BOD: before oral digestion; AOD: after oral digestion; AGD: after gastric digestion; AID: after intestinal digestion.

Table 3 shows the impact of in-vitro gastrointestinal digestion on the stability of OSCs in fresh and black onion. To better understand the impacts of the different digestive phases on the OSC concentration of fresh and black onion, a Principal Component Analysis (PCA) was performed (Figure 3). PC1, which explained 76.4% of the total variability, showed a clear discrimination between fresh and black onion samples (Figure 3A). This differentiation was ascribed principally to the presence of S-alk(en)yl-L-cysteine (SAC) derivatives, including methiin, propiin, isoalliin, S-(S-propyl) cysteine, propanethial S-oxide, and methionine sulfoxide, as well as γ-glutamyl-S-propyl cysteine sulfoxide (GSPC), in the black onion samples (Table 3, Figure 4). Meanwhile fresh onion was characterized by the presence of other specific γ-glutamyl-S-alk(en)yl-L-cysteine derivatives (GSAks) and SAC derivatives including γ-glutamyl-S-(2-carboxypropyl) cysteine-glycine, γ-glutamyl-S-allyl-L-cysteine (GSAC), γ-glutamyl-S-(2-propenyl) cysteine sulfoxide (G2PCS), γ-glutamyl-S-(propyl) cysteine sulfoxide, S-(2-carboxypropyl) cysteine-glycine, S-propyl-L-cysteine, S-allyl-L-cysteine (SAC), alliin, and isoalliin (Figure 3B). According to Moreno-Ortega et al. [6], during the thermal processing of black onion, many GSAk derivatives are transformed into simple and intermediate volatile compounds, thus decreasing their concentrations. It is worth noting that the total OSC content was significantly higher in black onion (66,452 nmol/g FW) than in fresh onion (8432 nmol/g FW) before the digestive process, mainly due to the high concentration of isoalliin, which totalled 1348 nmol/g FW in fresh onion and 53,117 nmol/g FW in black onion. PC2 explained 14.1% of the total variance and provided clear discrimination between samples belonging to the different gastrointestinal digestion phases of both kinds of onion (Figure 3A). In particular, the OSCs in fresh onion were more strongly affected by the oral phase, with the SAC derivatives (with 60.1% remaining, ranging from 45.8% for S-propyl-L-cysteine (deoxypropiin) to 85.9% for S-allyl-L-cysteine, SAC) being more greatly affected than the GSAk derivatives (with 70.2% remaining, ranging from 42.5% for γ-glutamyl-S-(propyl) cysteine sulfoxide to 95.0% for γ glutamyl-S-(propyl) cysteine, GSPC) (Figure 4). However, there were significant decreases in the recovery of compounds such as methiin (9.2%), propiin (4.6%), and alliin (not quantified), since they are the main substrates of the alliinase enzyme, which is found in the composition of the members of the *Allium* genus, as reported by Keusgen et al. [37]. The decrease in the total OSC content continued during gastric and intestinal digestion, with mean recoveries of 49.2 and 30.8%, respectively (Table 3). Meanwhile, the intestinal phase had the greatest impact on the OSC content in black onion. This was not due to the decrease in the total OSC content but mainly to the significant increases in specific OSCs such as methionine sulfoxide (168%) and GSMCS (160.3%). The decreases in methiin, propiin, and S-(S-propyl) cysteine during this stage were mainly due to the fact that these smaller structures are very unstable under the alkaline conditions present during intestinal digestion [38].

The methoxidation reaction of methionine during simulated gastrointestinal digestion could explain the significant increase in methionine sulfoxide [39], while the increase in the GSMCS level could be due to the oxidation of its precursor, γ-glutamyl-S-methyl-L-cysteine (GSMC), during the in-vitro process, a factor that has been identified previously in black onion [6,24].

Overall, fresh onion was significantly more greatly affected by the digestive process than black onion (Figure 4), with its OSC content decreasing by 69%, from 8432 to 2594 nmol/g FW. In black onion, the decrease in OSCs from 66,452 to 55,153 nmol/g represented a total loss of 17% (Table 3). The stability of the main OSCs during the stages of digestion could be explained by the inactivation of the alliinase enzyme during the production process of black onion, in which temperatures above 60 °C are reached, preventing its interaction with these compounds, as reported by Méndez et al. [40]. Moreover, the fresh onion was identified as γ-glutamyl-S-(1-propenyl)-L-cysteine sulfoxide (G1PCS) (206.3%), S-(S-propyl) cysteine (66.7%), and methionine sulfoxide (65.5%), with G2PCS, γ-glutamyl-S-(2-carboxypropyl) cysteine–glycine, GSAC, isoalliin, propanethial sulfoxide, deoxypropiin, and S (2 carboxypropyl) cysteine–glycine being the main OSCs remaining

after in-vitro digestion. GSMCS and methionine sulfoxide were identified as the most bioaccessible OSCs in black onion, although isoalliin was predominant, accounting for 80.1% of the total content. These results suggest that these OSCs, as occurs with the polyphenols, will potentially cross the small intestine and reach the colon, where they will undergo microbiota-mediated metabolism prior to absorption.

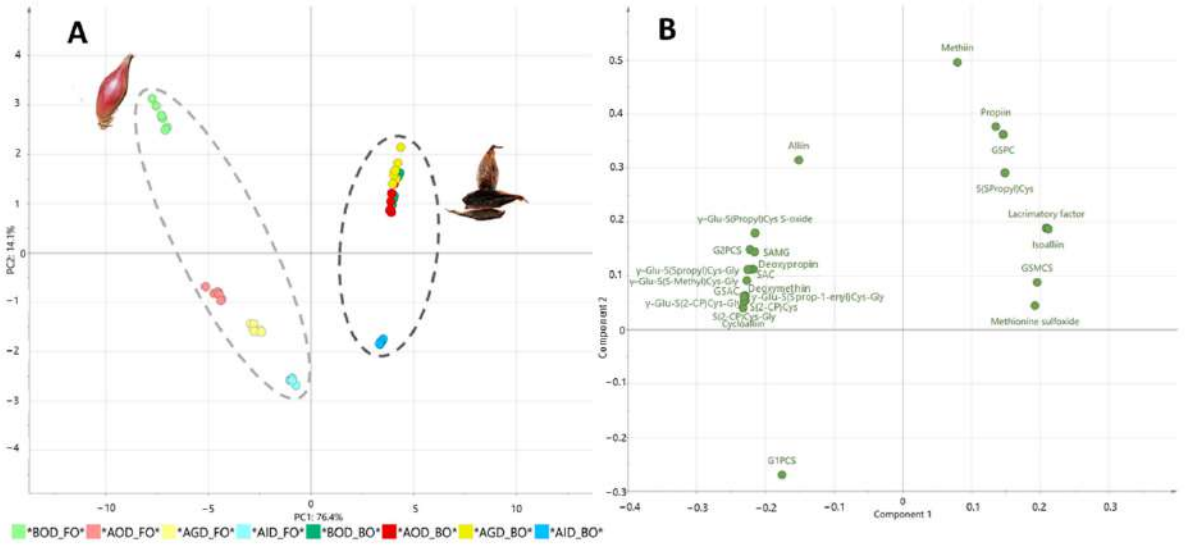

Figure 3. Scores (**A**) and loadings (**B**) of the PCA comparing data from organosulfur compounds of fresh and black onion during simulated gastrointestinal digestion.

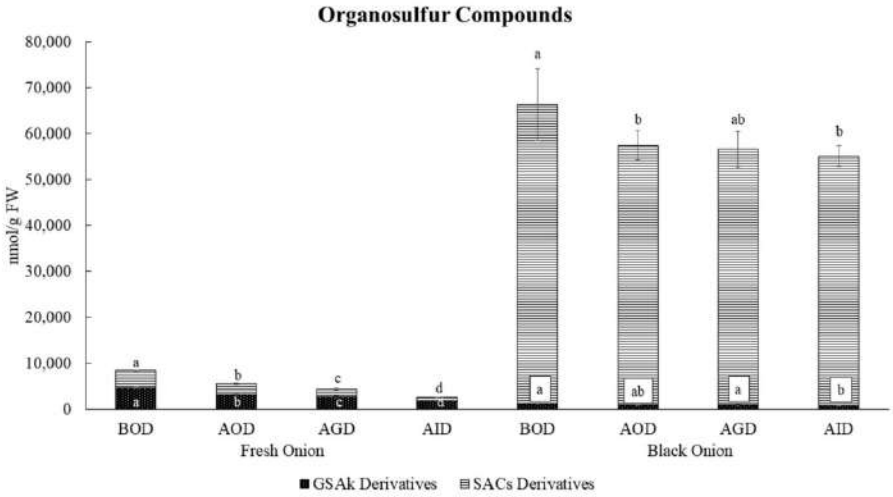

Figure 4. Quantities of organosulfur compounds during in-vitro simulated gastrointestinal digestion of fresh and black onion. Data are expressed in nmol/g FW as mean values ($n = 3$). Different letters (one-way ANOVA) denote statistically significant differences between the stages of simulated gastrointestinal digestion (p-value < 0.05).

4. Conclusions

This study evaluated the effects of simulated gastrointestinal digestion on the bioaccessibility of polyphenols and OSCs in black onion compared with its fresh counterpart. During the digestive process, there was a decrease in the concentration of gly-

cosylated flavonoids in fresh onion but a significant increase in the contents of free quercetin and isorhamnetin, the bioavailable forms of these compounds at the colonic level. These polyphenols showed the highest bioaccessibility indexes in fresh onion (165 and 210.7%, respectively) along with gallic acid (316.7%), vanillic acid (70.4%), and ferulic acid (62.5%). Meanwhile in black onion, the lower initial polyphenol content compared with that of fresh onion progressively decreased during in-vitro digestion, showing a total bioaccessibility index of 41.1%. The OSC content of the fresh onion was affected to a greater extent during the oral and intestinal stages than in the gastric stage, mainly because the alliinase enzyme was more active at the neutral pH found during the oral and intestinal stages. However, during the digestion of black onion, different behaviours were observed among the three stages, with a more stable trend being found for the OSC concentrations and a total bioaccessibility index of 83.3%. This greater stability could be explained by the fact that the temperatures used to produce black onion may inactivate the alliinase enzyme. Therefore, it seems that the black onion production process has a positive effect on the bioaccessibility of OSCs, with propanethial sulfoxide, isoalliin, GSMCS, and methionine sulfoxide being the OSCs that were most readily absorbed and transformed in the large intestine.

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Abbreviations

OSCs: organosulfur compounds. 2VD: 2-vinyl-4H-1,3-dithiin. DADS: diallyl disulphide. DATS: diallyl trisulfide. UHPLC-HRMS: ultra-high-performance liquid chromatography coupled to high resolution mass spectrometry. BI: bioaccessibility index. FA: formic acid. RH: relative humidity. FW: fresh weight. BOD: before oral digestion. AOD: after oral digestion. AGD: after gastric digestion. AID: after intestinal digestion. GSAk: γ-glutamyl-S-alk(en)yl-L-cysteine. GSMCS: γ-glutamyl-S-methyl-L-cysteine sulfoxide. G1PCS: γ-glutamyl-S-(1-propenyl) cysteine sulfoxide. G1PCS: γ-glutamyl-S-(2-propenyl) cysteine sulfoxide. GSPC: γ-glutamyl-S-(propyl) cysteine. GSAC: γ-glutamyl-S-allyl-L-cysteine. SACs: S-alk(en)yl-L-cysteine. SAC: S-allylcysteine.

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Artículo 5

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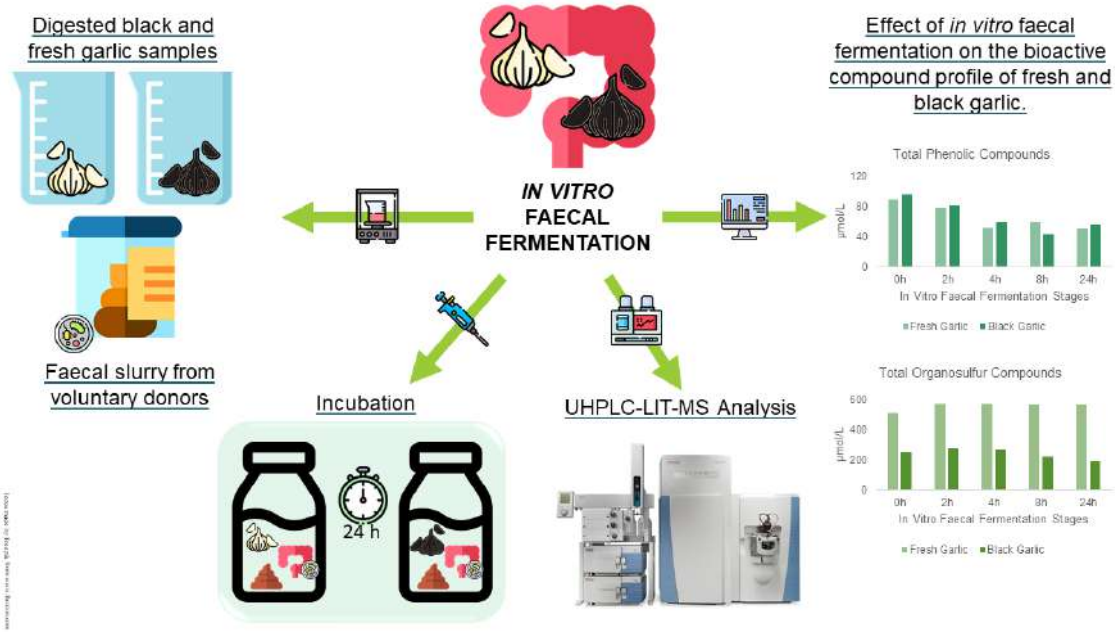

In vitro colonic fermentation of fresh and black garlic

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Abstract

The beneficial properties associated with garlic consumption have been related to the presence of recognised bioactive compounds including (poly)phenols and organosulfur compounds (OSCs). This study aims to assess the effect of *in vitro* colonic fermentation on fresh and black garlic by determining the stability and transformation of these compounds through ultra-high-performance liquid chromatography coupled to mass spectrometry with a linear ion trap (uHPLC-LIT-MS). Colonic fermentation had a similar influence on the phenolic content of fresh and black garlic, with total respective decreases of 43.8% and 41.7% regarding the initial content. Meanwhile, *in vitro* colonic fermentation resulted in a significant decrease (33%) in OSCs in black garlic, but not in the fresh variety. Compounds such as 4-hydroxybenzoic acid (16.2 μmol/L), benzoic acid (7.8 μmol/L), *S*-allylcysteine (SAC) (326 μmol/L), methionine sulfoxide (158 μmol/L), and γ −glutamyl-*S*-allyl-L-cysteine (GSAC) (31 μmol/L) were the phenolic compounds and OSCs with the highest concentration in fresh garlic after the *in vitro* fermentation. Meanwhile, the main compounds found after the fermentation of black garlic were 4′-hydroxyphenylacetic acid (16.0 μmol/L), 4-hydroxybenzoic acid (14.1 μmol/L), methionine sulfoxide (158 μmol/L) and *S*-allylcysteine (18 μmol/L). These compounds, potentially present at colonic level, might be responsible for the systemic health benefits associated with the consumption of black and fresh garlic.

Keywords: colonic fermentation; human microbial metabolism; (poly)phenols; organosulfur compounds

1. Introduction

Garlic (*Allium sativum* L.) is a plant food that originated in Asia and whose consumption is widespread throughout the world, being a fundamental ingredient in the diet of the Mediterranean basin (Vallverdú-Queralt et al., 2013). Historically, the regular consumption of garlic has been associated with a multitude of health benefits, making it an interesting subject of study for many researchers (Ansary et al., 2020; Rana et al., 2011). For instance, regular garlic consumption has been related to a reduction in the risk of cardiovascular disease and metabolic syndrome by decreasing serum cholesterol and triglycerides, and increasing fibrinolytic activity (Banerjee & Maulik, 2002; Choudhary et al., 2018) . Similarly, the consumption of this plant food has been found to have a potential preventive or therapeutic effect against prostate (Devrim & Durak, 2007), liver (X. Liu et al., 2019), ovarian (Xu et al., 2013), colorectal and stomach cancers (Fleischauer et al., 2000). These beneficial properties associated with garlic consumption have been related to the presence of compounds with recognised bioactivity: (poly)phenols and organosulfur compounds (OSCs) (S. Chen et al., 2013; Santhosha et al., 2013).

The popularity of black garlic has increased in recent years among the population. It is a derived product with quite a different composition obtained from fresh garlic under specific temperature and humidity conditions (Kimura et al., 2017; Ryu & Kang, 2017). Compared with fresh garlic, it presents higher contents of reducing sugars, organic acids, and bioactive compounds, particularly *S*-allyl-cysteine (SAC) and coumaric acid (Colín-González et al., 2012b; Martinez-Casas et al., 2017). This results in a product with different organoleptic characteristics, such as a darker colour, sweeter taste, softer texture and less pungent flavour, making it much more attractive to consumers. Despite the differences between them, the consumption of black garlic has been shown to potentially improve human health (Shaikh et al., 2019; S. Tanaka et al., 2006; Williams et al., 2005; Wlosinska et al., 2020), while the mechanisms by which phenolics and OSCs of fresh and black garlic may exert their health effects are not fully elucidated (Ryu & Kang, 2017).

The bioactive compounds present in both fresh and black garlic must first be released from the food matrix during gastrointestinal digestion to generate a physiological response, for which their bioaccessibility needs to be evaluated. In this respect, Moreno-Ortega et al. (Moreno-Ortega, Pereira-Caro, Ordóñez, Moreno-Rojas, et al., 2020) reported the bioaccessibility of (poly)phenols and OSCs of fresh and black garlic during simulated gastrointestinal digestion, showing that OSCs presented a bioaccessibility of

15 and 55%, respectively, while (poly)phenols showed a bioaccessibility of 59 and 47%, respectively. Thus, bioaccessible compounds can be absorbed through the stomach and small intestine, or most of them could reach the colon and be extensively transformed into small molecules by the gut microbiota before absorption.

To date, the colonic metabolism of the characteristic OSCs of the genus *Allium* remains to be understood. Therefore, the aim of this study is to assess the effect of *in vitro* colonic fermentation on fresh and black garlic by determining the stability and transformation of their phenolic and OSC profile by ultra-high-performance liquid chromatography coupled to mass spectrometry with a linear ion trap (UHPLC-LIT-MS).

2. Materials and Methods

2.1. Chemicals

For colonic fermentation, bile salts, soluble starch, (+)-arabinogalactan, tryptone, yeast extract, xylan from birchwood, L-cysteine hydrochloride monohydrate, guar gum, inulin, Tween 80, buffered peptone water, Dulbecco's phosphate buffer saline (PBS), casein sodium salt from bovine milk, pectin from citrus fruits, mucin from porcine stomach-type III, $CaCl_2$, KCl, NaCl, $NaHCO_3$, anhydrous K_2HPO_4, KH_2PO_4, $MgSO_4$ monohydrate, $FeSO_4$ heptahydrate, resazurin redox indicator were purchased from Sigma-Aldrich (St. Louis, MO, USA). Reference phenolic compounds, including 3-(3′-hydroxyphenyl)propanoic acid, 3-hydroxybenzoic acid, 3-phenylpropanoic acid, 4′-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, 3-(3′,4′-dihydroxyphenyl)propanoic acid (aka dihydrocaffeic acid), kaempferol, 3-phenylacetic acid, benzene-1,3,5-triol (aka phloroglucinol), 3,4-dihydroxybenzoic acid (aka protocatechuic acid), quercetin and rutin and 3′,4′-dihydroxycinnamic acid (aka caffeic acid), 3,4,5-trihydroxybenzoic acid (aka gallic acid), 4′-hydroxy-3′-methoxycinnamic acid (aka ferulic acid), 4′-hydroxycinnamic acid (aka *p*-coumaric acid), 4-hydroxy-3-methoxybenzoic acid (aka vanillic acid), luteolin, 3′-hydroxy-4′-methoxycinnamic acid (aka isoferulic acid), 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid (aka dihydroferulic acid), 4′-hydroxy-3′-methoxyphenylacetic acid (aka homovanillic acid), 3-hydroxymandelic acid, 3-(3′-hydroxy-4′-methoxyphenyl)-propanoic acid, benzoic acid, 3-hydroxy-4-methoxybenzoic acid (aka isovanillic acid) were obtained from Sigma-Aldrich (St Louis, MO, USA).

Reference OSCs, including alliin, γ-glutamyl-*S*-allyl-L-cysteine (GSAC), *S*-allyl-L-cysteine (SAC), and *S*-methyl-L-cysteine were purchased from Merck Life Science (Milan, Italy), while *S*-methyl-l-cysteine sulfoxide (methiin) was acquired from Prodotti Gianni (Milan, Italy).

For the UHPLC analysis, all solvents and reagents were UHPLC-grade and were purchased from VWR International (Milan, Italy), unless otherwise indicated. Ultrapure water from MilliQ system (Millipore, Bedfort, MA, USA) was used throughout the experiment.

Note, the names for the phenolic acids used in this paper are based on the nomenclature rules of Kay et al. 2020 (Kay et al., 2020).

2.2. Sample Preparation

Fresh and black garlic (Spring White Garlic) provided by a local supplier (La Abuela Carmen®, Córdoba, Spain) were subjected to *in vitro* gastrointestinal digestion following the protocol previously described (Moreno-Ortega, Pereira-Caro, Ordóñez, Moreno-Rojas, et al., 2020). Subsequently, the digested samples were freeze-dried, milled (Freezer Mill model 6870, Fisher Scientific, Waltham, MA USA) and stored at -80ºC until the colonic fermentation process.

2.3. Faecal Slurry and Growth Medium Preparation

Faecal samples were collected from three healthy volunteers without intestinal diseases who had not consumed antibiotics during the previous 3 months. The volunteers followed a rigorous diet without (poly)phenols and OSCs for 48 h before the faecal collection. After collection, faecal samples were stored in anaerobic conditions and processed within 2 h. Volunteer faeces were pooled in equal amounts (10 g of each one) and homogenized with 300 mL of 1% (*w/v*) sterilized Dulbecco's PBS to obtain a 10% (*w/w*) faecal slurry (Di Pede et al., 2020). The growth medium (1 L) was prepared adopting the composition reported by Bresciani et al. 2018 (Bresciani et al., 2018) and was sterilised at 121 °C for 15 min in glass vessels (12 mL).

2.4. In vitro Colonic Fermentation

The *in vitro* colonic fermentation was performed as previously reported Dall'Asta et al., 2012(Dall'Asta et al., 2012) with slight modifications. The fermentation was performed in 12-mL sterilised glass vessels to which 1.8 mL of growth medium, 1.8 mL of faecal slurry and 400 µL of a solution of digested fresh or black garlic were added, reaching a total volume of 4 mL(Tan et al., 2015). Before filling each vessel, 100 mg of digested and freeze-dried fresh and black garlic was dissolved in a Dulbecco's PBS solution containing bile salts for 2 hours under stirring at room temperature(Di Pede et al., 2020).

2.5. Phenolic and Organosulfur Compounds and Faecal Metabolites Extraction

Phenolic compounds, OSCs and their related faecal metabolites produced during the *in vitro* colonic fermentation of digested fresh and black garlic were extracted as previously was reported by Bresciani et al. 2018 (Bresciani et al., 2018, 2021), with minor modifications. (Poly)phenols and OSCs were extracted following the same procedure but with different solvents, ethyl acetate (0.1% (*v/v*) formic acid) and ethanol (0.1% (*v/v*) formic acid), respectively. Briefly, 300 µL of fermented sample was mixed with 1.2 mL of the solvent and vortexed for 30 s, sonicated for 10 min in an ultrasonic bath, and vortexed again for 30 s, re-sonicated for 5 min, and then centrifuged (Centrisart® A-14C Refrigerated Micro-Centrifuge and Rotor YCSR-A1C, Sartorius Lab Instruments GmbH and Co. KG, Goettingen, Germany) at 14,000 rpm for 10 min. The supernatant was collected in a clean microfuge tube, while the pellet was re-extracted using 500 µL of the same solvent following the same protocol described. Finally, the supernatants were pooled and stored at -80 ºC. The fermented samples extracted with ethyl acetate (0.1% (*v/v*) formic acid) were brought to dryness though a centrifugal concentrator (SpeedVac Savant SPD121P, Thermo Fisher Scientific Inc., San José, CA, USA), to be reconstituted in 300 µL methanol-acidified water (0.1 % (*v/v*) formic acid) (50:50, *v/v*). Finally, these samples were vortexed for 30 s and centrifuged at 14,000 rpm for 5 min and stored at -80 ºC until the UHPLC-LIT-MS analysis.

2.6. Phenolic and Organosulfur Compounds and faecal Metabolites Analysis

Changes in (poly)phenols and OSCs during the colonic fermentation were analysed by ultra-high performance liquid chromatography (uHPLC) coupled with mass spectrometry (MS), using an Accela uHPLC 1250 apparatus equipped with linear ion trap MS (LIT-MS) (LTQ XL, Thermo Fisher Scientific Inc.) fitted with a heated-electrospray ionization (H-ESI-II) probe (Thermo Fisher Scientific Inc.). For the analysis of (poly)phenols and their metabolites in fermented fresh and black garlic extracts, separation was carried out by means of a Kinetex Evo C18 column (100 x 2.1 mm; 2.6 µm particle size, Phenomenex, CA, USA) installed with a precolumn cartridge (Phenomenex). The volume injection was 5 µL and the column oven was set to 35 °C. The mobile phases, A-acidified acetonitrile 0.01% (*v/v*) formic acid and B-acidified water 0.01% (*v/v*) formic acid, were pumped at a flow of 500 µL/min with a 14 min gradient, starting with 5% A and maintained for 0.5 min, then rising to 40% A in 7 min, before rising 80% A in 1 min and maintained for 2 min. Next, the column was equilibrated to the initial conditions within 0.5 min and maintained for 3 min to re-equilibrate it. The (poly)phenols and their metabolites in the fermented fresh and black garlic extracts were analysed by adopting

the mass spectrometry method following a previously described methodology (Di Pede et al., 2020).

A modified methodology was developed for the analysis of the OSCs and their metabolites in the extracted fermented samples (Moreno-Rojas et al., 2018). Analyte separation was performed using an ACQUITY UPLC® BEH AMIDE (2.1 x 150 mm, 1.7 µm particle size) column (Waters, Milford, MA, USA). The column oven was set to 35 °C. The autosampler temperature and the injection volume were set at 10 °C and 2 µL, respectively. The mobile phases consisted of a mixture of 0.1 % (*v/v*) formic acid in acetonitrile (phase A), 0.1 % (*v/v*) formic acid in water (phase B), and ammonium formate (20 mmol/L) containing 1% (*v/v*) formic acid (phase C). The flow rate was set at 400 µL/min with the following gradient: starting from 0 to 1 min of 5% phase B and 5% phase C in phase A, the proportion of phase B was increased linearly to 30% over a period of 7 min, then increased to 45% over 4 min. The start condition was re-established in 3 min and kept for 4 min to re-equilibrate the column. Phase C was maintained at 5% during the run (total run: 19 min). The analytical mass spectrometry conditions were optimized by infusion of a pure standard of alliin, working in a positive ionization mode. The H-ESI-II interface was set to a capillary temperature of 275 °C and the source heater temperature was set at 200 °C. The sheath gas (N_2) flow rate was set at 50 (arbitrary units), while auxiliary and sweep gases (N_2) were set at 15 and 0, respectively (arbitrary units). The source voltage was 3.8 kV, the capillary voltage was 6 V and tube lens voltage was 50 V. Ultra-pure helium gas (99.9999%) was used in MS/MS analyses. The OSCs and their metabolites in the fermented samples were analysed using a full-scan, data dependent MS^2 from *m/z* 100 to 500. Based on the reference compounds, GSAC, SAC, alliin, methiin and *S*-methyl-L-cysteine were monitored through full MS/MS experiments with a collision induced dissociation (CID) equal to 35 (arbitrary units) (Table 1).

XCalibur software v. 2.1 (Thermo Fisher Scientific Inc.) was employed to acquire the chromatograms and spectral data and quantification was performed with linear regression analysis with R^2 values of >0.998 of the available reference compounds. The limits of quantification (LOQs) and limits of detection (LODs) for each standard used were calculated based on the minimum accepted signal-to-noise (S/N) ratio values of 10 and 3, respectively. The quantification limits were from 0.03 to 25 µmol/L for the (poly)phenols and from 0.25 to 1 µmol/L for the OSCs, and the limits of detection were from 0.01 to 5 µmol/L for (poly)phenols and from 0.03 to 0.25 µmol/L for OSCs.

2.7. Statistical Analysis

Statistical analyses were performed based on three replicates of each sample. A one-way ANOVA was performed using R software (v. 3.6.3, R Core Team, Vienna, Austria) to determine significant differences for the same fermented sample at the different incubation period (0, 2, 4, 8 or 24 h), significance being accepted for a p-value < 0.05. Next, Fisher's LSD pairwise comparison was performed on the data. A principal component analysis (PCA) was performed as an unsupervised method using SIMCA software (v.17.0.1) to determine whether the overall changes in the profiles of the phenolic compounds and OSCs were different enough to distinguish between the *in vitro* colonic fermentation stages and matrices (fresh or black garlic).

3. Results and Discussion

3.1 Evolution of Phenolic Compounds of Fresh and Black Garlic during Colonic Fermentation

Table 1.- UHPLC-LIT-MSn-based identification of phenolic and organosulfur compounds detected in fresh and black garlic during in vitro colonic fermentation.

Compound	RT (min)	m/z	Full Scan	MS^2 Fragmentation Pattern	MSI MI Level*	Occurrence
Phenolic Compounds		**[M-H]-**				
Flavonol						
Quercetin	6.12	301		**179**, 151, 273, 257, 193, 239, 107, 283	1	FG; BG
Kaempferol	6.97	285		**151**, 229, 257, 213, 243, 185, 169, 107, 143	1	FG; BG
Rutin	4.29	609		**301**, 300, 343, 271, 179, 591	1	FG; BG
Isorhamnetin	9.13	315		**297**, 279, 171, 241, 227, 151	2	FG; BG
Flavone						
Luteolin	6.13	285		**241**, 175, 199, 217, 241, 257, 151, 107	1	BG
Hydroxycinnamic acids						
4'-Hydroxycinnamic acid	3.31	163		**119**, 135	1	FG; BG
3',4'-Dihydroxycinnamic acid	2.51	179		**135**, 151, 117, 91	1	FG; BG
4'-Hydroxy-3'-methoxycinnamic acid	3.7	193		**149**, 178, 134, 107	1	FG; BG
Phenylpropionic acids derivatives						
3-(3′,4′-Dihydroxyphenyl) propanoic acid	1.85	181		**137**, 119, 59, 163	1	FG; BG
3-(4′-Hydroxy-3′-methoxyphenyl) propanoic acid	3.08	195		**136**, 151, 177, 123, 59	1	FG; BG
3-(3'-Hydroxyphenyl) propanoic acid	3.1	165		**121**, 147, 165, 97	1	FG; BG
3-Phenylpropanoic acid	4.8	149		**105**, 131	1	FG; BG
Phenylacetic acids derivatives						
4'-Hydroxyphenylacetic acid	1.85	151		**107**, 120, 93	1	FG; BG
3-Phenylacetic acid	3.36	135		**91**	1	FG; BG
Benzoic acids derivatives						
Benzoic acid	3.5	121		**77**	1	FG; BG
4-Hydroxy-3-methoxybenzoic acid	2.31	167		**123**, 152, 108, 79	1	FG; BG
3,4,5-Trihydroxybenzoic acid	0.74	169		**125**, 97, 81	1	FG; BG
3,4-Dihydroxybenzoic acid	1.16	153		**109**, 135, 81	1	FG; BG
3-Hydroxybenzoic acid	2.08	137		**93**, 115	1	FG; BG
4-Hydroxybenzoic acid	1.78	137		**93**	1	FG; BG
3-Hydroxy-4-methoxybenzoic acid	2.44	167		**152**, 123, 99	1	BG

Hydroxycarboxilic acids derivatives						
3'-Hydroxymandelic acid	0.87	167		**123**, 105, 85	1	FG; BG
Benzenotriol						
Benzene-1,3,5-triol	0.65	125		**83**, 57	1	FG; BG
Organosulfur Compounds		**[M-H]+**				
γ-Glutamyl-S-Alk(en)yl-L-Cysteine Sulfoxides						
γ −Glutamyl-*S*-methyl-L-cysteine sulfoxide (GSMCS)	4.85	281	**121**, 132, 173, 207, 235, 245		2	FG; BG
γ −Glutamyl-*S*-(2-propenyl) cysteine sulfoxide (G2PCS)	3.89	307	**229**, 269, 171, 146, 130		2	FG; BG
γ −Glutamyl-*S*-(1-propenyl) cysteine sulfoxide (G1PCS)	7.92	307	**176**, 207, 134, 235, 269		2	FG; BG
γ-Glutamyl-S-Alk(en)yl-L-Cysteine						
γ −Glutamyl-L-cysteine	4.06	251		**223, 2**34, 166, 178, 110, 206, 138	2	FG; BG
γ −Glutamyl-*S*-methyl-L-cysteine (GSMC)	3.7	265	**231**, 105, 171, 167, 212, 130, 145, 196		2	FG
γ −Glutamyl-*S*-(*S*-methyl) cysteine-glycine	4.59	354	**244**, 118, 145, 260, 203		2	BG
γ −Glutamyl-*S*-(2-carboxypropyl) cysteine	6.95	335	**120**, 161, 203, 229, 290, 302		2	BG
γ −Glutamyl-*S*-allyl-L-cysteine (GSAC)	6.19	291		**162**, 145, 170, 274, 245, 122, 84	1	FG; BG
γ -Glutamyl-*S*-(2-propenyl) cysteine (GS2PC)	7.09	291	**148**, 291, 365, 203		2	FG; BG
S-Alk(en)yl-L-Cysteine Sulfoxides						
Alliin	6.33	178		**88**, 91, 116, 73, 132, 160	1	FG; BG
Isoalliin	6.86	178		**88**, 91, 116, 73, 132, 160	2	FG; BG
S-Methyl-L-cysteine sulfoxide (Methiin)	7.47	152		147, 105, 118, 122, 132	1	FG
S-Propyl-L-cysteine sulfoxide (Propiin)	8.68	180		**162**, 144, 134	2	BG
Methionine sulfoxide	4.7	166		**121**, 149, 131	2	FG; BG
S-Alk(en)yl-L-Cysteine						
S-Allylcysteine (SAC)	4.5	162		**145**, 115, 134, 76, 99	1	FG; BG
S-Allylmercapto-L-cysteine (SAMC)	3.92	194	**171**, 154, 130, 136, 118		2	FG; BG
S-Propylmercapto-L-cysteine (SPMC)	3.78	196	**104**, 171, 145, 110, 130, 184		2	FG
S-(2-Carboxypropyl) cysteine	5.96	208	**105**, 171, 182, 132, 118, 146, 159		2	FG; BG
S-(2-Carboxypropyl) cysteine-glycine	6.95	265	**120**, 161, 230, 171, 244, 258		2	FG; BG
S-Allylglutathione (SAG)	4.69	348	**229**, 203, 244, 105, 118, 270		2	BG

**Metabolite standards initiative (MSI) metabolite identification (MI) levels*(Sumner et al., 2007)*. Reference compounds were available for all compounds identified at MSI MI level 1. RT: Retention time; FG: Fresh Garlic; BG; Black Garlic; Compounds for which there was a reference compound were quantified with them, while for the rest, the most structurally*

similar reference compound was used. Isorhamnetin was quantified with quercetin. γ-Glutamyl-S-Alk(en)yl-L-Cysteine Sulfoxides and γ-Glutamyl-S-Alk(en)yl-L-Cysteine were quantified with GSAC; Isoalliin was quantified with Alliin and Propiin with Methiin. S-Alk(en)yl-L-Cysteine were quantified with SAC.

A total of 21 and 23 phenolic compounds were identified and quantified during the different stages of the *in vitro* colonic fermentation (0, 2, 4, 8, and 24h) in the fresh and black garlic, respectively (Table 1). Among them, 4 flavonols, 1 flavone, 3 hydroxycinnamic acids, 4 phenylpropanoic acid derivatives, 2 phenylacetic acid derivatives, 7 benzoic acid derivatives, 1 hydroxycarboxylic acid and 1 benzenotriol were identified and quantified in the fermented samples. The time-course profiles of the phenolic compounds identified during the colonic fermentation of the fresh and black garlic are shown in Figure 1 and in Table S1 in Supporting Information. Colonic fermentation influenced the phenolic content of the fresh and black garlic similarly, with a significant decrease of 43.8% and 41.7% during the process, respectively. Both matrices showed a progressive decrease in their phenol content during *in vitro* colonic fermentation, although the fresh garlic experienced a slight increase after 8 hours and then continued to decrease until the end of the process, and black garlic showed a significant increase in its phenol content after 24h of colonic fermentation compared to the previous stage. At the beginning of the colonic fermentation, phenylacetic acid and benzoic acid derivatives were the main phenolic families in fresh (43.5 and 24.7%, respectively) and black garlic (45.8 and 25%), followed by derivatives of benzenetriol (17% in fresh garlic and 15.9% in black garlic) and phenylpropanoic acid (13.2% in fresh garlic and 10.6% in black garlic).

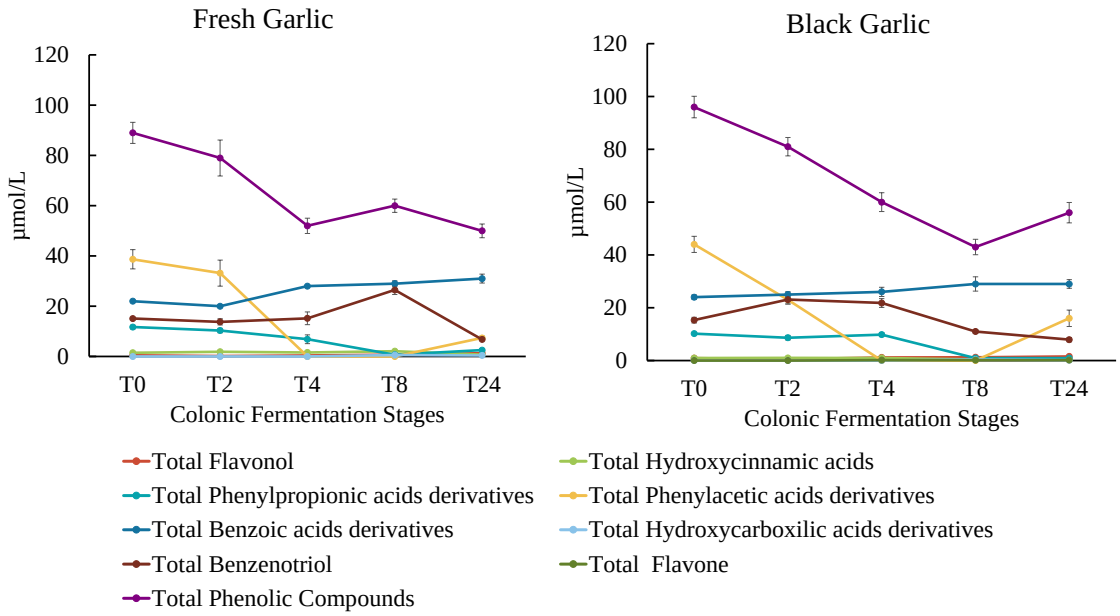

Figure 1. Time-course profiles of phenolic families found during in vitro colonic fermentation of fresh and black garlic at 0, 2, 4, 8 and 24h (T0, T2, T4, T8 and T24, respectively). Data are expressed as mean values ± standard deviation of three different replicates (n=3).

The individual phenolic compound profile of fresh and black garlic during the *in vitro* colonic fermentation is represented by Figure 2 and in Table S1 in Supporting Information.

In the fresh garlic, the concentration of the total phenylpropanoic acid derivatives decreased during the fermentation process, reaching a final concentration of 2.55 µmol/L, which represented 21.7% of its initial content (Figure 1). 3-(3′,4′-Dihydroxyphenyl)propanoic acid and 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid were the only phenylpropanoic acids present after 24 h of fermentation. The total content of phenylacetic acid derivatives showed a significant decrease in concentration after 4 h of colonic fermentation, increasing its final content after 24 h to 7.4 µmol/L (Figure 1), mainly due to the formation of 4-hydroxyphenylacetic acid during the last step of the fermentation (Figure 2).

The total content of benzoic acid derivatives increased significantly during the *in vitro* colonic fermentation, reaching 140.9% regarding the initial content (Figure 1). This is mainly due to the fact that benzoic acid derivatives are final catabolites of the degradation pathway of phenolic compounds during colonic fermentation (Selma et al., 2009). Indeed, among them, 3,4-dihydroxybenzoic acid probably derives from the α-oxidation of 3-(3′,4′-dihydroxyphenyl)propanoic acid (Ludwig et al., 2013) and 4-hydroxybenzoic acid potentially was derived from the dehydroxylation of 3,4-dihydroxybenzoic acid (Di Pede et al., 2020).

The total flavonol content increased progressively after 2 h of colonic fermentation, showing its highest concentration after 24 h, with a 2.9-fold increase compared with the beginning of the process (Figure 1). This may be due to the release of these compounds with the hydrolysis of their glycosides, as well as their release from the food matrix during fermentation (Amaretti et al., 2015; Hollman et al., 1995, 1997). Concerning hydroxycinnamic acids, the total content increased from 4 h of incubation until it reached its maximum amount at 8 h, decreasing by 50.7% after 24 h of fermentation (Figure 1).

Of note was that benzene-1,3,5-triol experienced a significant increase in concentration after 8 h of colonic fermentation, to be subsequently degraded after 24 h, with a final concentration of 6.8 µmol/L, being one of the main catabolites found after *in vitro* colonic fermentation (Figure 1; Table S1). This catabolite is reported to be derived from quercetin, resulting from the breakdown of one of the rings of the quercetin structure by the action of colonic microbiota (A.-M. Aura, 2008; Dueñas et al., 2015). This is the presumed cause for the increase in the concentration of this catabolite during the first

hours of the fermentation. Subsequently, benzene-1,3,5-triol probably continues its degradation to short-chain fatty acids until the end of colonic fermentation (Braune & Blaut, 2016).

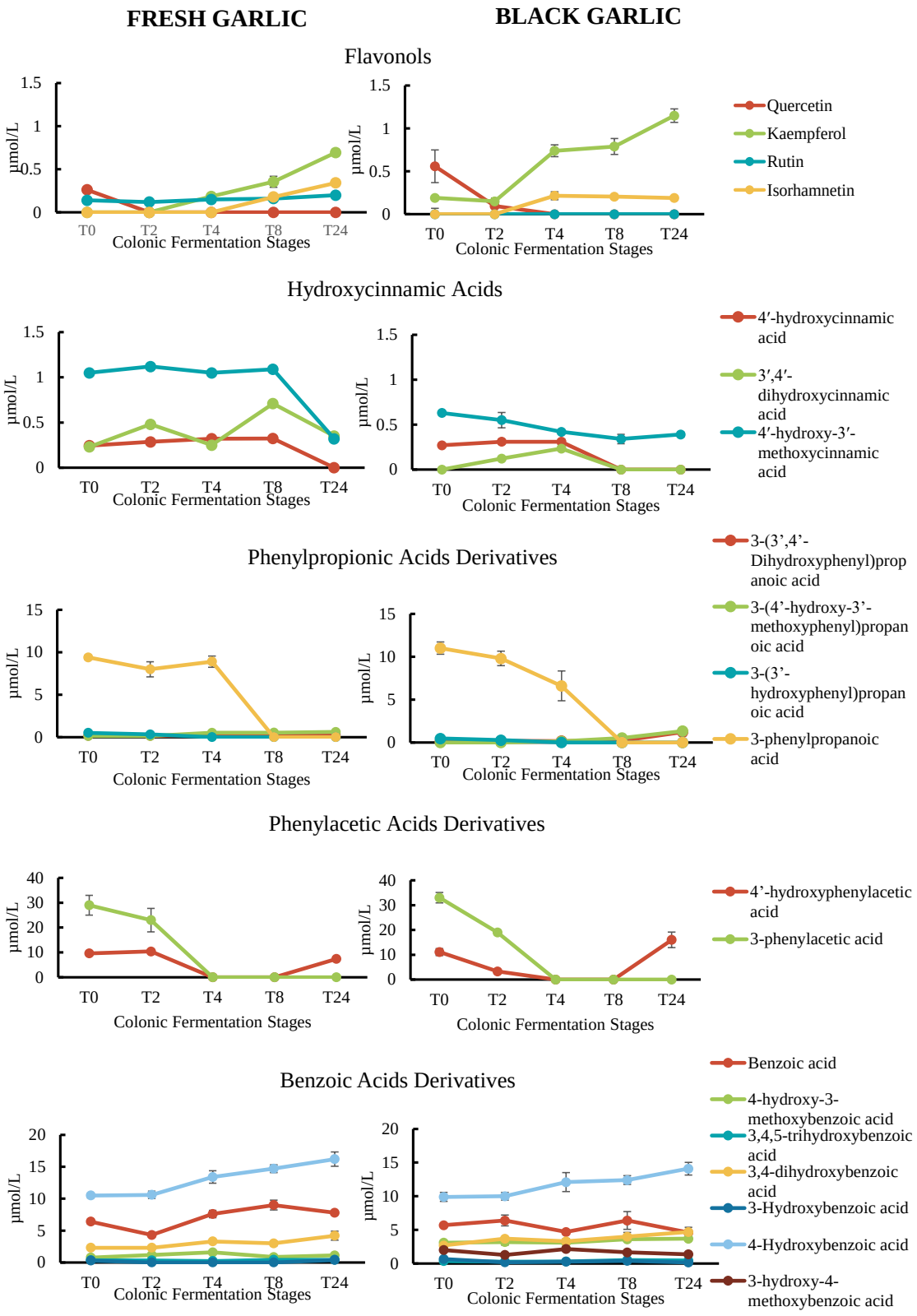

Figure 2. Evolution of phenolic compounds and catabolites during the in vitro colonic fermentation of fresh and black garlic at 0, 2, 4, 8 and 24h (T0, T2, T4, T8 and T24, respectively). Data are expressed as mean values ± standard deviation of three different replicates (n=3).

Regarding black garlic, the evolution of the phenolic profile of the different families showed similar trends to those presented by fresh garlic (Figure 2). 3-(3′,4′-Dihydroxy(phenyl)propanoic acid and 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid were the main phenylpropanoic acid derivatives detected after 24 hours of fermentation, their concentrations increasing during the entire process to reach 188 and 443% of their initial content, respectively. Similarly, the total content of phenylacetic acid derivatives decreased throughout the colonic fermentation despite a significant increase in 4-hydroxyphenylacetic acid, which reached a concentration of 16 µmol/L. Serra et al. (2012) (Serra et al., 2012), among other authors, reported that phenylacetic acids are one of the main catabolites of the colonic degradation of quercetin and other flavonols.

During *in vitro* colonic fermentation, benzoic acid derivatives remained stable, increasing non-significantly after 8 h and remaining stable after 24 h. This was mainly due to the significant increase in 4-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid after the final stage, representing 142 and 174% of the initial concentration.

The concentration of benzene-1,3,5-triol, a microbial metabolite of quercetin, increased considerably after 4 h of colonic fermentation, after which it started to decrease to a final concentration of 7.9 µmol/L. This compound may subsequently be degraded by the action of the microbiota to short-chain fatty acids (Braune & Blaut, 2016).

Finally, flavonols increased in concentration during *in vitro* colonic fermentation, likely as a result of the release of aglycones from more complex structures that have not been detected in this analysis, as well as the release of these compounds from the food matrix. Flavonol increase was mainly due to the increase of kaempferol, which presented a concentration 6.1-fold higher than at the beginning of fermentation, possible due to the dehydroxylation of quercetin by microbial action(A. M. Aura et al., 2002). Hydroxycinnamic acids remained stable during the first 4 h, after 8 h their concentration decreased by half, and this was continued until the end of colonic fermentation. These compounds are substrates for the colonic microbiota and precursors of other compounds that are produced during fermentation (phenylpropanoic acids derivatives, phenylacetic acids derivatives and benzoic acids derivatives) (Leonard et al., 2021).

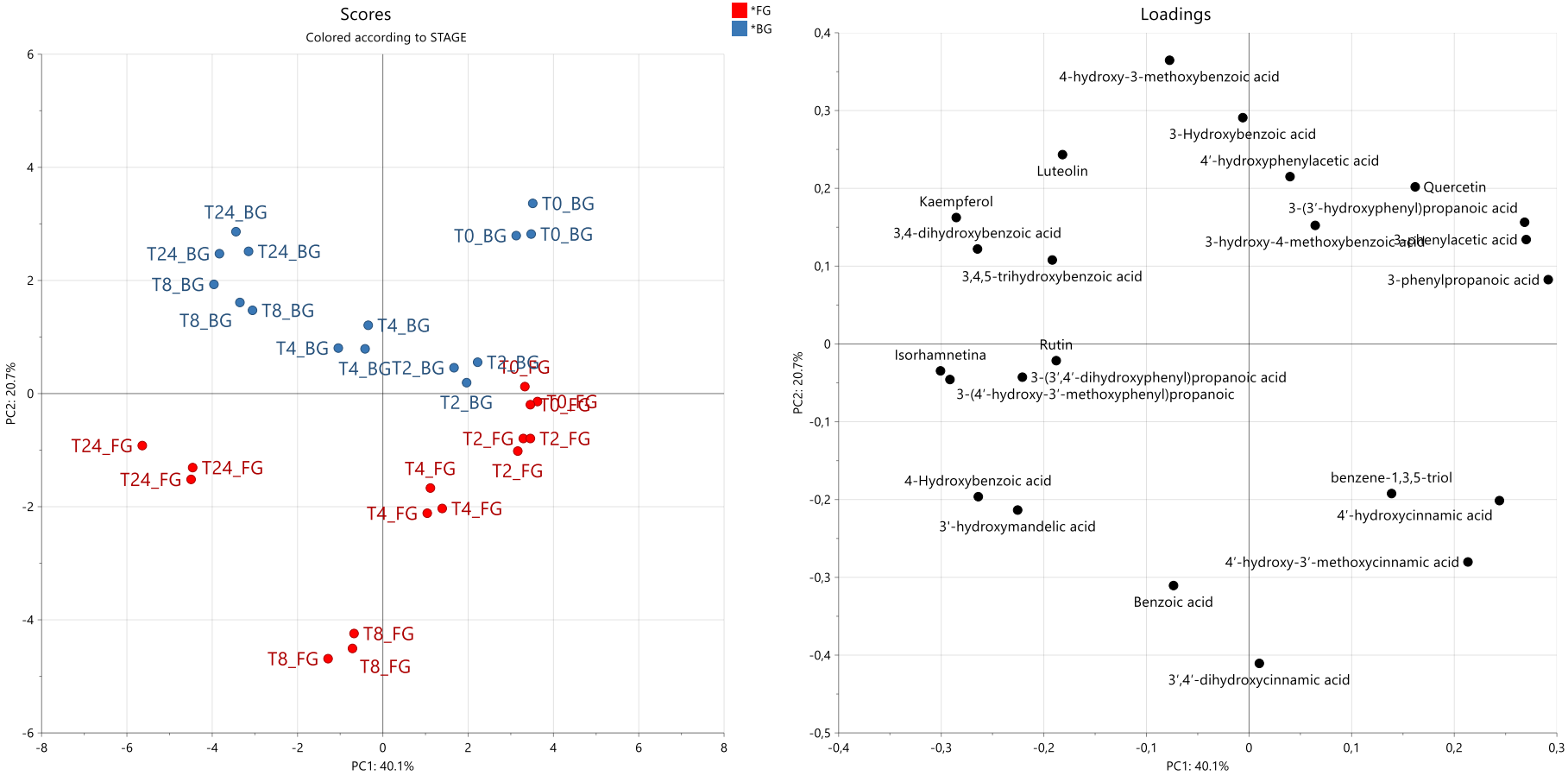

Figure 3.- Scores and loadings obtained in the principal component analysis (PCA) comparing data from phenolic compounds in fresh (FG = red plot) and black garlic (BG = blue plot) during *in vitro* colonic fermentation at 0, 2, 4, 8 and 24h (T0, T2, T4, T8 and T24, respectively).

To determine the effect of the *in vitro* colonic fermentation of fresh and black garlic on their phenolic profile, a principal component analysis (PCA) was applied (Fig. 3). Figure 3 shows the score plot according to the two principal components selected (PC). PC1, accounting for 40% of the total variance, clearly discriminated between the samples based on the different stages of the fermentation process, kaempferol, 3,4-dihydroxybenzoic acid, isorhamnetin and 3-(4′-hydroxy-3′-methoxyphenyl) propanoic acid being the most significant variables that contribute to sample discrimination as they were found in greater quantities at the end of the fermentation process (T24) in the fresh and black garlic. Meanwhile PC2 explained 20% of the total variance, clearly discriminating between the fresh and black garlic matrices. The loading plot reveals that 4-hydroxy-3-methoxybenzoic acid and 3-hydroxybenzoic acid strongly contributed to this separation by their greater presence in black garlic, while 3′,4′-dihydroxycinnamic acid and benzoic acid were found to a greater extent in fresh garlic.

Overall, the main end products in the fresh garlic were 4-hydroxybenzoic acid, benzoic acid, 4-hydroxyphenylacetic acid, benzene-1,3,5-triol and 3,4-dihydroxybenzoic acid, comprising 84.8% of the total phenolic compounds, while 4-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, benzene-1,3,5-triol, 3,4-dihydroxybenzoic acid, benzoic acid, and 4-hydroxy-3-methoxybenzoic acid were the main end products in the black garlic, comprising 91.1% of total phenolic compounds. It is of note that the presence of these compounds at the colonic level has a potentially beneficial effect on health, in particular by protecting against the proliferation of cancer cells. Dobani et al (2021) (Dobani et al., 2021) reported that the presence of phenolic compounds such as benzoic acid and 4-hydroxybenzoic acid, derived from the microbial degradation of raspberry (poly)phenols, had a protective effect against carcinogenesis due to the activation of detoxifying enzymes, mediated by nuclear factor erythroid 2-related factor 2 (Nrf2) activity. Regarding benzene-1,3,5-triol, it has been observed that treatment with a physiological concentration of this compound is capable of causing cell apoptosis in HT29 colon cancer cell lines, preventing their proliferation (M.-H. Kang et al., 2014b). Moreover, nanocarrier systems have been designed to allow 3,4-dihydroxybenzoic acid to specifically reach cancer cells at colon level to improve its bioefficacy (Abotaleb et al., 2020). Likewise, Gong et al. (2019) (Gong et al., 2019) reported that 4-hydroxy-3-methoxybenzoic acid is able to inhibit the proliferation of human colon cancer cells (HCT116) at a physiological concentration.

3.2 Evolution of Organosulfur Compounds of Fresh and Black Garlic during Colonic Fermentation

A total of 16 OSCs were identified and quantified in the fresh garlic during the different stages of the *in vitro* colonic fermentation, including 9 *S*-alk(en)yl-L-cysteine derivatives (SACs) and 7 γ-glutamyl-*S*-alk(en)yl-L-cysteine (GSAk) derivatives (Table 1). The basis of their identification is presented in Table 1. Among them, alliin (217.3 μmol/L), methionine sulfoxide (104 μmol/L) and SAC (72 μmol/L) were the main SAC derivatives at the beginning of the fermentation of the fresh garlic, accounting for the 83% of the SAC derivatives, while GSAC (19 μmol/L) and γ −glutamyl-*S*-(2-propenyl) cysteine sulfoxide (G2PCS) (12.2 μmol/L) were the main GSAk derivatives, accounting for the 78% of the total GSAk compounds (Table S2, Supporting Information). These results are in line with those reported by Moreno-Ortega et al. 2020 (Moreno-Ortega, Pereira-Caro, Ordóñez, Moreno-Rojas, et al., 2020), who found that alliin, GSAC and SAC were the major OSCs in fresh garlic after simulated gastrointestinal digestion. Regarding the black garlic, a total of 17 OSCs were identified and quantified during the colonic fermentation, of which 91.9% were SAC derivatives, where methionine sulfoxide (91 μmol/L), propiin (53.2 μmol/L) and SAC (35 μmol/L) were the main ones. The main GSAk derivatives were γ −glutamyl-L-cysteine (12.1 μmol/L) and γ −glutamyl-*S*-methyl-L-cysteine sulfoxide (GSMCS) (3.42 μmol/L), accounting for 6.1% of the total OSCs in the black garlic before the colonic fermentation. These data are in line with Moreno-Ortega et al. (2020) (Moreno-Ortega, Pereira-Caro, Ordóñez, Moreno-Rojas, et al., 2020), who also reported the presence of SAC, methionine sulfoxide and GSMCS in black garlic after the intestinal phase of *in vitro* digestion.

The time-course of the main families of OSCs of fresh and black garlic during colonic fermentation are presented in Figure 4. The total OSCs in the black garlic were strongly affected by *in vitro* colonic fermentation, while GSAk and SAC derivatives as well as the total organosulfur compounds in the fresh garlic remained stable during the whole process, accounting for 92.5, 111.6 and 110.1% at the end, respectively. Furthermore, after colonic fermentation the total content of OSCs in the fresh garlic increased by 10%, while the black garlic showed a decrease of 23%.

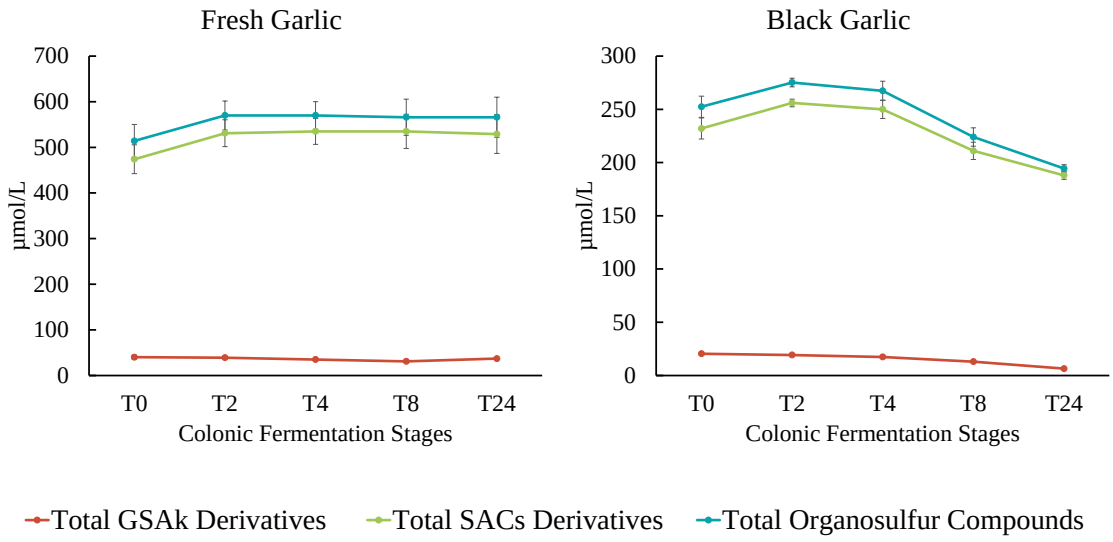

Figure 4. Time-course profiles of organosulfur compound groups found during in vitro colonic fermentation of fresh and black garlic at 0, 2, 4, 8 and 24h (T0, T2, T4, T8 and T24, respectively). Data are expressed as mean values ± standard deviation of three different replicates (n=3).

The total content of SAC derivatives in the fresh garlic did not vary significantly between the stages of the colonic fermentation process, a slight increase being found with regard to the initial content, mainly due to the increase in the concentration of methionine sulfoxide (1.5-fold from initial content) and SAC (4.5-fold from initial content) after 24 h of treatment (Figure 5, Table S2 in Supporting Information). As GSAC content increases, part of it is degraded, resulting in a concomitant increase in SAC, as reported previously by some authors (Colín-González et al., 2012a; Lawson & Hunsaker, 2018; Naoko Yoshimoto et al., 2015). The increase in methionine sulfoxide content during colonic fermentation may be due to the release of methionine from proteins and peptides and its reaction with reactive oxygen species (ROS), resulting in the formation of methionine sulfoxide (Campbell et al., 2016). *S*-Propylmercapto-L-cysteine (SPMC) and *S*-(2-carboxypropyl) cysteine-glycine showed a decrease in their content from the beginning to the end of fermentation, while alliin, isoalliin, *S*-methyl-L-cysteine sulfoxide (methiin) and *S*-(2-carboxypropyl) cysteine experienced a significant increase in their content during the first two hours of the process, accounting for 231.4, 25.6, 17.6 and 5.5 µmol/L, respectively. After the first two hours, the degradation of these compounds started. Likewise, the *S*-allylmercapto-L-cysteine (SAMC) content increased 2.8-fold during the first 8 hours of colonic fermentation, reaching 66.9 µmol/L, while after 24 h the concentration decreased, with a final concentration of 9.6 µmol/L.

The lowest amount of GSAk derivatives was found after 8 h of fermentation, when there was a decrease in GSMCS, G2PCS, γ −glutamyl-*S*-(1-propenyl) cysteine sulfoxide

(G1PCS), γ −glutamyl-*S*-methyl-L-cysteine (GSMC) and GSAC content (Figure 5, Table S2 in Supporting Information). These compounds continued to decrease after 24 h of fermentation, except for GSAC, which increased by 206.7% compared to the previous stage and by 163.2% compared to the initial content. Considering the metabolic pathways proposed by Yamaguchi et al. (2020) (Yamaguchi & Kumagai, 2020), the increase in GSAC during this process may be due to the degradation of more complex compounds usually present in this matrix such as glutathione, *S*-(2-carboxypropyl) glutathione and γ-glutamyl-*S*-(2-carboxypropyl) cysteine.

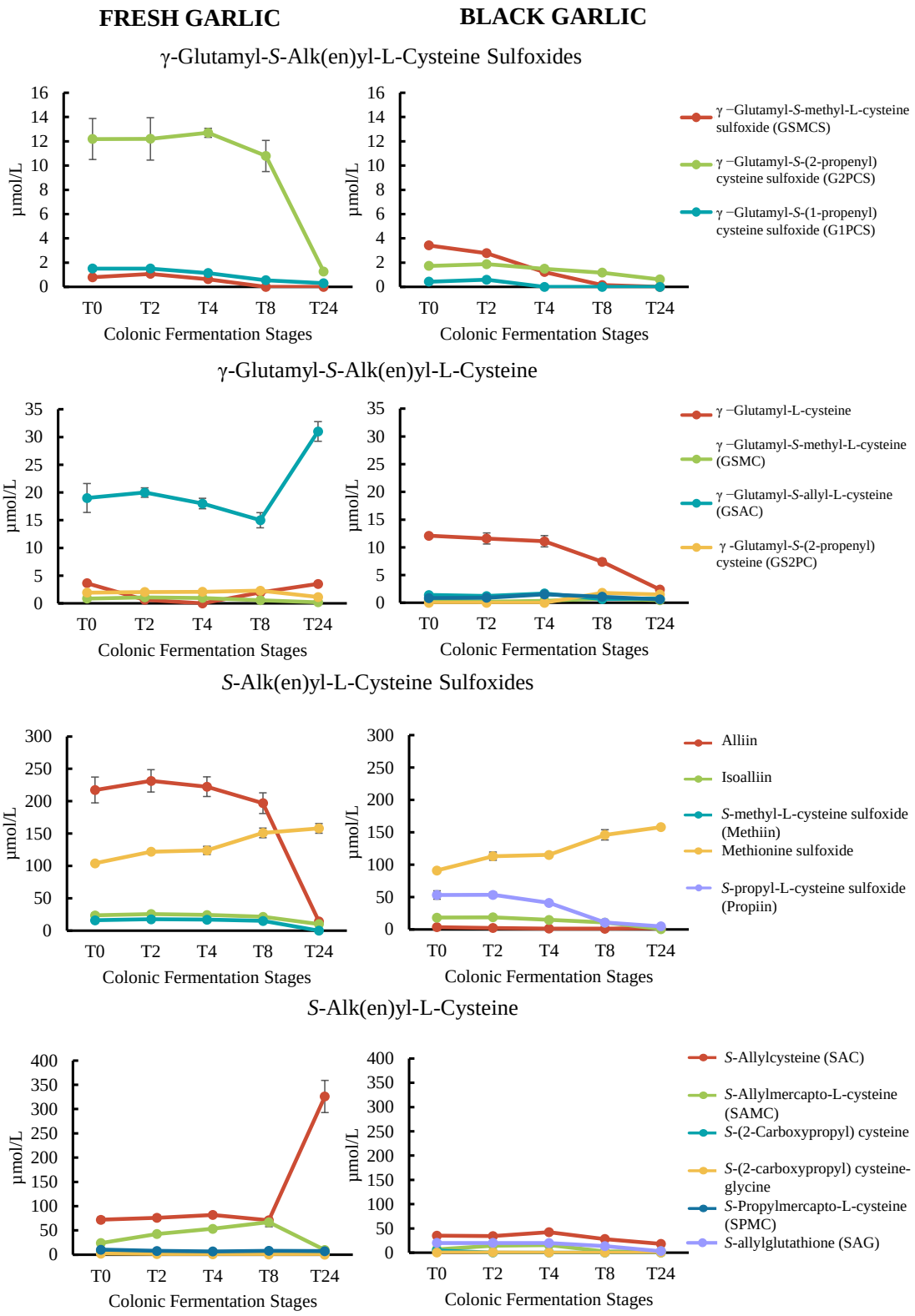

Figure 5. Evolution of organosulfur compounds during the in vitro colonic fermentation of fresh and black garlic at 0, 2, 4, 8 and 24h (T0, T2, T4, T8 and T24, respectively). Data are expressed as mean values ± standard deviation of three different replicates (n=3).

Regarding the colonic fermentation of the black garlic, the final concentration of SAC derivatives was 81% of the initial concentration, so they were less affected by colonic microbiota than the GSAk derivatives (Figure 5, Table S2 in Supporting Information). These compounds were affected by the fermentation process as there was a decrease in their content, except for methionine sulfoxide, the concentration of which progressively increased up to 1.7-fold during the whole process. The formation of this compound may be because methionine is a target compound for ROS, which react with sulphur to produce methionine sulfoxide (Campbell et al., 2016). ROS were probably found due to the incomplete reduction of oxygen during colonic fermentation (R. Jones et al., 2012).

S-(2-(2-carboxypropyl) cysteine was not detected after the start of fermentation, while the same occurred with *S*-(2-carboxypropyl) cysteine-glycine after the first two hours. However, propiin, SAG and isoalliin reached their highest concentrations after two hours of fermentation and started to decrease after this stage. Conversely, SAC and SAMC increased in concentration after 4 hours of fermentation, a decrease being observed thereafter, accounting for 18 and 2.5 µmol/L, respectively, at the end of the colonic fermentation.

Regarding GSAk derivatives, their total content decreased by 68.6% during the *in vitro* colonic fermentation (Figure 5, Table S2 in Supporting Information). GSMCS, γ−glutamyl-L-cysteine and γ−glutamyl-*S*-(2-carboxypropyl) cysteine presented their highest concentrations in the initial stage of colonic fermentation, 3.42 µmol/L, 12.1 µmol/L and 1.42 µmol/L, respectively. Their concentrations began to decline after that. The concentrations of G2PCS and G1PCS increased slightly, although not significantly, during the first two hours of fermentation, after which G1PCS was not detected and G2PCS started to decrease. Finally, GS2PC reached its maximum concentration after 4 hours of colonic fermentation while GSAC was detectable for the first time after 8 hours. The highest concentration of γ -glutamyl-*S*-(*S*-methyl) cysteine-glycine was also found after 8 hours of fermentation, reaching 0.76 µmol/L.

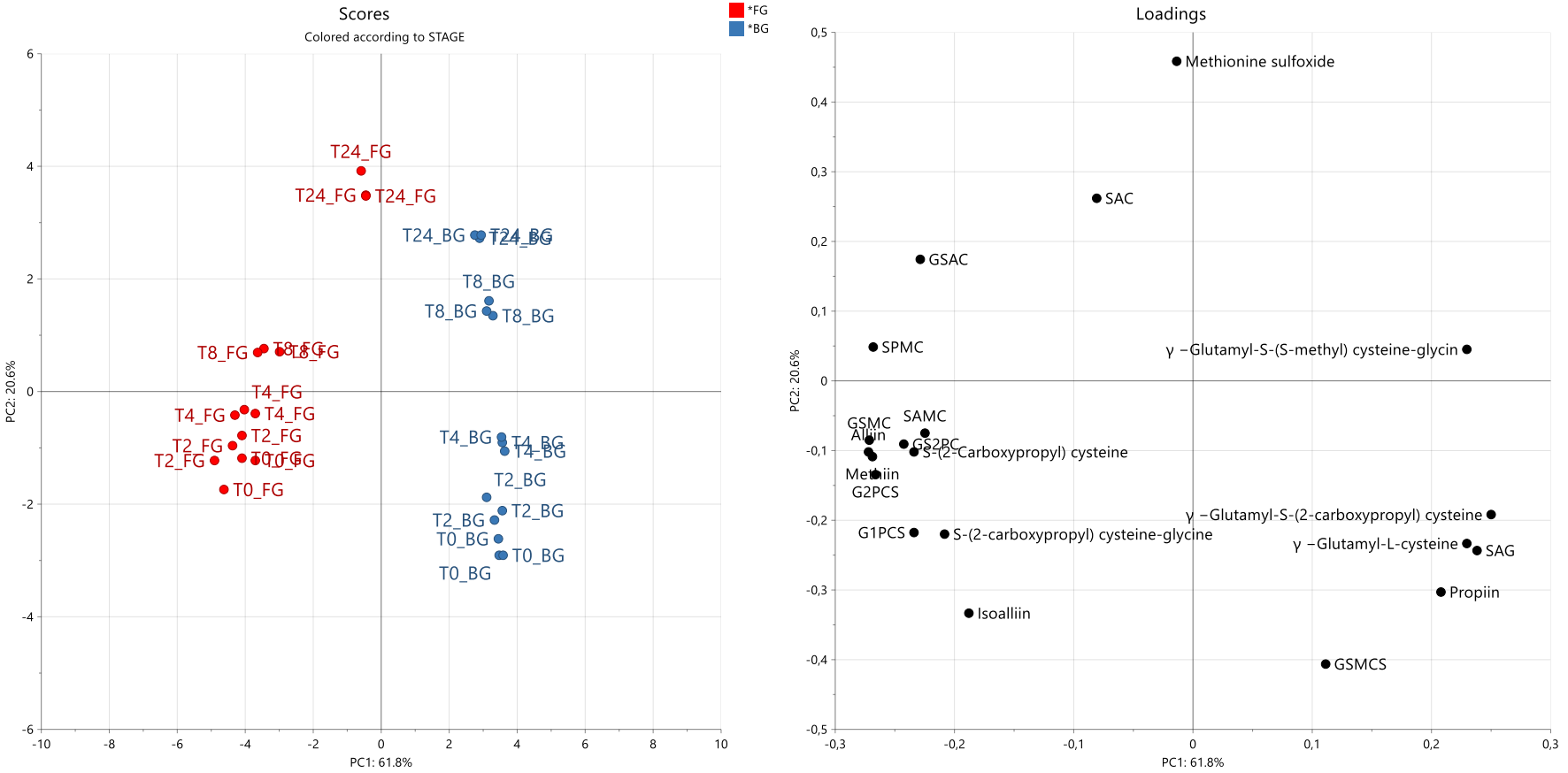

Figure 6.- Scores and loadings obtained in the principal component analysis (PCA) comparing data from organosulfur compounds in fresh (FG = red plot) and black garlic (BG = blue plot) during in vitro colonic fermentation at 0, 2, 4, 8 and 24h (T0, T2, T4, T8 and T24, respectively). GSMCS: γ −Glutamyl-S-methyl-L-cysteine sulfoxide; G2PCS: γ −Glutamyl-S-(2-propenyl) cysteine sulfoxide; G1PCS: γ −Glutamyl-S-(1-propenyl) cysteine sulfoxide; GSMC: γ −Glutamyl-S-methyl-L-cysteine; GSAC: γ −Glutamyl-S-allyl-L-cysteine; GS2PC: γ -Glutamyl-S-(2-propenyl) cysteine; SAC: S-Allylcysteine; SAMC: S-Allylmercapto-L-cysteine; SPMC: S-Propylmercapto-L-cysteine; SAG: S-allylglutathione;

A PCA was performed to obtain more in-depth knowledge of the impact of *in vitro* colonic fermentation on organosulfur compounds in fresh and black garlic (Figure 6). The PC1 described 61% of the total variability and showed a clear discrimination between the fresh and black garlic samples. This discrimination was attributed to specific compounds including SPMC, GSMC and methiin, which were identified exclusively in fresh garlic, and to propiin, SAG, γ -glutamyl-*S*-(*S*-methyl) cysteine-glycine and γ -glutamyl-*S*-(2-carboxypropyl) cysteine, which were identified in black garlic. PC2 explained 20% of the total variability and highlighted the significant influence of the fermentation process on the OSCs of both matrices, characterised by the presence of methionine sulfoxide, which significantly increased in fresh and black garlic during this process.

The main compounds found after the *in vitro* colonic fermentation of the fresh and black garlic were SAC and methionine sulfoxide, accounting for 91.5 and 93.6% of the total OSCs, respectively. The presence of SAC at the colonic level has been shown to have a protective effect against the development of colon cancer by inhibiting the development of 1,2-dimethylhydrazine (DMH)-induced tumours, as well as stimulating enzymatic activity to eliminate other carcinogenic agents (Sumiyoshi & Wargovich, 1990; S. Tanaka et al., 2006). Furthermore, Lin et al. (2020) (Lin et al., 2020) reported the protective effect of dietary SAC intake against the combined carcinogenic action of 2-amino-1-methyl-6-phenylimidazol[4,5-b]pyridine (PhIP) and dextran sulfate sodium salt (DSS) in mice, a dietary heterocyclic amine and colitis-inducing agent, respectively. Moreover, SAC improved the intestinal barrier and the homeostasis of the colonic microbiota. The formation of methionine sulfoxide allows methionine to act as an antioxidant agent that protects colonic cells from oxidative stress and the action of ROS, since methionine reacts with ROS to form methionine sulfoxide. This compound becomes a substrate for methionine sulfoxide transferase enzymes, which are present in the colonic microbiota. This prevents contact between ROS and the intestinal and colonic barrier and the potential cellular damage they could cause (B. C. Lee & Gladyshev, 2011; Luo & Levine, 2009; Pamplona & Barja, 2006).

4. Conclusions

This study aims to assess the effect of *in vitro* colonic fermentation on the profile of bioactive compounds in fresh and black garlic, allowing the two matrices to be compared, considering the limited information available. Colonic fermentation had a similar influence on the phenolic content of fresh and black garlic, with a decrease of 43.8% and 41.7% during the process, respectively. In fresh garlic, 4-hydroxybenzoic acid and benzoic acid were the main (poly)phenol remaining at the end of the fermentation

process, while the main ones in black garlic were 4′-hydroxyphenylacetic acid and 4-hydroxybenzoic acid. Regarding the OSC content, black garlic was strongly affected by *in vitro* colonic fermentation while fresh garlic remained stable during the whole process. The main OSCs found after *in vitro* colonic fermentation of fresh and black garlic were SAC and methionine sulfoxide, accounting for 91.5 and 93.6% of the total OSCs, respectively. The presence of these compounds at the colonic level has potential health benefits as they protect against the proliferation of cancer cells.

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SUPPLEMENTARY INFORMATION

In vitro colonic fermentation of fresh and black garlic

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Table S1. Concentration (µmol/L) of phenolic compounds presented in fresh and black garlic samples at different stages during the *in vitro* colonic fermentation. Data is expressed as mean values (n=3).

Phenolic Compounds	T0	T2	T4	T8	T24	p-value
	Fresh Garlic					
Flavonol						
Quercetin	0.262a	<LOD	<LOD	<LOD	<LOD	***
Kaempferol	<LOD	<LOD	0.185c	0.356b	0.695a	***
Quercetin-3-*O*-rutinoside (Rutin)	0.14bc	0.12c	0.15bc	0.16b	0.20a	***
Isorhamnetin	<LOD	<LOD	<LOD	0.179b	0.343a	***
Total Flavonol	**0.46c**	**0.21d**	**0.43c**	**0.74b**	**1.33a**	***
Hydroxycinnamic acids						
4'-Hydroxycinnamic acid	0.244b	0.286ab	0.321a	0.323a	<LOD	***
3',4'-Dihydroxycinnamic acid	0.23d	0.48b	0.25d	0.71a	0.35c	***
4'-Hydroxy-3'-methoxycinnamic acid	1.05a	1.12a	1.05a	1.09a	0.32b	***
Total Hydroxycinnamic acids	**1.52b**	**1.89ab**	**1.62b**	**2.13a**	**0.75c**	***
Phenylpropanoic acids derivatives						
3-(3',4'-Dihydroxyphenyl)propanoic acid	0.13b	0.19b	0.17b	0.21b	1.22a	***
3-(4'-Hydroxy-3'-methoxyphenyl)propanoic acid	<LOD	<LOD	0.126c	0.524b	1.336a	***
3-(3'-Hydroxyphenyl)propanoic acid	0.46a	0.27b	<LOD	<LOD	<LOD	***
3-Phenylpropanoic acid	11.0a	9.8a	6.6b	<LOD	<LOD	***
Total Phenylpropanoic acids derivatives	**11.73a**	**10.35a**	**6.88b**	**0.73c**	**2.55c**	***
Phenylacetic acids derivatives						
4'-Hydroxyphenylacetic acid	9.6a	10.4a	<LOD	<LOD	7.4b	***
3-Phenylacetic acid	29a	23a	<LOD	<LOD	<LOD	***
Total Phenylacetic acids derivatives	**38.7a**	**33.2a**	**<LOD**	**<LOD**	**7.4b**	***
Benzoic acids derivatives						
Benzoic acid	6.4c	4.3d	7.6bc	9.0a	7.8ab	***
4-Hydroxy-3-methoxybenzoic acid	0.77d	1.17b	1.58a	0.85cd	1.10bc	***
3,4,5-Trihydroxybenzoic acid	0.36a	0.32ab	0.22b	0.42a	0.42a	**
3,4-Dihydroxybenzoic acid	2.3c	2.3c	3.3ab	3.0bc	4.2a	***
3-Hydroxybenzoic acid	0.286a	<LOD	<LOD	<LOD	0.378a	***
4-Hydroxybenzoic acid	10.5c	10.6c	13.4b	14.7ab	16.2a	***
Total Benzoic acids derivatives	**22c**	**20c**	**28b**	**29ab**	**31a**	***
Hydroxycarboxilic acids derivatives						
3'-Hydroxymandelic acid	<LOD	<LOD	<LOD	0.59a	0.43b	***
Total Hydroxycarboxilic acids derivatives	**<LOD**	**<LOD**	**<LOD**	**0.59a**	**0.43b**	***
Benzenotriol						
Benzene-1,3,5-triol	15.1b	13.8b	15.2b	26.5a	6.8c	***
Total Benzenotriol	**15.1b**	**13.8b**	**15.2b**	**26.5a**	**6.8c**	***
Total Phenolic Compounds	**89a**	**79a**	**52b**	**60b**	**50b**	***
Black Garlic						
Flavonol						
Quercetin	0.560a	0.095b	<LOD	<LOD	<LOD	***
Kaempferol	0.19c	0.15c	0.74b	0.79b	1.15a	***
Quercetin-3-*O*-rutinoside (Rutin)	0.12	0.19	0.19	0.18	0.16	ns
Isorhamnetin	<LOD	<LOD	0.215a	0.204a	0.189a	***
Total Flavonol	**0.92b**	**0.46c**	**1.19b**	**1.22ab**	**1.55a**	***

Flavone						
Luteolin	<LOD	<LOD	0.15a	0.16a	0.16a	***
Total Flavone	**<LOD**	**<LOD**	**0.15a**	**0.16a**	**0.16a**	*******
Hydroxycinnamic acids						
4'-Hydroxycinnamic acid	0.27a	0.31a	0.31a	<LOD	<LOD	***
3',4'-Dihydroxycinnamic acid	<LOD	0.122b	0.234a	<LOD	<LOD	***
4'-Hydroxy-3'-methoxycinnamic acid	0.63a	0.55a	0.42b	0.34b	0.39b	***
Total Hydroxycinnamic acids	**0.94a**	**0.98a**	**0.96a**	**0.40b**	**0.45b**	*******
Phenylpropanoic acids derivatives						
3-(3',4'-Dihydroxyphenyl)propanoic acid	0.16b	0.15b	0.29a	0.37a	0.30a	***
3-(4'-Hydroxy-3'-methoxyphenyl)propanoic acid	0.14b	0.14b	0.52a	0.53a	0.62a	***
3-(3'-Hydroxyphenyl)propanoic acid	0.505a	0.335b	<LOD	<LOD	<LOD	***
3-Phenylpropanoic acid	9.4a	8.0b	8.9ab	<LOD	<LOD	***
Total Phenylpropanoic acids derivatives	**10.20a**	**8.63b**	**9.82ab**	**0.90c**	**0.91c**	*******
Phenylacetic acids derivatives						
4'-Hydroxyphenylacetic acid	11.1b	3.3c	<LOD	<LOD	16.0a	***
3-Phenylacetic acid	33a	19b	<LOD	<LOD	<LOD	***
Total Phenylacetic acids derivatives	**44a**	**23b**	**<LOD**	**<LOD**	**16c**	*******
Benzoic acids derivatives						
Benzoic acid	5.7	6.4	4.7	6.4	4.6	ns
4-Hydroxy-3-methoxybenzoic acid	3.1	3.2	3.1	3.6	3.7	ns
3,4,5-Trihydroxybenzoic acid	0.38bc	0.29c	0.32c	0.52a	0.45ab	***
3,4-Dihydroxybenzoic acid	2.7c	3.7ab	3.3bc	4.0ab	4.7a	***
3-Hydroxybenzoic acid	0.68a	0.20b	0.29b	0.40b	0.16b	***
4-Hydroxybenzoic acid	9.9c	10.0bc	12.1abc	12.4ab	14.1a	***
3-Hydroxy-4-methoxybenzoic acid	2.01a	1.29b	2.16a	1.66ab	1.37b	**
Total Benzoic acids derivatives	**24**	**25**	**26**	**29**	**29**	**ns**
Hydroxycarboxilic acids derivatives						
3'-Hydroxymandelic acid	<LOD	<LOD	<LOD	0.39a	0.13b	***
Total Hydroxycarboxilic acids derivatives	**<LOD**	**<LOD**	**<LOD**	**0.39a**	**0.13b**	*******
Benzenotriol						
Benzene-1,3,5-triol	15.3b	23.1a	21.8a	11.0c	7.9c	***
Total Benzenotriol	**15.3b**	**23.1a**	**21.8a**	**11.0c**	**7.9c**	*******
Total Phenolic Compounds	**96a**	**81b**	**60c**	**43d**	**56c**	*******

LOD: limit of detection; Different letters (one-way ANOVA) denote significant differences ($p<0.05$) among the five stages for the same compound

Table S2. Concentration (µmol/L) of organosulfur compounds presented in fresh and black garlic samples at different stages during the *in vitro* colonic fermentation. Data is expressed as mean values (n=3).

Organosulfur Compounds	T0	T2	T4	T8	T24	p-value
	Fresh Garlic					
γ-Glutamyl-*S*-Alk(en)yl-L-Cysteine Derivatives						
γ −Glutamyl-*S*-methyl-L-cysteine sulfoxide (GSMCS)	0.79b	1.07a	0.63b	<LOD	<LOD	***
γ −Glutamyl-*S*-(2-propenyl) cysteine sulfoxide (G2PCS)	12.19a	12.20a	12.70a	10.79a	1.26b	***
γ −Glutamyl-*S*-(1-propenyl) cysteine sulfoxide (G1PCS)	1.51a	1.51a	1.14b	0.55c	0.30c	***
γ −Glutamyl-L-cysteine	3.64a	0.66c	<LOD	1.96b	3.51a	***
γ −Glutamyl-*S*-methyl-L-cysteine (GSMC)	0.88a	1.09a	0.98a	0.57b	0.20c	***
γ −Glutamyl-*S*-allyl-L-cysteine (GSAC)	19bc	20b	18bc	15c	31a	***
γ -Glutamyl-*S*-(2-propenyl) cysteine (GS2PC)	1.95a	2.05a	2.09a	2.29a	1.15b	***
Total GSAk Derivatives	**40a**	**39ab**	**35ab**	**31b**	**37ab**	*
***S*-Alk(en)yl-L-Cysteine Derivatives**						
Alliin	217.3a	231.4a	222.3a	197.0a	14.2b	***
Isoalliin	23.5a	25.6a	24.2a	21.4a	10.1b	***
S-Methyl-L-cysteine sulfoxide (Methiin)	16.0a	17.6a	16.9a	15.0a	<LOD	***
Methionine sulfoxide	104c	122b	124b	151a	158a	***
S-Allylcysteine (SAC)	72b	76b	82b	71b	326a	***
S-Allylmercapto-L-cysteine (SAMC)	24.1c	42.6b	53.5b	66.9a	9.6d	***
S-Propylmercapto-L-cysteine (SPMC)	10.3a	7.9b	6.7b	8.1b	7.3b	**
S-(2-Carboxypropyl) cysteine	5.1ab	5.5a	5.0ab	4.4bc	3.9c	**
S-(2-Carboxypropyl) cysteine-glycine	2.30a	1.51b	0.74c	0.64c	<LOD	***
Total SACs Derivatives	**474**	**531**	**535**	**535**	**529**	**ns**
Total Organosulfur Compounds	**514**	**569**	**570**	**566**	**566**	**ns**
	Black Garlic					
γ-Glutamyl-*S*-Alk(en)yl-L-Cysteine Derivatives						
γ −Glutamyl-*S*-methyl-L-cysteine sulfoxide (GSMCS)	3.42a	2.78b	1.22c	0.15d	<LOD	***
γ −Glutamyl-*S*-(2-propenyl) cysteine sulfoxide (G2PCS)	1.73ab	1.87a	1.49bc	1.17c	0.61d	***
γ −Glutamyl-*S*-(1-propenyl) cysteine sulfoxide (G1PCS)	0.43a	0.59a	<LOD	<LOD	<LOD	***
γ −Glutamyl-L-cysteine	12.1a	11.6a	11.1a	7.4b	2.4c	***
γ −Glutamyl-*S*-(*S*-methyl) cysteine-glycine	0.43bc	0.19d	0.36c	0.76a	0.47b	***
γ −Glutamyl-*S*-(2-carboxypropyl) cysteine	1.42ab	1.23b	1.69a	0.68c	0.78c	***
γ −Glutamyl-*S*-allyl-L-cysteine (GSAC)	<LOD	<LOD	<LOD	1.80a	1.51b	***
γ -Glutamyl-*S*-(2-propenyl) cysteine (GS2PC)	0.86bc	0.92bc	1.52a	1.10b	0.61c	***
Total GSAk Derivatives	**20.4a**	**19.2a**	**17.4b**	**13.0c**	**6.4d**	***
***S*-Alk(en)yl-L-Cysteine Derivatives**						
Alliin	3.35a	2.07b	1.26cd	1.11d	1.71bc	***
Isoalliin	18.15a	18.62a	14.69b	10.54c	0.34d	***

S-Propyl-L-cysteine sulfoxide (Propiin)	53.2a	53.3a	40.9b	10.6c	4.7c	***
Methionine sulfoxide	91c	113b	115b	146a	158a	***
S-Allylcysteine (SAC)	35b	34bc	42a	28c	18d	***
S-Allylmercapto-L-cysteine (SAMC)	6.9b	14.5a	15.5a	2.3c	2.5c	***
S-(2-Carboxypropyl) cysteine	4.4a	<LOD	<LOD	<LOD	<LOD	***
S-(2-Carboxypropyl) cysteine-glycine	0.35a	0.48a	<LOD	<LOD	<LOD	***
S-Allylglutathione (SAG)	19.9a	20.0a	19.6a	13.4b	3.1c	***
Total SACs Derivatives	**232b**	**256a**	**250ab**	**211c**	**188d**	*******
Total Organosulfur Compounds	**252b**	**275a**	**267ab**	**224c**	**194d**	*******

LOD: limit of detection; Different letters (one-way ANOVA) denote significant differences ($p<0.05$) among the five stages for the same compound

Artículo 6

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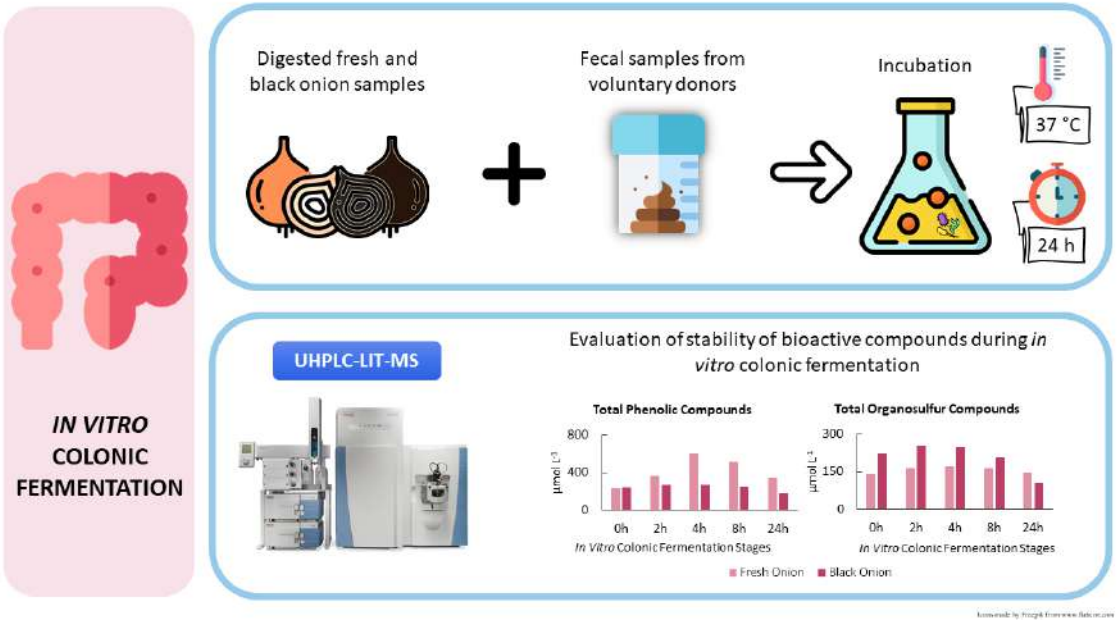

Effect of colonic fermentation on the stability of fresh and black onion bioactives

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Abstract

The health properties related to onion intake are attributed mainly to the presence of bioactive compounds, particularly phenolic and organosulfur compounds (OSCs). The aim of this study was to investigate the effect of an *in vitro* colonic fermentation on the stability of phenolic and OSCs of fresh and black onion by ultra-high-performance liquid chromatography coupled to mass spectrometry with a linear ion trap (UHPLC-LIT-MS). Throughout colonic fermentation, fresh onion showed an increase in total phenolic content of 45%, mainly due to an increase in the content of the flavonoid family, while the OSCs remained stable along the fermentation. Black onion presented a different behaviour, showing significantly decreases in total (poly)phenol and OSC content, 22 and 48%, respectively. The main compounds found after the *in vitro* colonic fermentation of fresh onion were isorhamnetin (141 µmol L^{-1}), quercetin (95 µmol L^{-1}), 3,4-dihydroxybenzoic acid (53 µmol L^{-1}), methionine sulfoxide (100 µmol L^{-1}) and *S*-allylcysteine (SAC) (21.7 µmol L^{-1}), whereas 3,4-dihydroxybenzoic acid (70 µmol L^{-1}), 4-hydroxyphenylacetic acid (68 µmol L^{-1}), methionine sulfoxide (82 µmol L^{-1}) and *S*-propylmercapto-*L*-cysteine (SPMC) (10.1 µmol L^{-1}) accounted for the highest concentrations of phenolics and OSCs in fermented black onion. These compounds, presumably present for their absorption and action at the colonic level, could be related to the health benefits of regular consumption of fresh and black onion.

Keywords: *in vitro* colonic fermentation; human microbiota; (poly)phenols; organosulfur compounds; onion

1. Introduction

Onion (*Allium cepa* L.) is a staple food belonging to the genus *Allium*, the consumption of which has historically been associated with beneficial effects on health [1]. The intake of onion, along with other *Allium* vegetables, has been related with a protective effect against heart and kidney diseases, as well as a preventive effect against type 2 diabetes mellitus and hypertension [2,3]. These health properties are attributed mainly to the presence of bioactive compounds, particularly the presence of (poly)phenols and organosulfur compounds (OSCs) [4,5]. Quercetin is the main phenolic compound found in onions, the consumption of which has been related to cardio-protective effects [6]. Isoalliin is the main OSC found in this food and its regular intake is associated with the prevention of cardiovascular diseases and some types of cancers through different pathways, including enhancing the immune system, inducing apoptosis, detoxifying carcinogens or reacting with reactive oxygen species (ROS) [7,8].

Recently, the food industry has developed a product derived from fresh onions, called black onion, obtained by an ageing process with controlled humidity and temperature conditions without the use of microorganisms or additives [9]. The ageing process has been shown to affect the phytochemical composition and sensory attributes of the initial product. Indeed, black onion shows a sweeter and less pungent taste compared with the original product, a dark brown colour, and a soft consistency. Main changes with regard to its composition are a lower phenolic compound content and significant increases in isoalliin, fructose, glucose, and tartaric acid [10]. The elimination of the undesirable characteristics of fresh onion at sensory and digestive levels enhances consumer acceptance of black onions, increasing the probability of their consumption. To date, there have been no studies relating the consumption of black onion to a possible beneficial effect on health although this is to be expected due to the composition of this new product. Indeed, isoalliin, the major OSC in both fresh and black onion, and other OSCs have been found to present health-promoting benefits[11]. To exert beneficial effects *in vivo*, (poly)phenols and OSCs from onion must be bioaccessible, be released from the food matrix, and be ready for absorption [12,13]. The bioaccessibility of (poly)phenols and OSCs in fresh onion and black onion has been previously evaluated by Moreno-Ortega et al. (2021) [14]. It has been shown that OSCs and (poly)phenols from black onion are more bioaccessible compared with fresh onion. Then, the bioaccesible compounds can be absorbed through the upper gastro-intestinal (GI) tract. Nevertheless, some of these compounds are not ready for absorption after passing through the upper GI tract and therefore reach the colon to be metabolised by the colonic microbiota [15]. Actually,

phenolic compounds from different matrices, including mango [16], black carrot [17], coffee [18], green pepper [19] and cardoon [20] have been extensively studied and therefore the metabolic pathways of most phenolic acids, flavonols, flavan-3-ols, and anthocyanins, among others, have been described [21–23]. However, up to date, there is no data on how human microbiota might be able to metabolize OSCs from fresh and black onion. Therefore, the aim of this study was to investigate the effect of an *in vitro* colonic fermentation on the stability of the individual profile of phenolics and OSCs contained in fresh and black onion by ultra-high-performance liquid chromatography coupled to mass spectrometry with a linear ion trap (UHPLC-LIT-MS).

2. Materials and Methods

2.1. Chemicals

For colonic fermentation bile salts, soluble starch, (+)-arabinogalactan, tryptone, yeast extract, xylan from birchwood, L-cysteine hydrochloride monohydrate, guar gum, inulin, Tween 80, buffered peptone water, Dulbecco's phosphate buffer saline (PBS), casein sodium salt from bovine milk, pectin from citrus fruits, mucin from porcine stomach-type III, $CaCl_2$, KCl, NaCl, $NaHCO_3$, anhydrous K_2HPO_4, KH_2PO_4, $MgSO_4$ monohydrate, $FeSO_4$ heptahydrate, resazurin redox indicator were purchased by Sigma-Aldrich (St. Louis, MO, USA). Reference phenolic compounds, including 3-(3′-hydroxyphenyl)propanoic acid, 3-hydroxybenzoic acid, 3-phenylpropanoic acid, 4′-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, 3-(3′,4′-dihydroxyphenyl)propanoic acid (aka dihydrocaffeic acid), kaempferol, 3-phenylacetic acid, benzene-1,3,5-triol (aka phloroglucinol), 3,4-dihydroxybenzoic acid (aka protocatechuic acid), quercetin, quercetin-3-*O*-rutinoside (rutin), 3′,4′-dihydroxycinnamic acid (aka caffeic acid), 3,4,5-trihydroxybenzoic acid (aka gallic acid), 4′-hydroxy-3′-methoxycinnamic acid (aka ferulic acid), 4′-hydroxycinnamic acid (aka *p*-coumaric acid), 4-hydroxy-3-methoxybenzoic acid (aka vanillic acid), luteolin, 3′-hydroxy-4′-methoxycinnamic acid (aka isoferulic acid), 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid (aka dihydroferulic acid), 4′-hydroxy-3′-methoxyphenylacetic acid (aka homovanillic acid), 3-hydroxymandelic acid, 3-(3′-hydroxy-4′-methoxyphenyl)propionic acid, benzoic acid, 3-hydroxy-4-methoxybenzoic acid (aka isovanillic acid) were obtained from Sigma-Aldrich (St Louis, MO, USA).

Reference OSCs, including alliin, γ-glutamyl-*S*-allyl-*L*-cysteine (GSAC), *S*-allyl-*L*-cysteine (SAC), *S*-methyl-*L*-cysteine were purchased from Merck Life Science (Milan, Italy) and *S*-methyl-l-cysteine sulfoxide (methiin) was acquired from Prodotti Gianni (Milan, Italy).

For UHPLC analysis, all solvents and reagents were UHPLC-grade and were purchased from VWR International (Milan, Italy), unless otherwise indicated. Ultrapure water from MilliQ system (Millipore, Bedfort, MA, USA) was used throughout the experiment.

The names for phenolic acids used in this paper are based on the nomenclature rules of Kay et al. [24].

2.2. Sample Preparation

Fresh and black onion (*Allium cepa* L.) provided by a local supplier (La Abuela Carmen®, Córdoba, Spain) were subjected to *in vitro* gastrointestinal digestion following the protocol previously described [14]. Subsequently, the digested samples were freeze-dried (FreeZone Plus 2.5 series, Labconco), milled (Freezer Mill model 6870, Fisher Scientific, Waltham, MA USA) and stored at -80 ºC until the *in vitro* colonic fermentation process.

2.3. Faecal Slurry and Growth Medium Preparation

Faecal samples were collected from three healthy volunteers without intestinal diseases and who had not consumed antibiotics for the previous 3 months. Volunteers followed a rigorous diet without (poly)phenols and OSCs for 48 h before faecal collection. After collection, faecal samples were stored in anaerobic conditions and were processed within 2 h. Volunteer faeces were pooled in equal amount (10 g of each one) and homogenized with 300 mL of 1% (*w/v*) sterilized Dulbecco's PBS to obtain a 10% (*w/w*) faecal slurry [25]. The growth medium (1 L) was prepared adopting the composition described by Bresciani et al. 2018 [26] before being sterilised at 121 °C for 15 min in 12 mL glass vessels.

2.4. In vitro Colonic Fermentation

The *in vitro* colonic fermentation was performed as previously reported Dall'Asta et al. 2012[27], with slight modifications. Fermentation was carried out in 12-mL sterilised glass vessels to which 1.8 mL of growth medium, 1.8 mL of fecal slurry and 400 µL of a solution of digested fresh or black onion were added reaching a total volume of 4 mL [28]. 100 mg of digested and freeze-dried fresh and black onion was dissolved in a Dulbecco's PBS solution containing bile salts for 2 h under stirring at room temperature before filling the vessels [25].

2.5. Phenolic and Organosulfur Metabolites Extraction

Phenolic and OSC metabolites produced during the *in vitro* colonic fermentation of digested fresh and black onion were extracted as previously was reported by Bresciani et al. 2018 and 2021 [26,29], with minor modifications. (Poly)phenols and OSCs were extracted with different solvents but following the same procedure, by using ethyl acetate acidified with formic acid (0.1%, *v/v*) and ethanol acidified with formic acid (0.1%, *v/v*), respectively. Briefly, 300 µL of fermented sample was mixed with 1.2 mL of the solvent and vortexed for 30 s, sonicated for 10 min in an ultrasonic bath, and vortexed again for 30 s, re-sonicated for 5 min, and then centrifuged (Centrisart® A-14C Refrigerated Micro-Centrifuge and Rotor YCSR-A1C, Sartorius Lab Instruments GmbH and Co. KG, Goettingen, Germany) at 14,000 rpm for 10 min. The supernatant was collected into a clean microfuge tube, while the pellet were re-extracted using 500 µL of the same solvent by following the same protocol described. Finally, supernatants were pooled and stored at -80 ºC. The fermented samples extracted with ethyl acetate acidified with formic acid (0.1%, *v/v*) were brought to dryness though a centrifugal concentrator (SpeedVac Savant SPD121P, Thermo Fisher Scientific Inc., San Jose, CA, USA), to be reconstituted in 300 µL methanol-water acidified with formic acid (0.1 %, *v/v*) (50:50, *v/v*). Finally, these samples were vortexed for 30 s and centrifuged at 14000 rpm 5 min and stored at -80ºC until UHPLC-LIT-MS analysis.

2.6. Analysis of Phenolic and Organosulfur Compound Metabolites

Changes in phenolic and OSCs during the colonic fermentation were analysed by UHPLC coupled with MS, using an Accela uHPLC 1250 apparatus equipped with linear ion trap MS (LIT-MS) (LTQ XL, Thermo Fisher Scientific Inc.), fitted with a heated-electrospray ionization (H-ESI-II) probe (Thermo Fisher Scientific Inc.). The analysis of (poly)phenols and their metabolites in fermented fresh and black garlic extracts were carried out following a previously described methodology [25]. Briefly, analyte separation was carried out by means of a Kinetex Evo C18 column (100 x 2.1 mm; 2.6 µmol L-1 particle size, Phenomenex, CA, USA) installed with a precolumn cartridge (Phenomenex). Volume injection was 5 µL and column oven was set to 35 °C. The mobile phases, A-acidified acetonitrile 0.01% (*v/v*) formic acid and B-acidified water 0.01% (*v/v*) formic acid, were pumped at a flow of 500 µL/min with a 14 min gradient, starting with 5% A and maintained during 0.5 min, then rising 40% A in 7 min, rising 80% A in 1 min and maintained during 2 min. After that, the column was equilibrated to the initial conditions within 0.5 min and maintained for 3 min to re-equilibrate it. The analysis of

(poly)phenols and their microbial metabolites in fermented fresh and black onion extracts was carried by adopting a previously described methodology[25].

A modified methodology was developed for the analysis of OSCs and their microbial metabolites in the extracted fermented samples [30]. Analytes were separated by using an ACQUITY UPLC® BEH AMIDE (2.1 x 150 mm, 1.7 µmol L-1 particle size) column (Waters, Milford, MA, USA). Column oven was set to 35 °C. The autosampler temperature and the injection volume were set at 10 °C and 2 µL, respectively. Mobile phases consisted of a mixture of 0.1 % (*v/v*) formic acid in acetonitrile (phase A), 0.1 % (*v/v*) formic acid in water (phase B), and ammonium formate (20 mmol L^{-1}) containing 1% (*v/v*) formic acid (phase C). The flow rate was set at 400 µL/min, with the following gradient. Starting from 0 to 1 min of 5% phase B and 5% phase C in phase A, the proportion of phase B was increased linearly to 30% over a period of 7 min, then increasing to 45% over 4 min. The start condition was re-established in 3 min and kept for 4 min to re-equilibrate the column. Phase C was maintained at 5% during the run (total run: 19 min). The analytical mass spectrometric conditions were optimized by infusion of a pure standard of alliin, working in a positive ionization mode. The H-ESI-II interface was set to a capillary temperature of 275 °C and the source heater temperature was set at 200 °C. The sheath gas (N_2) flow rate was set at 50 (arbitrary units), while auxiliary and sweep gases (N_2) were set at 15 and 0, respectively (arbitrary units). The source voltage was 3.8 kV, the capillary voltage was 6 V and tube lens voltage was 50 V. Ultra-pure helium gas (99.9999%) was used in MS/MS analyses. The analysis of OSCs and their metabolites in fermented samples were carried out using a full-scan, data dependent MS^2 from *m/z* 100 to 500. Based on the reference compounds, γ-glutamyl-*S*-allyl-*L*-cysteine (GSAC), *S*-allyl-*L*-cysteine (SAC), alliin, methiin and *S*-methyl-*L*-cysteine were monitored through full MS/MS experiments with a Collision Induced Dissociation (CID) equal to 35 (arbitrary units).

XCalibur software v. 2.1 (Thermo Fisher Scientific Inc.) was employed to acquire the chromatograms and spectral data and quantification was performed with linear regression analysis with R^2 values of >0.998 of the available reference compounds. The limits of quantification (LOQs) and limits of detection (LODs) for each standard used were calculated based on the minimum accepted signal-to-noise (S/N) ratio values of 3 and 10, respectively. The LOQs were from 0.03 to 25 µmol L^{-1} for (poly)phenols and from 0.25 to 1 µmol L^{-1} for OSCs, and the LODs were from 0.01 to 5 µmol L^{-1} for (poly)phenols and from 0.03 to 0.25 µmol L^{-1} for OSCs.

2.7. Statistical Analysis

Statistical analyses were performed based on three replicate measures of each sample. A one-way ANOVA was carried out using R software (v. 3.6.3, R Core Team, Vienna, Austria) to determine significant differences for same fermented sample at the different incubation period (0, 2, 4, 8 or 24 h), the significance being accepted for a *p*-value < 0.05. Next, Fisher's LSD pairwise comparison was performed on the data.

3. Results and Discussion

3.1 Changes in the Phenolic Compounds of Fresh and Black Onion during in vitro Colonic Fermentation

A total of 24 and 25 phenolic compounds were identified and quantified in fresh and black onion samples, respectively. The details of their identification are shown in Table 1. The phenolic compounds determined in both matrices belonged to 8 families, including 7 flavonols, 1 flavone, 3 hydroxycinnamic acids, 4 phenylpropanoic acid derivatives, 2 phenylacetic acid derivatives, 6 benzoic acid derivatives, 1 hydroxycarboxylic acid derivative and 1 benzenetriol. Flavonols and benzoic acid derivatives were the main phenolic families found in fresh digested onion at the beginning of fermentation, accounting for 94.9% of the total, while in the black onion samples, benzoic acid and phenylacetic acid derivatives comprised 90.9% of the total, being the main phenolic families found at the beginning of the process.

Table 1.- UHPLC-LIT-MS^n-based identification of phenolic and organosulfur compounds detected in fresh and black onion during in vitro colonic fermentation

Compound	RT (min)	Parent mass *(m/z)*	MS fragmentation pattern from full scan data dependent analyses (*m/z*)	MS^2 fragmentation pattern from full MS/MS analyses (*m/z*)	MSI MI Level*	Sample
Phenolic Compounds		**[M-H]$^-$**				
Flavonols						
Quercetin	6.12	301		**179**, 151, 273, 257, 193, 239, 107, 283	1	FO; BO
Kaempferol	6.97	285		**151**, 229, 257, 213, 243, 185, 169, 107, 143	1	FO; BO
Quercetin-3-*O*-rutinoside (rutin)	4.29	609		**301**, 300, 343, 271, 179, 591	1	FO; BO
Isorhamnetin	9.13	315		**297**, 279, 171, 241, 227, 151	2	FO; BO
Quercetin-3-*O*-glucoside	5.20	463		**301**, 343, 419, 373	2	FO; BO
Quercetin-4-*O*-glucoside	4.20	463		**373**, 351, 419, 342, 445, 301, 217	2	FO
Isorhamnetin glucoside	5.15	477		**315**, 396, 459, 357, 260, 408	2	FO
Flavone						
Luteolin	6.13	285		**241**, 175, 199, 217, 241, 257, 151, 107	1	FO; BO
Hydroxycinnamic acids						
4′-Hydroxycinnamic acid	3.31	163		**119**, 135	1	FO; BO
3′,4′-Dihydroxycinnamic acid	2.51	179		**135**, 151, 117, 91	1	FO; BO
4′-Hydroxy-3′-methoxycinnamic acid	3.70	193		**149**, 178, 134, 107	1	FO; BO
Phenylpropanoic acids derivatives						
3-(3′,4′-Dihydroxyphenyl)propanoic acid	1.85	181		**137**, 119, 59, 163	1	FO; BO
3-(4′-Hydroxy-3′-methoxyphenyl)propanoic acid	3.08	195		**136**, 151, 177, 123, 59	1	FO; BO
3-(3′-Hydroxy-4′-methoxyphenyl)propanoic acid	3.42	195		**119**, 151,149, 136, 180, 93	1	BO
3-(3′-Hydroxyphenyl)propanoic acid	3.10	165		**121**, 147, 165, 97	1	FO; BO
3-Phenylpropanoic acid	4.80	149		**105**, 131	1	FO; BO
Phenylacetic acids derivatives						
4′-Hydroxyphenylacetic acid	1.85	151		**107**, 120, 93	1	FO; BO
3-Phenylacetic acid	3.36	135		**91**	1	BO
Benzoic acids derivatives						
Benzoic acid	3.50	121		**77**	1	FO; BO
4-Hydroxy-3-methoxybenzoic acid	2.31	167		**123**, 152, 108, 79	1	FO; BO
3,4-Dihydroxybenzoic acid	1.16	153		**109**, 135, 81	1	FO; BO
3-Hydroxybenzoic acid	2.08	137		**93**, 115	1	FO; BO

4-Hydroxybenzoic acid	1.78	137		**93**	1	FO; BO
3-Hydroxy-4-methoxybenzoic acid	2.44	167		**152**, 123, 99	1	FO; BO
3,4,5-Trihydroxybenzoic acid	0.74	169		**125**, 97, 81	1	BO
Hydroxycarboxilic acids derivatives						
3′-Hydroxymandelic acid	0.87	167		**123**, 105, 85	1	FO; BO
Benzenetriol						
Benzene-1,3,5-triol	0.65	125		**83**, 57	1	FO; BO
Organosulfur Compounds		**[M-H]$^+$**				
γ-Glutamyl-S-Alk(en)yl-L-Cysteine Sulfoxides						
γ −Glutamyl-*S*-methyl-*L*-cysteine sulfoxide (GSMCS)	4.85	281	**121**, 132, 173, 207, 235, 245		2	FO; BO
γ −Glutamyl-*S*-(2-propenyl) cysteine sulfoxide (G2PCS)	3.89	307	**229**, 269, 171, 146, 130		2	FO
γ −Glutamyl-*S*-(1-propenyl) cysteine sulfoxide (G1PCS)	7.92	307	**176**, 207, 134, 235, 269		2	FO; BO
γ-Glutamyl-S-Alk(en)yl-L-Cysteine Derivatives						
γ −Glutamyl-*L*-cysteine	4.06	251		**223, 2**34, 166, 178, 110, 206, 138	2	FO; BO
γ −Glutamyl-*S*-(S-methyl) cysteine-glycine	4.59	354	**244**, 118, 145, 260, 203		2	FO; BO
γ −Glutamyl-*S*-(2-carboxypropyl) cysteine-glycine	7.06	394	**148**, 365, 218, 291, 263		2	FO
γ −Glutamyl-*S*-allyl-L-cysteine (GSAC)	6.19	291		**162**, 145, 170, 274, 245, 122, 84	1	FO; BO
γ -Glutamyl-*S*-(2-propenyl) cysteine (GS2PC)	7.09	291	**148**, 291, 365, 203		2	FO; BO
γ −Glutamyl-*S*-(2-carboxypropyl) cysteine	6.95	335	**120**, 161, 203, 229, 290, 302		2	BO
S-Alk(en)yl-L-Cysteine Sulfoxides Derivatives						
Isoalliin	6.86	178		**88**, 91, 116, 73, 132, 160	2	FO; BO
Methionine sulfoxide	4.70	166		**121**, 149, 131	2	FO; BO
S-propyl-*L*-cysteine sulfoxide (Propiin)	8.68	180		**162**, 144, 134		BO
S-Alk(en)yl-L-Cysteine Derivatives						
S-Allylcysteine (SAC)	4.50	162		**145**, 115, 134, 76, 99	1	FO; BO
S-Allylmercapto-*L*-cysteine (SAMC)	3.92	194	**171**, 154, 130, 136, 118		2	FO
S-Propyl-*L*-cysteine (Deoxypropiin)	9.38	164	**103**, 122, 132, 146		2	FO
S-Propylmercapto-*L*-cysteine (SPMC)	3.78	196	**104**, 171, 145, 110, 130, 184		2	FO; BO
S-(2-carboxypropyl) cysteine-glycine	6.95	265	**120**, 161, 230, 171, 244, 258		2	FO
S-(2-Carboxypropyl) cysteine	5.96	208	**105**, 171, 182, 132, 118, 146, 159		2	BO
S-Allylglutathione (SAG)	4.69	348	**229**, 203, 244, 105, 118, 270		2	BO

**Metabolite standards initiative (MSI) metabolite identification (MI) levels[31]. Reference compounds were available for all compounds identified at MSI MI level 1. RT: Retention time; m/z = mass to charge ratio; quantifier ions are reported in bold; FO: Fresh Onion; BO; Black Onion. Compounds for which there was a reference compound were quantified with them, while for the rest, the most structurally similar reference compound was used. Isorhamnetin was quantified with quercetin. γ-Glutamyl-S-Alk(en)yl-L-Cysteine Sulfoxides and γ-Glutamyl-S-Alk(en)yl-L-Cysteine were quantified with GSAC; Isoalliin was quantified with Alliin and Propiin with Methiin. S-Alk(en)yl-L-Cysteine were quantified with SAC.*

The time-course profiles of total and individual phenolic compounds during *in vitro* colonic fermentation are shown in Figure 1 and Table 2, respectively.

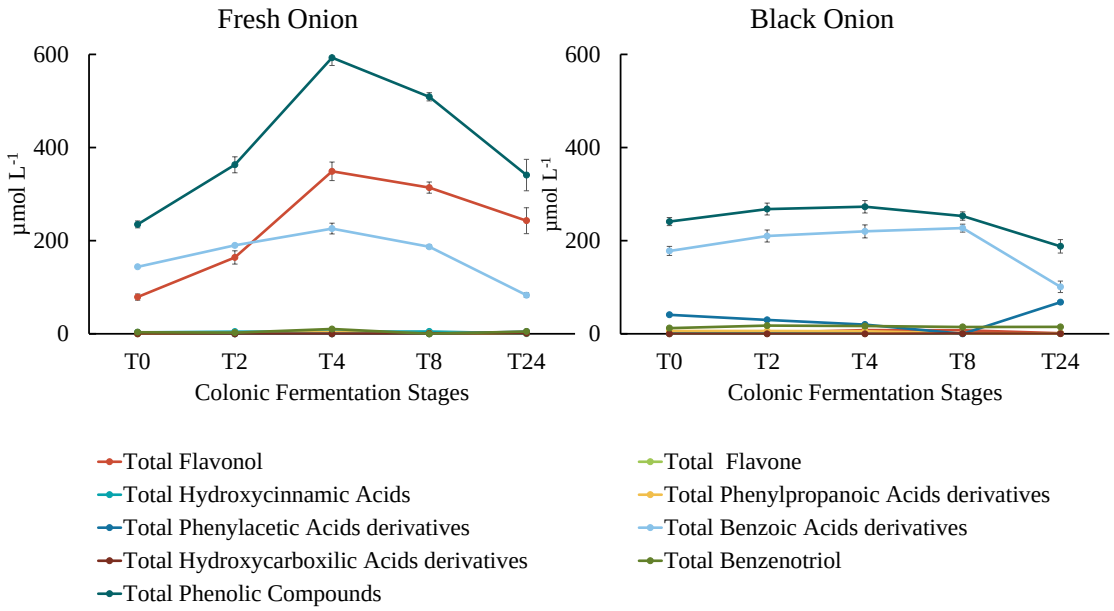

Figure 1. Time-course profiles of phenolic families found during in vitro colonic fermentation of fresh and black onion at 0, 2, 4, 8 and 24h (T0, T2, T4, T8 and T24, respectively). Data are expressed as mean values ± standard deviation of three different determinations (n=3).

Overall, the total phenolic content in fresh onion showed an increase of 145.1% of the total phenolics at the end of the faecal fermentation in comparison with the initial amount (Figure 1, Table 2). This was mainly due to the significantly increased in total flavonols content during the fermentation, showing an increase of 3.1-fold compared with the initial amount. This increase could be partially due to the hydrolysis of the glycosylated quercetin (quercetin-3-*O*-glucoside and quercetin-4-*O*-glucoside) and isorhamnetin (isorhamnetin-*O*-glucoside) during the faecal fermentation by the action the colonic microbiota which released the aglycones and increased their content. In line with our results, Kawabata et al. (2019) [32] and Cassidy et al. (2017) [33] reported microbial action on the glycosylated forms of phenolic compounds and their hydrolysis process for the release of aglycones. This is in keeping with other authors, such as Fernández-Jalao et al. (2021) [34] who subjected onion to a dynamic GI digestion and colon fermentation simulator, and they observed that in the ascending colon there was an increase in free quercetin content, probably due to this deglycosylation caused by the enzymatic action of colonic microbiota. Another explanation is the limitation in the quantification of the quercetin-glucosides and isorhamnetin glucosides with the aglycone which can lead to understimations or overestimations of the quantification as has been reported by Ottaviani et al., 2018 [35].

Regarding total hydroxycinnamic acids, although their concentration is marginal with respect to other families, their content increased in the first 2 h of fermentation, being maintained for the rest of 8 h, likely as a result of the release of non-quantified compounds from the food matrix during the colonic fermentation. After 24 h of fermentation, the total content of hydroxycinnamic acids suffered a significantly decreased representing more than 4-fold reduction compared to the previous stage (from 5.02 to 1.19 μmol L^{-1}, Table 2).

During colonic fermentation, the total content of phenylpropanoic and phenylacetic acid derivatives showed an increase of 166 and 168%, respectively. Among them, only 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid, 3-(3′,4′-dihydroxyphenyl)propanoic acid and 4-hydroxyphenylacetic acid were present after the 24 h faecal fermentation, showing an increase in their concentration responsible for the total increase of both families of compounds. These compounds could be formed from the microbial degradation of 4′-hydroxy-3′-methoxycinnamic acid and 3′,4′-dihydroxycinnamic acid [36], presented in fresh onion.

Benzoic acid derivatives were one of the major families of compounds determined during the *in vitro* faecal fermentation of fresh onion. Particularly, the total content of benzoic acids increased gradually for the first 4 h of fermentation, reaching a maximum concentration of 226 μmol L^{-1}. After 4 h, the concentration of this family decreased by 63% with respect to the initial content, reaching 83 μmol L^{-1} after 24 h. During the colonic fermentation, benzoic acid and 4-hydroxybenzoic acid increased their concentration 1.8-fold and 2.1-fold, respectively. The occurrence and increase of these two compounds during the fermentation process could be due to some extent to the colonic transformation of phenylpropanoic and phenylacetic acid derivatives, as it has been previously described by other authors (Russell et al. (2007) [37] and Pereira-Caro et al. (2017) [38]). On the other hand, 3,4-dihydroxybenzoic acid, which decreased its concentration by 58.6%, could be converted into other compounds as 4-hydroxybenzoic acid [25,39] or catechol [40] by the action of the microbiota, accounting for the significant increase in the concentration of both phenolics by the end of the *in vitro* faecal incubation.

Finally, benzene-1,3,5-triol, compound widely recognized to be one of the main catabolic product of flavonols, due to A-ring breakage [27,41], showed its highest concentration after 4 h of fermentation (10.4 μmol L^{-1}). After 24 h, although its content decreased considerably, the final concentration was still high compared with the initial content (145.7% with respect to the initial content). The decrease during the last h of the *in vitro* fermentation may be due to what was reported by Schoefer et al. (2003) [42], who

described that benzene-1,3,5-triol is used by the colonic microbiota for the synthesis of short-chain fatty acids.

Table 2.- Concentration (µmol L^{-1}) of phenolic compounds presented in fresh and black onion samples at different stages during the in vitro colonic fermentation. Data is expressed as mean values (n=3).

Phenolic Compounds (µmol L^{-1})	T0	T2	T4	T8	T24	p-value
Flavonol	*Fresh Onion*					
Quercetin	14.5c	22.7c	98.2b	123.2a	95.3b	***
Kaempferol	1.33d	2.77c	4.78b	6.55a	4.60b	***
Quercetin-3-*O*-rutinoside (Rutin)	0.079a	0.073a	0.021b	<LOD	<LOD	***
Isorhamnetin	18d	59c	156ab	177a	141b	***
Quercetin-3-*O*-glucoside	38.85b	70.59a	79.46a	6.03c	0.91c	***
Quercetin-4-*O*-glucoside	3.22b	4.48b	6.63a	0.63c	0.13c	***
Isorhamnetin glucoside	2.494c	4.265a	3.156b	0.481d	<LOD	***
Total Flavonol	**79d**	**164c**	**349a**	**314a**	**243b**	*******
Flavone						
Luteolin	0.087b	0.099b	0.081b	0.094b	0.149a	***
Total Flavone	**0.087b**	**0.099b**	**0.081b**	**0.094b**	**0.149a**	*******
Hydroxycinnamic acids						
4′-Hydroxycinnamic acid	1.19b	1.43ab	1.53a	1.61a	0.19c	***
3′,4′-Dihydroxycinnamic acid	0.15d	0.45c	0.62b	0.85a	0.26d	***
4′-Hydroxy-3′-methoxycinnamic acid	1.83b	2.82a	2.58a	2.57a	0.75c	***
Total Hydroxinnamic acids	**3.17b**	**4.70a**	**4.73a**	**5.02a**	**1.19c**	*******
Phenylpropanoic acids derivatives						
3-(3′,4′-Dihydroxyphenyl)propanoic acid	<LOD	<LOD	<LOD	0.092b	0.312a	***
3-(4′-Hydroxy-3′-methoxyphenyl)propanoic acid	0.14d	0.29cd	0.58c	0.98b	2.92a	***
3-(3′-Hydroxyphenyl)propanoic acid	0.218a	<LOD	<LOD	<LOD	<LOD	***
3-Phenylpropanoic acid	1.53b	1.24b	2.93a	<LOD	<LOD	***
Total Phenylpropanoic acids derivatives	**1.95b**	**1.66bc**	**3.57a**	**1.07c**	**3.23a**	*******
Phenylacetic acids derivatives						
4′-Hydroxyphenylacetic acid	2.8b	<LOD	<LOD	<LOD	4.7a	***
Total Phenylacetic acids derivatives	**2.8b**	**<LOD**	**<LOD**	**<LOD**	**4.7a**	*******
Benzoic acids derivatives						
Benzoic acid	<LOD	<LOD	4.1b	3.4b	7.3a	***
4-Hydroxy-3-methoxybenzoic acid	3.8	3.2	4.0	3.3	3.9	ns
3,4-Dihydroxybenzoic acid	128c	173b	204a	164b	53d	***
3-Hydroxybenzoic acid	0.192a	<LOD	<LOD	<LOD	<LOD	***
4-Hydroxybenzoic acid	5.8c	7.4bc	8.1b	11.0a	12.0a	***
3-Hydroxy-4-methoxybenzoic acid	6.5	6.5	5.6	5.6	6.5	ns
Total Benzoic acids derivatives	**144c**	**190b**	**226a**	**187b**	**83d**	*******
Hydroxycarboxilic acids derivatives						
3′-Hydroxymandelic acid	<LOD	<LOD	<LOD	0.81b	1.08a	***
Total Hydroxycarboxilic acids derivatives	**<LOD**	**<LOD**	**<LOD**	**0.81b**	**1.08a**	*******
Benzenetriol						
Benzene-1,3,5-triol	3.5c	2.7c	10.4a	6.4b	5.1b	***
Total Benzenetriol	**3.5c**	**2.7c**	**10.4a**	**6.4b**	**5.1b**	*******
Total Phenolic Compounds	**235d**	**363c**	**593a**	**515b**	**341c**	*******

Black Onion						
Flavonol						
Quercetin	0.53b	1.02b	3.41a	3.46a	0.47b	***
Kaempferol	0.17c	0.12c	0.71a	0.53b	<LOD	***
Quercetin-3-*O*-rutinoside (Rutin)	0.19a	0.18a	0.18a	<LOD	<LOD	***
Isorhamnetin	0.27c	1.09b	3.73a	3.61a	0.80bc	***
Quercetin-3-*O*-glucoside	0.15b	0.26a	<LOD	<LOD	<LOD	***
Total Flavonol	**1.30c**	**2.67b**	**8.03a**	**7.60a**	**1.28c**	***
Flavone						
Luteolin	0.33b	0.59a	0.64a	0.62a	0.44b	***
Total Flavone	**0.33b**	**0.59a**	**0.64a**	**0.62a**	**0.44b**	***
Hydroxycinnamic acids						
4′-Hydroxycinnamic acid	0.18b	0.33a	0.22b	<LOD	<LOD	***
3′,4′-Dihydroxycinnamic acid	0.24b	0.48a	0.56a	0.34b	0.49a	***
4′-Hydroxy-3′-methoxycinnamic acid	0.48b	0.64a	0.50b	0.46b	0.44b	**
Total Hydroxinnamic acids	**0.90b**	**1.45a**	**1.28a**	**0.80b**	**0.92b**	***
Phenylpropanoic acids derivatives						
3-(3′,4′-Dihydroxyphenyl)propanoic acid	0.15b	0.23b	0.22b	0.46a	0.54a	***
3-(4′-Hydroxy-3′-methoxyphenyl)propanoic acid	0.21c	0.34c	0.75a	0.75a	0.57b	***
3-(3′-Hydroxy-4′-methoxyphenyl) propanoic acid	0.13a	<LOD	<LOD	<LOD	<LOD	***
3-(3′-Hydroxyphenyl) propanoic acid	0.58a	0.53a	0.25b	<LOD	<LOD	***
3-Phenylpropanoic acid	5.0a	5.0a	4.4a	<LOD	<LOD	***
Total Phenylpropanoic acids derivatives	**6.11a**	**6.15a**	**5.62a**	**1.21b**	**1.11b**	***
Phenylacetic acids derivatives						
4′-Hydroxyphenylacetic acid	7.4b	3.4bc	<LOD	<LOD	68.0a	***
3-Phenylacetic acid	34a	26b	20c	<LOD	<LOD	***
Total Phenylacetic acids derivatives	**41b**	**30c**	**20d**	**<LOD**	**68a**	***
Benzoic acids derivatives						
Benzoic acid	8.9a	10.1a	7.8ab	2.3c	5.2b	***
4-Hydroxy-3-methoxybenzoic acid	6.3b	9.5a	7.5b	6.0b	4.1c	***
3,4,5-Trihydroxybenzoic acid	0.131a	0.052b	0.122a	0.152a	0.137a	**
3,4-Dihydroxybenzoic acid	137c	162bc	177ab	192a	70d	***
3-Hydroxybenzoic acid	0.41a	0.26b	0.14c	0.25b	0.10c	***
4-Hydroxybenzoic acid	19a	21a	21a	21a	17a	ns
3-Hydroxy-4-methoxybenzoic acid	6.7ab	7.3a	6.7ab	5.8b	3.6c	***
Total Benzoic acids derivatives	**178b**	**210a**	**220a**	**227a**	**101c**	***
Hydroxycarboxilic acids derivatives						
3′-Hydroxymandelic acid	<LOD	<LOD	<LOD	0.70a	0.43b	***
Total Hydroxycarboxilic acids derivatives	**<LOD**	**<LOD**	**<LOD**	**0.70a**	**0.43b**	***
Benzenetriol						
Benzene-1,3,5-triol	12.4c	17.7a	16.7a	14.8b	15.0b	***
Total Benzenetriol	**12.4c**	**17.7a**	**16.7a**	**14.8b**	**15.0b**	***
Total Phenolic Compounds	**241b**	**268ab**	**273a**	**253ab**	**188c**	***

*Different letters (one-way ANOVA) denote significant differences (p<0.05) among the five stages for the same compound. ns non-significant; * p-value < 0.05; ** p-value < 0.01; ***p-value < 0.001. LOD Limit of detection.*

In vitro colonic fermentation of black onion resulted in a substantially different evolution of phenolic compounds compared to that observed with fresh onion (Figure 1, Table 2). In fact, in black onion, the total flavonol content did not show statistically significant differences between the initial and final concentration, although a significant increase was observed after 4 h of fermentation. This increase was partially attributed to the release of quercetin and isorhamnetin from their glycosides during the initial time of the fermentation and also because the inaccurate quantification of the glucoside derivatives of quercetin and isorhamnetin using their aglycones [35]. After 24 h, the content of free quercetin and isorhamnetin decreased significantly, possibly due to their degradation by colonic microbiota to simpler phenolic compounds [25].

Similarly, hydroxycinnamic acids did not show statistically significant differences between the first and the last stage, but there was an increase in total content after 2 h. This increase was 1.6-fold the initial content, which may be due to, as previously mentioned, as a result of the release of non-quantified compounds from the food matrix during the colonic fermentation. The total content of hydroxycinnamic acids decreased during the remained stages, as these compounds are presumably precursors of other compounds that are produced by the action of colonic microbiota, such as phenylpropanoic, phenylacetic and benzoic acid derivatives [43].

After 24 h of colonic fermentation, the catabolites 3-(4′-hydroxy-3′-methoxyphenyl)propanoic acid and 3-(3′,4′-dihydroxyphenyl)propanoic acid were the main phenylpropanoic acid derivatives determined, showing an increase in their content from 0.21 and 0.15 µmol L-1 to 0.57 and 0.54 µmol L-1, respectively. These compounds are argue formed from the microbial degradation of caffeic and ferulic acid, or via ring fission of quercetin, isorhamnetin derivatives [44],as discussed above for fresh onion. Likewise, phenylpropanoic acid derivatives are a substrate for colonic microbiota, producing other catabolites (phenylacetic and/or benzoic acids), which is why the total content decreased by 81.8% during the whole fermentation process.

Meanwhile, 4-hydroxyphenylacetic acid showed a significant increased with a final concentration 9.2-fold higher than the initial concentration. Indeed, this compound is one of the main catabolites of the hydrolysis of 3,4-dihydroxyphenylacetic acid, the predominant catabolite of quercetin [45], although it was not detected in our analyses.

The total content of benzoic acid derivatives increased by approximately 27.5% during the first 8 h of colonic fermentation, decreasing thereafter to show a final concentration of 101 µmol L^{-1}, 55.5% lower than in the previous stage. The only compound that

significantly increased its content during this process was 3,4-dihydroxybenzoic acid, which showed an increase of 140.1% during the first 8 h, since it is a common catabolite in the metabolism of phenolic compounds in different food matrices [27,46]. However, after 24 h, we found a marked decrease, showing a final concentration of 70 µmol L^{-1} (36.5% less than at 8 h). This is potentially due to the fact that 3,4-dihydroxybenzoic acid is also a precursor of other molecules such as 4-hydroxybenzoic acid or catechol, among others [21].

Finally, among the benzenetriol family, only the presence of benzene-1,3,5-triol was detected, showing a significant increase after 2 h of colonic fermentation and accounting for 121% of the initial content after 24 h. It has been shown that this compound is an end product of quercetin degradation, in line with that reported by Fernández-Jalao et al. (2021) [34].

Overall, the main end products in fresh onion after *in vitro* faecal fermentation were isorhamnetin (141 µmol L^{-1}), quercetin (95.3 µmol L^{-1}), 3,4-dihydroxybenzoic acid (53 µmol L^{-1}) and 4-hydroxybenzoic acid (12 µmol L^{-1}), comprising 88.4% of total phenolic compounds, while 3,4-dihydroxybenzoic acid (70 µmol L^{-1}), 4-hydroxyphenylacetic acid (68 µmol L^{-1}), 4-hydroxybenzoic acid (17 µmol L^{-1}) and benzene-1,3,5-triol (15 µmol L^{-1}), comprising 90.4% of total phenolic compounds, were the main phenolic compound after black onion fermentation. These phenolic compounds might be responsible for the potential beneficial effects associated with the consumption of black and fresh onion. In this sense, it has been reported that quercetin is able to inhibit the growth of colon cancer cells as well as inducing apoptosis in HT-29 cells or blocking the cell cycles by which these colon cancer cells can reproduce, among others [47–49]. On the other hand, Jaramillo et al. (2010) [50] reported the possible chemo-preventive and therapeutic capabilities of isorhamnetin against colon cancer, since they demonstrated that isorhamnetin could inhibit cell growth and induce apoptosis in HCT-116 human colon cancer cells. With respect to 3,4-dihydroxybenzoic acid, a chemopreventive effect against chemically induced carcinogenesis in the colon, among other tissues, has been observed [51]. Furthermore, Dobani et al. (2021) [52] showed that phenolic catabolites from raspberry-enriched ileal fluid fermentates, including 4-hydroxybenzoic acid, could reduce DNA damage in normal colonocytes. Finally, Kang et al. (2014) [53] reported the action of benzene-1,3,5-triol to induce apoptosis via pathways regulated by insulin-like growth factor-1 (IGF-1), which is a cell growth promoter and an important marker of cancer cell proliferation, in HT29 colon cancer cells. Obviously, all this evidence comes from in vitro

studies so any claim of the beneficial effects of these compounds in the prevention of cancer development should be verified in more robust experimental models.

3.2 Changes in Organosulfur Compounds of Fresh and Black Onion during in vitro Colonic Fermentation

A total of 15 and 14 OSCs were identified and quantified in fresh and black onion, respectively, during the different stages of the *in vitro* colonic fermentation, including 9 γ-glutamyl-*S*-alk(en)yl-*L*-cysteine derivatives (GSAk) and 10 *S*-alk(en)yl-*L*-cysteine derivatives (SAk) and (Table 1). Among them, γ −glutamyl-*S*-(2-propenyl) cysteine sulfoxide (G2PCS) (21.1 µmol L^{-1}) and γ -glutamyl-*S*-(2-propenyl) cysteine (GS2PC) (7.35 µmol L^{-1}) were the main GSAk, while methionine sulfoxide (65 µmol L^{-1}), *S*-allylcysteine (SAC) (14.5 µmol L^{-1}), *S*-(2-carboxypropyl)cysteine-glycine (10.79 µmol L^{-1}) and isoalliin (9.9 µmol L^{-1}) were the main SAk in digested fresh onion samples. These results are in line with those reported by Moreno-Ortega et al. 2021 [14], who found G2PCS, isoalliin, *S*-(2-carboxypropyl) cysteine-glycine and SAC as major compounds after *in vitro* intestinal digestion of fresh onion. Moreover, digested black onion presented the 89.7% of SAk, where isoalliin (43.1%), methionine sulfoxide (31.0%) and *S*-propyl-*L*-cysteine sulfoxide (propiin) (10.3%) were the main ones. While the main GSAk were γ −glutamyl-*L*-cysteine (43.1%), γ −glutamyl-*S*-(*S*-methyl) cysteine-glycine (31.2%) and GSMCS (9.1%). These data are in keeping with Moreno-Ortega et al. (2021) [14], who also reported the presence of GSMCS, isoalliin and methionine in black onion after the simulated gastrointestinal digestion.

The evolution of total SAk and GSAk during the *in vitro* colonic fermentation of fresh and black onion was illustrated in Figure 3. In fresh onion, the content of GSAk decreased significantly (58.8%), while SAk showed an increase during colonic fermentation (120.8%), resulting in the total OSCs remaining stable throughout the fermentation process (105.7%). Conversely, derivatives of GSAk and SAk from black onion significantly decreased during *in vitro* colonic fermentation, accounting for 26.2% and 50.3% of the initial content, respectively. Although the total content of OSCs at the beginning of the process was 1.6-fold higher in black onion than in fresh onion, after the fermentation process, the content in fresh onion was 1.4-fold higher than in black onion.

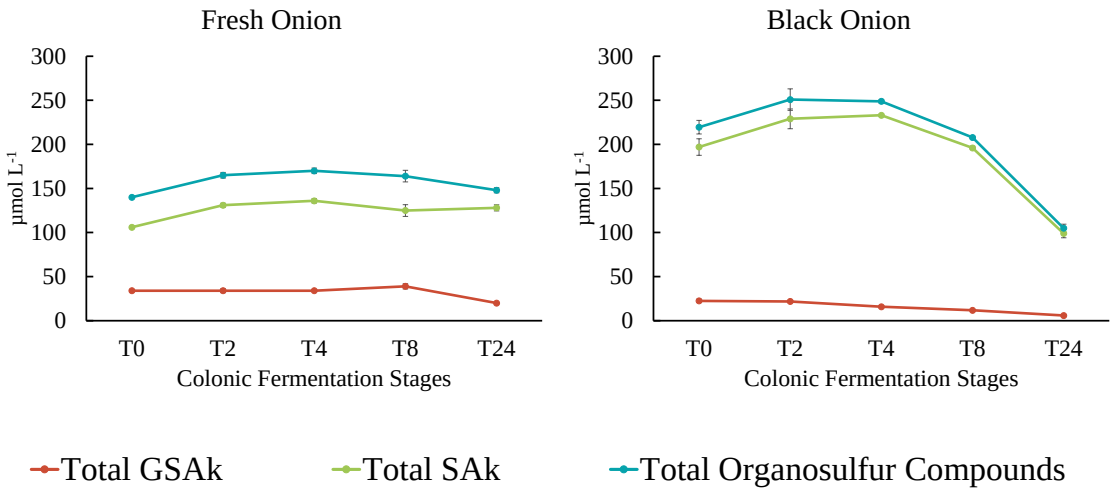

Figure 2.- Time-course profiles of organosulfur compounds groups and total organosulfur compounds found during in vitro colonic fermentation of fresh and black onion at 0, 2, 4, 8 and 24h (T0, T2, T4, T8 and T24, respectively). Data are expressed as mean values ± standard deviation of three different determinations (n=3).

The time-course of the individual OSCs of fresh onion during *in vitro* colonic fermentation are presented in Table 3. The total content of GSAk remained stable during the first 4 h. After 8 h, it showed a significant increase, mainly due to the increase in γ−glutamyl-*S*-allyl-*L*-cysteine (GSAC), which increases up to 9.6-fold compared to the previous stage. This increase could be due to the degradation by the colonic microbiota of more complex OSCs (Moreno-Ortega et al. [10,14]). For instance, γ -glutamyl-*S*-(2-carboxypropyl) cysteine-glycine (aka *S*-(2-carboxypropyl) glutathione) which via elimination of glycine produces γ -glutamyl-*S*-(2-carboxypropyl) cysteine and after decarboxylation and oxidation of the carboxypropyl group produces GSAC [54]. During the first 8 h, there was also a significant increase in GS2PC content (147.2%), probably caused by the reduction of G2PCS, which during colonic fermentation decreased until it was not detected at the end of fermentation [55]. The remaining OSCs derived from GSAk decreased progressively during the whole process, being especially affected after the last stage, 24 h of colonic fermentation.

Regarding total SAk, an increase in the total content was observed after 2 of colonic fermentation of fresh onion. A significant decrease however was observed after 8 h. Particularly, the compound isoalliin remained however stable until 8 h, decreasing significantly after 24 h of incubation, while *S*-(2-carboxypropyl)-cysteine-glycine showed a significant decrease after the first 2 h of incubation, decreasing again in concentration after 8 and 24 h and presenting a final concentration of 7.3 and 50.5% of the initial one, respectively. *S*-(2-carboxypropyl) cysteine-glycine could presumably lose glycine during

colonic fermentation and become a substrate for SAC synthesis, through decarboxylation and oxidation of the carboxypropyl group, producing an allyl group [56]. Isoalliin, in contrast, tends to be metabolised to form volatile compounds such as propanethial sulfoxide [57].

Moreover, *S*-allylmercapto-*L*-cysteine (SAMC) and *S*-propyl-*L*-cysteine (deoxypropiin) increased significantly their concentration after 2 h of colonic fermentation, while *S*-propylmercapto-*L*-cysteine (SPMC) continues to increase after 4 h. All of them decreased significantly after 8 h of fermentation, with 6.6% of the initial SAMC content, 37.7% of the initial deoxypropiin content and the SPMC content was below the detection limit.

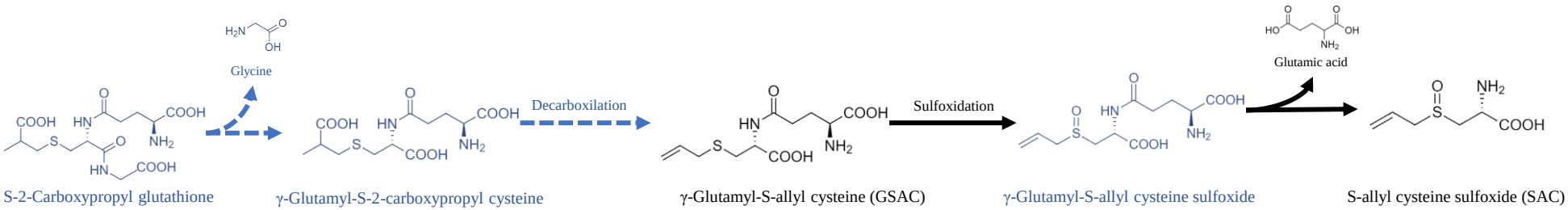

Figure 3.- Putative pathway for the catabolism of γ-Glutamyl-S-allyl cysteine (GSAC) and S-allyl cysteine (SAC)[54]

Finally, the SAk that increased their concentration during the fermentation process were methionine sulfoxide and SAC, both showing a final content 1.5-fold higher than the initial one. Lawson et al. (2018) [58] and Colín-González (2012) [59] reported that SAC could be originated by the action of γ-glutamyl transferase of GSAC, in this case, degradation of GSAC precursor compounds, such as *S*-(2-carboxypropyl) glutathione and γ-glutamyl-*S*-(2-carboxypropyl) cysteine, may presumably be occurring at a higher rate than the enzyme γ-glutamyl transferase transforms GSAC into SAC, resulting in an increase in the content of both compounds. Whereas, the increase in methionine sulfoxide may be due to the reaction of methionine, released from the protein matrix, with reactive oxygen species (ROS), causing oxidation of this amino acid and resulting in an increase in its concentration [60].

Table 3.- Concentration (µmol L^{-1}) of organosulfur compounds presented in fresh and black garlic samples at different stages during the in vitro colonic fermentation. Data is expressed as mean values (n=3).

Organosulfur Compounds (µmol L^{-1})	**T0**	**T2**	**T4**	**T8**	**T24**	**p-value**
	Fresh Onion					
γ-Glutamyl-*S*-Alk(en)yl-*L*-Cysteine Sulfoxides						
γ −Glutamyl-*S*-methyl-*L*-cysteine sulfoxide (GSMCS)	0.83a	0.80a	0.55b	<LOD	<LOD	***
γ −Glutamyl-*S*-(2-propenyl) cysteine sulfoxide (G2PCS)	21.10a	19.70a	17.20b	0.90c	<LOD	***
γ −Glutamyl-*S*-(1-propenyl) cysteine sulfoxide (G1PCS)	<LOD	<LOD	0.354a	0.247b	0.085c	***
γ-Glutamyl-*S*-Alk(en)yl-*L*-Cysteine Derivatives						
γ −Glutamyl-*L*-cysteine	1.99b	1.78b	2.93a	1.65b	1.53b	***
γ −Glutamyl-*S*-(*S*-methyl) cysteine-glycine	0.16c	0.25c	0.36b	0.55a	0.35b	***
γ −Glutamyl-*S*-(2-carboxypropyl) cysteine-glycine	1.08ab	0.94ab	1.16a	0.89b	0.61c	***
γ −Glutamyl-*S*-allyl-*L*-cysteine (GSAC)	1.78c	1.55c	2.49c	23.94a	16.15b	***
γ -Glutamyl-*S*-(2-propenyl) cysteine (GS2PC)	7.35b	8.49b	8.95ab	10.82a	1.62c	***
Total GSAk	**34ab**	**34b**	**34b**	**39a**	**20c**	*******
***S*-Alk(en)yl-*L*-Cysteine Sulfoxides Derivatives**						
Isoalliin	9.9a	9.7a	9.5a	8.7a	5.0b	***
Methionine sulfoxide	65c	82b	81b	97a	100a	***
***S*-Alk(en)yl-*L*-Cysteine Derivatives**						
S-Allylcysteine (SAC)	14.5b	16.1b	18.1ab	15.9b	21.7a	**
S-Allylmercapto-*L*-cysteine (SAMC)	2.57c	5.48[a]	4.26b	0.36d	0.37d	***
S-Propyl-*L*-cysteine (Deoxypropiin)	3.23b	4.43[a]	3.44b	1.67c	0.36d	***
S-Propylmercapto-*L*-cysteine (SPMC)	0.63c	4.53b	10.85a	<LOD	<LOD	***
S-(2-Carboxypropyl) cysteine-glycine	10.79a	8.95b	8.33b	1.49c	0.79c	***
Total SAk	**106c**	**131ab**	**136a**	**125b**	**128ab**	*******
Total Organosulfur Compounds	**140b**	**165[a]**	**170a**	**164a**	**148b**	*******
	Black Onion					
γ-Glutamyl-*S*-Alk(en)yl-*L*-Cysteine Derivatives						
γ −Glutamyl-*S*-methyl-*L*-cysteine sulfoxide (GSMCS)	2.05a	1.57b	0.43c	<LOD	<LOD	***
γ −Glutamyl-*S*-(1-propenyl) cysteine sulfoxide (G1PCS)	0.78a	0.56ab	0.41bc	0.22c	0.23c	***

γ −Glutamyl-*L*-cysteine	9.7a	8.1ab	7.8ab	7.1b	4.2c	***
γ −Glutamyl-*S*-(S-methyl) cysteine-glycine	7.03a	6.70[a]	2.81b	0.90bc	0.49c	***
γ −Glutamyl-*S*-(2-carboxypropyl) cysteine	1.35b	2.22[a]	2.09a	1.01bc	0.66c	***
γ −Glutamyl-*S*-allyl-*L*-cysteine (GSAC)	<LOD	<LOD	<LOD	0.51a	<LOD	***
γ -Glutamyl-*S*-(2-propenyl) cysteine (GS2PC)	1.58c	2.65[a]	2.27ab	2.10b	0.32d	***
Total GSAk	**22.5a**	**21.8[a]**	**15.8b**	**11.8c**	**5.9d**	***
***S*-Alk(en)yl-*L*-Cysteine Derivatives**						
Isoalliin	84.96b	106.71[a]	108.12a	75.64b	1.90c	***
S-Propyl-*L*-cysteine sulfoxide (Propiin)	20.3a	25.4[a]	22.6a	13.4b	<LOD	***
Methionine sulfoxide	61c	65bc	72ab	80a	82a	***
S-Allylcysteine (SAC)	5.51a	3.68b	3.41b	1.80c	1.43c	***
S-Propylmercapto-*L*-cysteine (SPMC)	10.4b	12.3[a]	12.0a	12.7a	10.1b	*
S-(2-Carboxypropyl) cysteine	5.3b	6.8[a]	3.8c	4.8b	2.2d	***
S-Allylglutathione (SAG)	9.73b	8.17bc	11.57a	7.49c	1.26d	***
Total SAk	**197b**	**229[a]**	**233a**	**196b**	**99c**	***
Total Organosulfur Compounds	**219b**	**250[a]**	**249a**	**208b**	**105c**	***

*Different letters (one-way ANOVA) denote significant differences (p<0.05) among the five stages for the same compound. ns non-significant; * p-value < 0.05; ** p-value < 0.01; ***p-value < 0.001. LOD Limit of detection. GSAk: γ-Glutamyl-S-Alk(en)yl-L-Cysteine Derivatives; SAk: S-Alk(en)yl-L-Cysteine Derivatives.*

Regarding black onion, the total GSAk content decreased by 73.8% during colonic fermentation, and it was significantly affected after 4, 8 and 24 h of incubation. Compounds including GSMCS, G1PCS, γ−glutamyl-*L*-cysteine and γ −glutamyl-*S*-(*S*-methyl) cysteine-glycine showed their highest concentration before *in vitro* fermentation, accounting for 2.05, 0.78, 9.7 and 7.03 µmol L-1, respectively. Among them, GSMCS was not detected after 8 h, while the others decreased their concentration to 29.5% for G1PCS, 43.3% for γ-glutamyl-*L*-cysteine and 7.0% for γ -glutamyl-*S*-(*S*-methyl) cysteine-glycine after 24 h. In contrast, γ-glutamyl-*S*-(2-carboxypropyl) cysteine and GS2PC significantly increased their concentration after 2 h fermentation up to 2.22 and 2.65 µmol L-1, respectively, and then started to decrease until the end of the process. The increase of these compounds may be due to the degradation of their precursors γ-glutamyl-*S*-(2-carboxypropyl)-cysteine-glycine and GS2PCS, respectively [54], not determined in the present study, although they were reported to be present in black onion [10,30].

Focusing on SAk, the total content showed a significant increase during the first 4 h, with a decrease of 57.5% in the last two stages. In this sense, it was observed that isoalliin showed a significant increase in concentration during the first 2 and 4 h of fermentation. After 24 h, the isoalliin content decreased drastically to 2.2% of the initial content, since it is presumably a substrate for enzymes that would result in the formation of volatile compounds [57].

After the first 4 h of fermentation, propiin, *S*-(2-carboxypropyl)cysteine and *S*-allylglutathione (SAG) showed an increase in their content, while continued to increase after 4 h and *S*-propylmercapto-*L*-cysteine (SPMC) up to 8 h after the start of fermentation. Despite this, propiin was not detected after 24 h of incubation, while the final content of *S*-(2-carboxypropyl)cysteine, SAG and SPMC was 41.5, 12.9 and 97.1% of the initial content, respectively. Moreover, SAC showed a progressive decrease from the beginning of fermentation, with a final concentration of 1.43 µmol L-1. Conversely, methionine sulfoxide increased its content during the whole process up to 134%, probably due to the interaction of free methionine with ROS, causing its oxidation.

Finally, methionine sulfoxide, SAC and GSAC were the main OSCs after the *in vitro* colonic fermentation of fresh onion, accounting for 93.1% of the total OSCs, while for black onion, the main ones were methionine sulfoxide and SPMC with 93.0% of the total content. The presence of these compounds at the colonic level may have a potentially beneficial effect on health. For instance, methionine sulfoxide is formed by the reaction of methionine with ROS, preventing ROS contact with colonic cells and the possible cell damage they cause. Subsequently, methionine sulfoxide is metabolised by methionine sulfoxide transferase enzymes present in colonic bacteria [61,62]. Moreover, Agbana et al. 2020 [63] have described the role of SAC against various cancers, reporting its action against colon cancer studied in mice and rats. This compound may have the capacity to inhibit colonic tumorigenesis by its detoxifying action against some types of carcinogenic compounds. Concerning SPMC, it is a compound that is not stable at 35 °C and neutral pH for many h and could therefore be used as a microbial substrate for the formation of disulfides and volatile compounds [64]. Lai et al. (2013) [65] reported the capacity of some volatile compounds, such as diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS), to inhibit tumour cell invasion in colon cancer by downregulating some types of matrix metalloproteinases (MMPs), enzymes whose action is correlated with cancer cell invasiveness. But, again, all this evidence is very limited and further efforts should be carried out to address the potential chemopreventive actions of OSCs in adequate, physiologically-plausible contexts.

4. Conclusions

Given the limited information available, this research aimed to evaluate the impact of *in vitro* colonic fermentation on the profile of phenolic and OSCs of fresh and black onion. *In vitro* colonic fermentation significantly affected the phenolic content, resulting in an

increase of 45% in fresh onion and a decrease of 22% in black onion. Concerning OSCs, the total content in black onion showed a significant decrease after colonic fermentation (48%), while in fresh onion it remained stable during the fermentation process. Most of these compounds could be responsible for the potential health benefits related to fresh and black onion consumption, but further research should be conducted to clarify their bioactivity.

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Conflicts of interest: There are no conflicts to declare.

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CAPÍTULO 5

CAPÍTULO 5: RESULTADOS OBJETIVO 4

Artículo 7

Bioavailability of Organosulfur Compounds After the Ingestion of Black Garlic by Healthy Humans

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Bioavailability of Organosulfur Compounds After the Ingestion of Black Garlic by Healthy Humans

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Abstract

Regular consumption of garlic and its derivative, black garlic, is related to a decreased risk of many human diseases due to the presence of compounds with recognised bioactivity such as organosulfur compounds. However, information on the metabolization of these compounds in humans is limited. This study aims to identify and quantify the organosulfur compounds excreted in human urine 24 h after the acute intake of black garlic by ultra-high-performance liquid chromatography coupled to a high-resolution mass spectrometry (UHPLC-HRMS) analysis. Thirty-three organosulfur compounds were identified and quantified in urine 24 hours after ingestion, being the main ones methiin (3433 ± 1150 nmol), isoalliin (3027 ± 2133 nmol), *S*-(2-carboxypropyl)-L-cysteine (2199 ± 1913 nmol) and *S*-propyl-L-cysteine (deoxypropiin) (1294 ± 253 nmol). Additionally, N-acetyl-*S*-allyl-L-cysteine (NASAC), N-acetyl-*S*-allyl-L-cysteine sulfoxide (NASACS) and N-acetyl-*S*-(2-carboxypropyl)-L-cysteine (NACPC) were detected in urine samples, which are recognised metabolites of *S*-allyl-L-cysteine (SAC), alliin and *S*-(2-carboxypropyl)-L-cysteine, respectively. These compounds are potentially N-acetylated in liver and kidney and metabolic degradation pathways have been proposed in order to explain the presence of the major compounds in urine. In total, the excretion of organosulfur compounds 24 hours after the ingestion of black garlic was 13197 ± 6657 nmol, equivalent to 2.65% of the 498.8 µmol intake of organosulfur compounds, being the 97.5% *S*-alk(en)yl-L-cysteine derivatives (SAk) and 2.5% γ-glutamyl-*S*-alk(en)yl-L-cysteine derivatives (GSAk). The identification of these organosulfur compounds as urinary metabolites of black garlic ingestion provides opportunities to study the role of black garlic in human health.

Keywords: black garlic, bioavailability, human urine, organosulfur compounds; phenolic compounds

1. Introduction

Garlic (*Allium sativum* L.) is a basic ingredient appreciated in the world's cuisines for its organoleptic characteristics and the health properties associated with its consumption (Amagase & Petesch, 2003). In this sense, regular consumption of garlic has been related to a protective effect against some types of cancer such as colorectal, lung, gastric and bladder cancer, by regulating the metabolism of carcinogenic substances, inducing apoptosis or inhibiting the proliferation of cancer cells, among other mechanisms (Bagul et al., 2015; Shin et al., 2017; Xu et al., 2013). Moreover, garlic consumption has been associated with a decrease in blood pressure and total plasma cholesterol, both of which are risk factors for cardiovascular disease (Asdaq & Inamdar, 2010; Khatua et al., 2017; Supakul et al., 2013). Similarly, the consumption of this bulb has been related to a protective effect against other types of diseases such as neurodegenerative, digestive or renal diseases (Ebrahimzadeh-Bideskan et al., 2016; Nasiri et al., 2016; Zardast et al., 2016). These health properties associated with their consumption are mainly due to the presence of compounds with recognised bioactivity in their composition, specifically phenolic and organosulfur compounds (Ansary et al., 2020).

Black garlic, a garlic-derived product, has generated growing interest in recent years for its organoleptic qualities and physico-chemical characteristics. This product is obtained by subjecting fresh garlic to controlled temperature and humidity conditions (Jang et al., 2008; Kang, 2016; Kim et al., 2013). The elaboration process results in a final product with a brownish colour, sweeter taste, softer texture and low pungency (Ríos-Ríos et al., 2019; Toledano Medina et al., 2016). In addition, compositional changes are produced, with caffeic and coumaric acid and S-allyl-L-cysteine (SAC) being the major phenolic and organosulfur compounds in black garlic, respectively (Moreno-Ortega et al., 2020). The described changes in the profile of phytochemical compounds during the processing of black garlic have attracted scientific interest, leading to multiple studies aimed at assessing the potential health effect of regular consumption of black garlic (Ahmed & Wang, 2021; Alkreathy et al., 2020; Dong et al., 2014; Gomez et al., 2019). However, the mechanisms by which phenolic and organosulfur compounds in black garlic may exert these positive effects on health status need to be further elucidated.

Previous *in vitro* studies have provided information on the bioaccessibility of phytochemical compounds in black garlic, as well as on the evolution of these compounds during fermentation at the colonic level. In this sense, Moreno-Ortega et al. (2022) reported that 4′-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, methionine

sulfoxide and SAC were the main phenolic and organosulfur compounds remained after 24h of *in vitro* colonic fermentation. After colonic microbiota action, these molecules are potentially absorbed, distributed, metabolised and excreted, and their bioavailability could then be determined. Regarding this, some studies have been carried out assessing the bioavailability of organosulfur compounds in fresh garlic, mainly those of a volatile nature (Qin et al., 2020; Rosen et al., 2000; Scheffler et al., 2016, 2019a, 2019b). However, to the best of our knowledge, there are no studies evaluating the bioavailability of organosulfur compounds after ingestion of black garlic.

Based on these observations, the aim of the present study was to identify and quantify the organosulfur compounds excreted in urine 24 h after the acute intake of black garlic by ultra-high-performance liquid chromatography coupled to a high-resolution mass spectrometry (UHPLC-HRMS) analysis.

2. Materials and methods

2.1. Chemicals

The reference standard compounds including *S*-allyl-L-cysteine sulfoxide (aka alliin) and *S*-allyl-L-cysteine (SAC) were purchased from Sigma-Aldrich (Madrid, Spain). Ammonium acetate, ammonium formate and ethanol were obtained from Sigma-Aldrich. Acetonitrile, methanol and water were of LC-MS grade.

2.2. Materials

Fresh and black garlic were kindly provided by a local supplier Abuela Carmen®, Córdoba, Spain.

2.3. Study design

Twelve healthy volunteers (seven females and five males, mean age 29.0 ± 8.9 years) with a body mass index (BMI) of 23.5 ± 2.2 kg/m^2 (mean ± SD) participated in this study. Exclusion criteria were pregnancy or lactation, drug allergies, any chronic medication and any antibiotic treatment during the 6 months prior to the study. Written informed consent was provided by all participants. The study protocol was approved by the Ethical Committee of the Human Clinical Research Unit at the Arnau Vilanova University Hospital, Lleida, Spain (Approval Number: CEiC-1790).

Before attending the clinic, participants were asked to follow a diet absent of organosulfur compounds for two days by avoiding *Allium* vegetables. On the morning of the feeding trial, the volunteers brought their 12 h overnight urine (basal) and were invited to eat a

portion of 20 g of black garlic after fasting overnight and to continue with the organosulfur-free diet that day and until the next morning, when the last urine sample was collected. Urine samples were obtained at the interval times of 0-2, 2-4, 4-8, 8-24 h after the black garlic intake. The total volume of each sample was recorded before storing the aliquots (2 mL) at -80ºC until the chromatographic analysis.

2.4. Processing of urine samples

Urine samples were defrosted, vortexed, centrifuges at 15000 rpm for 10 min at 4ºC and the supernatant was collected and stored at -80ºC until UHPLC-HRMS analysis. Each sample was prepared in duplicate.

2.5. Analysis of organosulfur compounds in urine samples

Aliquots of human urine samples were analysed using a UHPLC-PDA-MS mass spectrometer system (Thermo Scientific, San José, CA, USA) comprising of a UHPLC pump, a PDA detector scanning from 200 to 600 nm, and an autosampler operating at 4 °C (Dionex Ultimate 3000 RS, Thermo Corporation). The chromatographic conditions were previously described by Moreno-Rojas et al. (2018) (Moreno-Rojas et al., 2018). Briefly, separation of organosulfur compounds was performed on a 2.1 × 150 mm ACQUITY UPLC 1.7 µm BEH amide column (equipped with an ACQUITY UPLC BEH amide 1.7 µm van-guard pre-column) (Waters, Spain), which was maintained at 35 °C and eluted at a flow rate of 0.4 ml/min with a 20 min gradient using two mobile phases: A: deionized water with 5 mM of ammonium acetate, 5 mM ammonium formate and 1% formic acid and B: acetonitrile. The gradient started with 5% of A rising 46% in 13 min and finally return to 5% of A and was maintained 4 min to equilibrate the column.

After passing through the flow cell of the PDA detector, the column eluate was directed to an Exactive Orbitrap mass spectrometer (Thermo Scientific, San José, CA, USA) fitted with a Heated Electrospray Ionization Probe (HESI) operating in positive ionization mode for the determination of organosulfur compounds (Moreno-Rojas et al., 2018). Data acquisition and processing were carried out using Xcalibur 3.0 software.

Identification of organosulfur compounds in human urine samples was achieved by comparing the exact mass and the retention time with available standards. In the absence of standards, compounds were tentatively identified by comparing the exact theoretical mass of the molecular ion with the measured accurate mass of the molecular ion and searched against metabolite databases, including Metlin, Human Metabolome DataBase (HMDB), and more general chemical databases, such as PubChem and ChemSpider. The quantification of organosulfur compounds was carried out by selecting

the exact theoretical mass of the molecular ion by reference to standard curves, obtaining a linear regression analysis with R^2 values of >0.998 (n = 6). In the absence of reference compounds, they were quantified by reference to the calibration curve of a closely related parent compound.

2.6. Statistical analysis

A one-way ANOVA was carried out using R software (v.3.6.3., R Core Team, Vienna, Austria) to determine significant differences between the human urine samples at different interval times after intake of black garlic. The significance being accepted for a *p*-value < 0.05. Next, Fisher's LSD pairwise comparison was performed on the data.

3. Results and discussion

3.1. Characterization of Organosulfur Compounds in Black Garlic

UHPLC-HRMS analysis detected and quantified sixteen organosulfur compounds in black garlic, in agreement with our previous analysis of black garlic (Moreno-Ortega et al., 2020) (Table 1). S-allyl-L-cysteine (SAC) (153 µmol), alliin (112 µmol), ɣ −glutamyl-S-allyl-L-cysteine (GSAC) (87.9 µmol) and γ-glutamyl-S-allyl-L-cysteine sulfoxide (GSACS) (69.0 µmol) were the main ones, accounting for 85% of total organosulfur content, followed by S-allylsulfenic acid, ɣ−glutamyl-S-allylthio-L-cysteine and methionine sulfoxide with 18.70, 10.04 and 10.08 µmol, respectively. Small quantities of other organosulfur compounds, such as S-allylmercapto-L-cysteine (8.16 µmol), ɣ −glutamyl-S-methyl-L-cysteine sulfoxide (7.60 µmol) and methiin (4.86 µmol) were also present. A total of 499 µmol of organosulfur compounds was present in 20 g of black garlic ingested by the volunteers, of which 181 µmol corresponded to the total amount of γ-glutamyl-S-alk(en)yl-L-cysteine derivatives (≈ 36% of the total organosulfur content) and 318 µmol to S-alk(en)yl-L-cysteine derivatives (≈ 64% of the total organosulfur content). Figure 1 shows the chemical structures of the main organosulfur compounds found in black garlic.

Table 1.- Organosulfur compounds identified and quantified in black garlic samples expressed as mean ± SD

Black Garlic	µmol	% in Black Garlic
γ-Glutamyl-*S*-alk(en)yl-L-cysteine derivatives (GSAk)		
γ-Glutamyl-S-methyl-L-cysteine sulfoxide (GSMCS)	7.60±0.02	1.5
γ-Glutamyl-S-allyl-L-cysteine sulfoxide (GSACS)	69.04±0.29	13.8
γ-Glutamyl-S-allyl-L-cysteine (GSAC)	87.86±0.36	17.6
γ-Glutamyl-S-methyl-L-cysteine (GSMC)	3.98±0.00	0.8

γ-Glutamyl-(2-carboxyethyl)-L-cysteine-glycine	2.38±0.01	0.5
γ-Glutamyl-S-allylthio-L-cysteine	10.08±0.02	2.0
Total GSAk	**180.94±0.69**	**36.3**
***S*-alk(en)yl-L-cysteine derivatives (SAk)**		
S-Allyl-L-cysteine sulfoxide (Alliin)	112.22±0.56	22.5
S-Methyl-L-cysteine sulfoxide (Methiin)	4.86±0.03	1.0
S-Methyl-L-cysteine (Deoxymethiin)	3.36±0.02	0.7
S-Allyl-L-cysteine (SAC)	153.66±0.30	30.8
S-(2-Carboxypropyl)-L-cysteine	2.30±0.00	0.5
S-Allylmercapto-L-cysteine (SAMC)	8.16±0.02	1.6
S-Propylmercapto-L-cysteine (SPMC)	4.58±0.01	0.9
S-Allylsulfenic acid	18.70±0.05	3.7
Methionine sulfoxide	10.04±0.03	2.0
Total SAk	**317.88±0.91**	**63.7**
Total Organosulfur Compounds	**498.82±1.58**	**100.0**

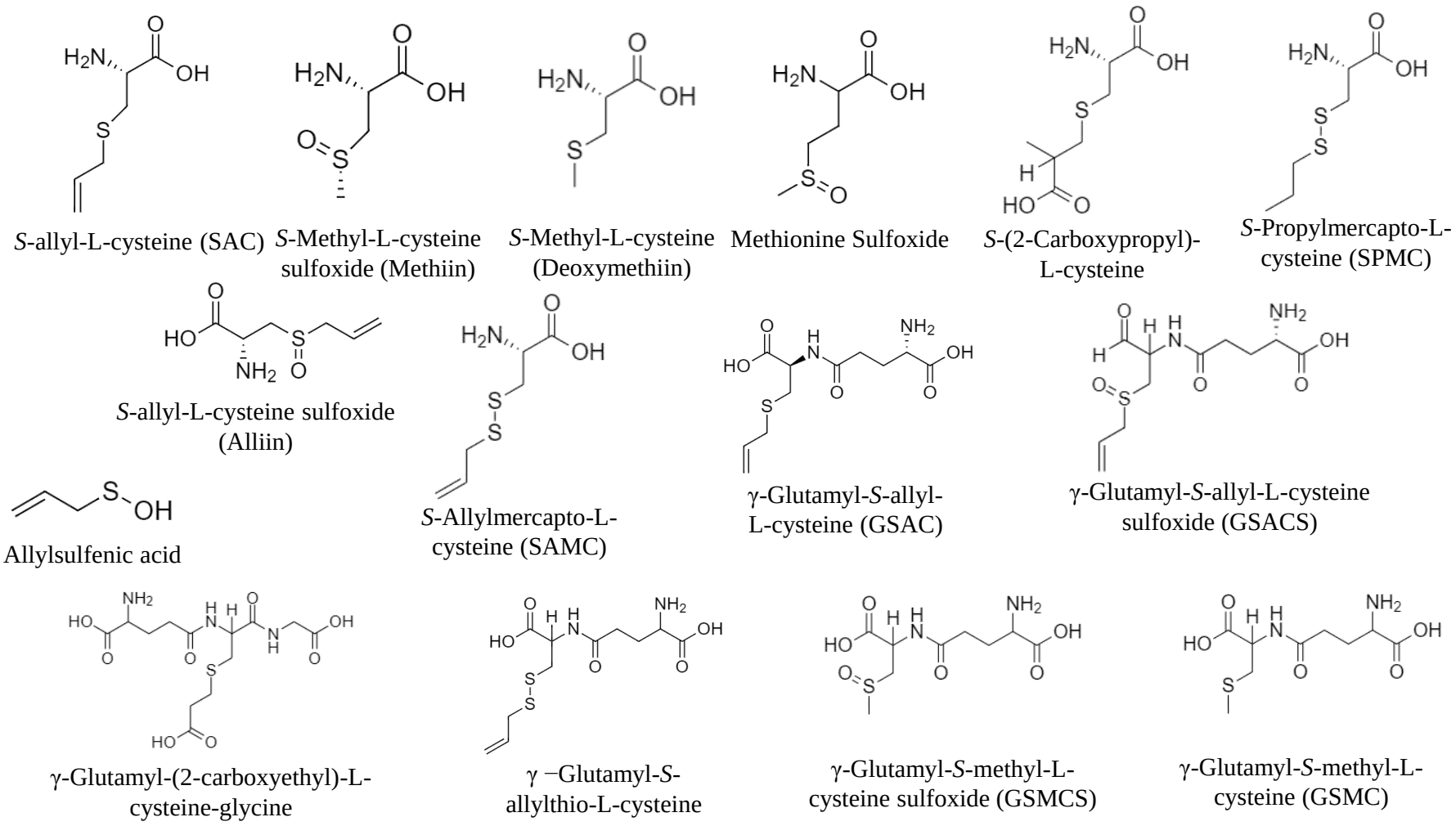

Figure 1.- Chemical structures of the main organosulfur compounds found in black garlic

3.2. Identification and quantification of organosulfur compounds and their metabolites in urine

The HPLC-HRMS analysis revealed a total of thirty-three organosulfur compounds excreted in urine 0-24 h following an acute intake of 20 g of black garlic (Table 2). From the total of 33 compounds identified in urine, 12 were GSAk derivatives and 21 were SAk derivatives. Table 3 shows the urinary excretion of the organosulfur compounds identified and quantified 0-24 h after black garlic consumption. None of these compounds were presented in urine 12 h pre-supplementation of black garlic. Moreover, except for ɣ-glutamyl-*S*-(2-carboxyethyl)-L-cysteine-glycine, ɣ-glutamyl-S-allylthio-L-cysteine, deoxymethiin, *S*-allylmercapto-L-cysteine, *S*-propylmercapto-L-cysteine and *S*-allylsulfenic acid which were absent in urine, the rest of parent organosulfur compound present in black garlic were detected in urine after the ingestion of this product. In total, the excretion of organosulfur compounds 0-24h after the ingestion of black garlic was 13197 ± 6657 nmol, equivalent to 2.65% of the 498.8 µmol intake of organosulfur compounds (Table 3), being the 97.5% SAk derivatives and 2.5% GSAk derivatives.

Table 2.- UHPLC-HRMS-based identification of phenolic and organosulfur compounds detected in fresh and black garlic in human urine samples

Peak	Retention Time (min)	Compound	Chemical Formula $[m/z]^-$	Experimental Mass $[m/z]^-$	δ (ppm)	MSI MI level[a]
		γ-Glutamyl-*S*-alk(en)yl-L-cysteine derivatives (GSAk)				
1	4.34	γ −Glutamyl-S-(propyl) cysteine sulfoxide	C11H21N2O6S	309.1115	-2.1191	2
2	6.34	γ −Glutamyl-*S*-(*S*-1-propenyl) cysteine-glycine	C13H22N3O6S2	380.0944	0.8362	2
3	6.43	γ −Glutamyl-*S*-(2-carboxypropyl) cysteine	$C12H19N2O7S^{-2}$	335.0907	-0.0579	2
4	7.86	γ −Glutamyl-*S*-(*S*-methyl) cysteine-glycine	C11H20N3O6S2	354.0788	0.6916	2
5	7.88	γ −Glutamyl-*S*-methyl-L-cysteine (GSMC)	C9H17N2O5S	265.0853	0.1777	2
6	8.53	Glutathione	C10H18N3O6S	308.0911	0.7967	2
7	8.55	γ −Glutamyl-*S*-allyl-L-cysteine (GSAC)	C11H19N2O5S	291.1009	0.4285	2
8	8.71	γ −Glutamyl-L-cysteine	C8H15N2O5S	251.0696	-0.9444	2
9	9.00	γ −Glutamyl-*S*-allyl-L-cysteine sulfoxide (GSACS)	C11H19N2O6S	307.0958	-0.4247	2
10	9.60	γ −Glutamyl-*S*-methyl-L-cysteine sulfoxide (GSMCS)	C9H17N2O6S	281.0801	-0.8092	2
11	9.60	γ −Glutamyl-*S*-(propyl) cysteine	C11H21N2O5S	293.1165	-0.3883	2
12	10.00	γ −Glutamyl-*S*-allyl cysteine sulfoxide	C11H19N2O6S	307.0958	-1.0128	2
		***S*-alk(en)yl-L-cysteine derivatives (SAk)**				
13	1.39	*S*-(2-Propenyl)-allyl-L-cysteine sulfoxide	C9H16NO3S	218.0845	-1.8770	2
14	3.92	N-Acetyl-*S*-allyl-L-cysteine sulfoxide (NASACS)	C8H14NO4S	220.0638	-1.2877	2
15	3.96	*S*-Butanoyl-L-cysteine sulfoxide (Butiin)	C7H16NO3S	194.0845	-0.8589	2
16	4.21	N-Acetyl-*S*-allyl-L-cysteine (NASAC)	C8H14NO3S	204.0689	-1.9576	2
17	4.87	*S*-Allylcysteine (SAC)	C6H12NO2S	162.0583	-1.0605	1
18	5.03	trans-*S*-(1-Propenyl)-L-cysteine (S1PC)	C6H12NO2S	162.0583	-0.5898	2
19	5.60	*S*-Propyl-L-cysteine (Deoxypropiin)	C6H14NO2S	164.0740	-4.2089	2
20	6.18	*S*-Allyl-L-cysteine sulfoxide (Alliin)	C6H12NO3S	178.0532	1.5374	1
21	6.70	*S*-(1-Propenyl)-L-cysteine sulfoxide (Isoalliin)	C6H12NO3S	178.0532	2.5793	2
22	7.10	Cycloalliin	C6H12NO3S	178.0532	2.3222	2
23	7.13	*S*-(2-Carboxypropyl)-L-cysteine	C7H14NO4S	208.0638	1.7404	2
24	7.26	*S*-Methyl-L-cysteine sulfoxide (Methiin)	C4H10NO3S	152.0376	3.5869	2
25	7.56	Methionine sulfoxide	C5H12NO3S	166.0532	-0.6264	2
26	7.87	*S*-Propyl-L-cysteine sulfoxide (Propiin)	C6H14NO3S	180.0689	2.4960	2
27	8.09	*S*-Carboxymethyl-L-cysteine (Carbocysteine)	C5H10NO4S	180.0325	2.5441	2
28	8.43	*S*-(2-Carboxypropyl)-L-cysteine-glycine	C9H17N2O5S	265.0853	0.6382	2
29	8.55	*S*-Ethyl-L-cysteine sulfoxide (Ethiin)	C5H12NO3S	166.0532	-0.0752	2

30	9.50	Ajoene	C9H15OS3	235.0280	-1.1224	2
31	9.58	N-Acetyl-*S*-(2-carboxypropyl)-L-cysteine (NACPC)	C9H16NO5S	250.0744	2.5686	2
32	9.74	*S*-Allylmercaptoglutathione	C13H22N3O6S2	380.0945	0.2443	2
33	10.89	Allixin	C12H19O4	227.1278	-1.6378	2

[a]*Metabolite standards initiative (MSI) metabolite identification (MI) levels* (Sumner et al., 2007). *Reference compounds were available for all compounds identified at MSI MI level 1. Compounds for which there was a reference compound were quantified with them, while for the rest, the most structurally similar reference compound was used.*

The main urinary organosulfur compounds were methiin (3433±1150 nmol), isoalliin (3027±2133 nmol), *S*-(2-carboxypropyl)-L-cysteine (2199±1913 nmol) and *S*-propyl-L-cysteine (deoxypropiin) (1294±253 nmol), accounting for 75.4% of the total organosulfur compounds excreted in urine after black garlic intake. Substantial amount of SAC (622.8±242.3 nmol), tran-*S*-(1-propenyl)-L-cysteine (S1PC) (477.9±199.8 nmol), methionine sulfoxide (446.0±79.2 nmol) and alliin (343.3±271.7 nmol) were also quantified in urine after black garlic consumption. These results are in keeping with previous studies on black garlic where alliin and SAC have been found after simulated gastrointestinal digestion and methionine sulfoxide, SAC and propiin after colonic fermentation *in vitro* (Moreno-Ortega et al., 2020). Regarding the total content of organosulfur compounds, 70% were excreted between 8 and 24 hours after ingestion of black garlic, which likely means that the absorption of these compounds mainly occurs at the intestinal level (Figure 2).

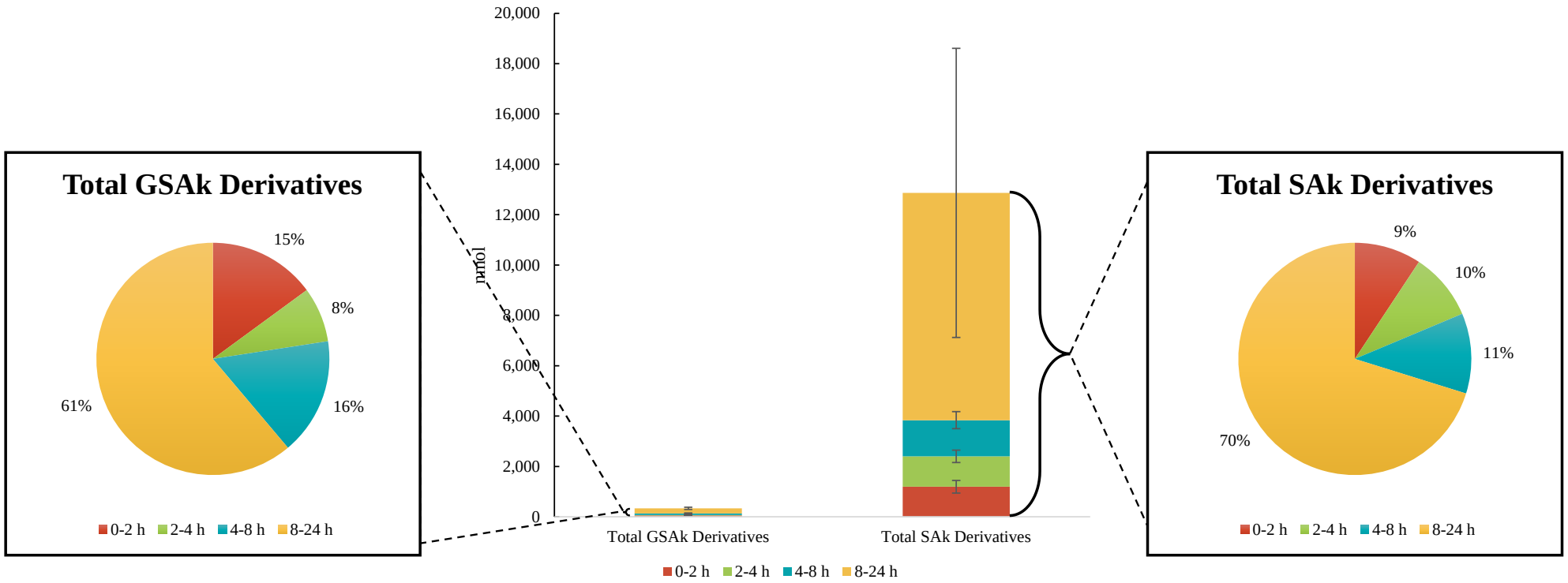

Figure 2.- Time-course profiles of the organosulfur compounds identified during the urinary excretion after the intake of black garlic

Table 3.- Time-course profiles of the organosulfur compounds identified during the urinary excretion after the intake of black garlic

Black Garlic (nmol)	Basal 24h	0-2h	2-4h	4-8h	8-24h	0-24h	p-value	% Excretion
γ-Glutamyl-*S*-alk(en)yl-L-cysteine derivatives (GSAk)								
Glutathione	<LOD	3.8±0.8c	1.3±0.4c	3.6±1.1c	11.2±2.3b	19.8±4.6a	***	0.00
γ −Glutamyl-L-cysteine	<LOD	6.5±2.1b	2±0.7b	3.7±1.6b	19.8±4.4a	32±8.8a	***	0.01
γ −Glutamyl-S-methyl-L-cysteine (GSMC)	<LOD	12.1±2.5b	6.6±0.9b	16.3±2.3b	75.8±16.7a	110.8±22.3a	***	0.02
γ −Glutamyl-S-methyl-L-cysteine sulfoxide (GSMCS)	<LOD	7.3±2.8b	3.7±1.7b	9.2±4.2b	38.1±8.9a	58.3±17.6a	***	0.01
γ −Glutamyl-S-allyl-L-cysteine (GSAC)	<LOD	4.1±1.2bc	1.9±0.6bc	2.5±0.9bc	6.6±1.4b	15±4a	***	0.00
γ −Glutamyl-S-(propyl)-L-cysteine	<LOD	2.5±0.9bc	1.1±0.3c	2.2±0.7bc	7.8±1.7b	13.5±3.5a	***	0.00
γ −Glutamyl-S-allyl-L-cysteine sulfoxide (GSACS)	<LOD	1.9±0.7bc	1±0.4bc	1±0.4bc	3.9±0.9b	7.7±2.4a	***	0.00
γ −Glutamyl-S-(propyl)-L-cysteine sulfoxide	<LOD	2.2±2.2	3.2±2.1	5.8±3.7	0.7±0.7	12±8.6	ns	0.00
γ −Glutamyl-S-(2-carboxypropyl)-L-cysteine	<LOD	3±0.6b	0.9±0.2b	1.5±0.5b	5.8±2.8ab	11.2±4.2a	***	0.00
γ −Glutamyl-S-(S-methyl)-L-cysteine-glycine	<LOD	6.2±1.1c	3.8±1.1c	8.6±1.2c	34.6±5.9b	53.2±9.3a	***	0.01
γ −Glutamyl-S-(S-1-propenyl)-L-cysteine-glycine	<LOD	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	ns	0.00
Total GSAk	**<LOD**	**49.8±15c**	**25.6±8.4c**	**54.5±16.7c**	**204.4±45.7b**	**334.2±85.8a**	*******	**0.07**
***S*-alk(en)yl-L-cysteine derivatives (SAk)**								
S-Methyl-L-cysteine sulfoxide (Methiin)	<LOD	384±81.1b	327.8±68.7b	456±82.8b	2265.6±917.6ab	3433.4±1150.1a	***	0.69
S-Allyl-L-cysteine (SAC)	<LOD	39.2±8.6b	40.8±8.2b	67.3±15.4b	475.5±210ab	622.8±242.3a	**	0.12
trans-S-(1-Propenyl)-L-cysteine (S1PC)	<LOD	<LOQ	<LOQ	38.1±19.7ab	439.7±180.1ab	477.9±199.8a	**	0.10
S-Propyl-L-cysteine (Deoxypropiin)	<LOD	273.2±40.7bc	195.4±25.4c	218.6±46.6bc	607±139.7b	1294.2±252.5a	***	0.26
Methionine sulfoxide	<LOD	46.6±7.6c	68.2±12.5c	107.3±19.4bc	223.9±39.7b	446±79.2a	***	0.09
S-Ethyl-L-cysteine sulfoxide (Ethiin)	<LOD	12.6±3.7c	6.1±1.6c	14.9±4.9c	48.3±8b	81.9±18.2a	***	0.02
S-Allyl-L-cysteine sulfoxide (Alliin)	<LOD	20.3±4	30.7±8.5	19.2±3.3	273.1±255.9	343.3±271.7	ns	0.07
S-(1-Propenyl)-L-cysteine sulfoxide (Isoalliin)	<LOD	168.7±35.4	350.9±74.6	282.7±86.3	2225.2±1937.3	3027.5±2133.6	ns	0.61
Cycloalliin	<LOD	22.4±3.2bcd	41.6±6.1b	32.4±7.2bc	9.6±1.7cd	106±18.1a	***	0.02
S-(2-Carboxypropyl-L-cysteine-glycine	<LOD	50.1±20.4bc	17.3±5.5bc	39.4±15.7bc	99.3±16.5b	206±58.1a	***	0.04
S-Carboxymethyl-L-cysteine (Carbocysteine)	<LOD	11.3±1.9c	10.4±1.8c	15.8±3c	49±10.4b	86.6±17a	***	0.02
S-Propyl-L-cysteine sulfoxide (Propiin)	<LOD	36.1±9.2c	26.2±5.3c	39.6±6c	99.1±15.9b	201±36.4a	***	0.04

S-Butanoyl-L-cysteine sulfoxide (Butiin)	<LOD	2.2±0.8bc	3.8±2.7bc	0.9±0.7c	18.2±6.6ab	25±10.8a	***	0.01
S-Allylmercaptoglutathione	<LOD	<LOQ	<LOQ	<LOQ	0.3±0.2ab	0.5±0.3a	**	0.00
S-(2-Carboxypropyl)-L-cysteine	<LOD	73.1±14.8	51±9.5	65.8±10.3	2009±1878.1	2198.8±1912.8	ns	0.44
S-(2-Propenyl)-allyl-L-cysteine sulfoxide	<LOD	28.8±7.8b	13±3.9b	5.4±4.7b	31.3±15.1b	78.6±31.5a	***	0.02
N-Acetyl-S-allyl-L-cysteine (NASAC) (Allymercapturic Acid)	<LOD	<LOQ	0.7±0.4a	0.2±0.1a	0.4±0.3a	1.3±0.8a	*	0.00
N-Acetyl-S-allyl-l-cysteine sulfoxide (NASACS)	<LOD	18.3±8.1	19±10.2	24.4±8.5	132.6±100.6	194.3±127.3	ns	0.04
N-Acetyl-S-(2-carboxypropyl) cysteine (NACPC)	<LOD	5.4±1.5c	1.5±0.7c	3.2±1.7c	16.4±2.8b	26.5±6.6a	***	0.01
Allixin	<LOD	<LOQ	0.5±0.5ab	1.3±0.7ab	0.1±0.1ab	1.9±1.3a	*	0.00
Ajoene	<LOD	1±0.4b	0.8±0.2b	2.9±0.5b	4.4±1.8ab	9.1±2.9a	***	0.00
Total SAk	**<LOD**	**1193±249a**	**1205±246a**	**1435±337a**	**9028±5738a**	**12862±6571a**	*****	**2.58**
Total Organosulfur Compounds	**<LOD**	**1243±264ab**	**1231±254ab**	**1490±354.2ab**	**9232±5784ab**	**13197±6657a**	*****	**2.65**

*Results are expressed as mean ± SE (standard error). LOD: Limit of Detection; LOQ: Limit of Quantification. Different letters (one-way ANOVA) denote significant differences (p < 0.05) among the stages for the same compound. Ns, non-significant; * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001.*

Among the organosulfur compounds present in urine, N-acetyl-*S*-allyl-L-cysteine (NASAC), N-acetyl-*S*-allyl-L-cysteine sulfoxide (NASACS) and N-acetyl-*S*-(2-carboxypropyl)-L-cysteine (NACPC) are the N-acetylated forms of alliin, SAC and *S*-(2-carboxypropyl)-L-cysteine, respectively. NASAC and NASACS have been previously described as urinary biomarkers of garlic consumption and are considered to be metabolites of SAC. This was observed by Nagae et al 1994, who administered SAC orally and intravenously to rats, mice and dogs, and subsequently determined the concentration of this compound and its metabolites (NASAC and NASACS) in plasma, organs, urine and bile (Nagae et al., 1994). Moreover, Verhagen et al. 2001 conducted a human study in which it was shown that those who took the garlic-supplemented diet had NASAC in their urine, while it was not found in the control diet volunteers (Verhagen et al., 2001). However, these compounds have a complex metabolism. Amano et al. (2015) (Amano et al., 2015)showed that N-acetylation of these compounds occurred in liver and kidney by amino acid transported located in the intestinal lumen of mammalian species (Bröer, 2008; Munck & Munck, 1994). In this sense, they evaluated the N-acetylation activity of the liver and kidney of rats and dogs, observing that in rats N-acetylation occurred to a greater extent, while in dogs, the kidney showed significantly higher NASACS deacetylation activity than N-acetylation. The results obtained in our study suggest that significant deacetylation activity can also be produced in human liver and kidney, since the concentration of SAC (622.8±242.3 nmol) found in urine after 24 hours was significantly higher than that of NASAC (1.3±0.8 nmol) and NASACS (194.3±127.3 nmol). It can be explained taking into account that amino acids are actively reabsorbed from the urine via several types of transporters in the kidney (Bröer, 2008; Silbernagl et al., 1975). Additionally, SAC could be present in urine due to the degradation of more complex molecules such as GSAC, GSACS, alliin or S-(2-carboxypropyl) cysteine, as was described by Yamaguchi et al. 2020 (Yamaguchi & Kumagai, 2020).

Furthermore, Praticò et al. (Praticò et al., 2018) in their review reported a human study in which a significant increase in urinary N-acetyl-*S*-(2-carboxypropyl)-L-cysteine (NACPC) content was observed after ingestion of garlic and onion, being this compound formed by the N-acetylation of *S*-(2-carboxypropyl)-L-cysteine, and considered as a biomarker for the consumption of *Allium* vegetables.

A potential pathway for the in vivo metabolism of organosulfur compounds in humans is summarized in Figure 3. The parent compound could be γ-glutamyl-*S*-(2-carboxypropyl)-L-cysteine-glycine, present in black garlic, which after hydrolysis can lose glutamic acid or glycine, forming *S*-(2-carboxypropyl)-L-cysteine-glycine or γ-glutamyl-*S*-(2-

carboxypropyl)-L-cysteine, respectively. Both compounds can potentially be degraded by a second hydrolysis to *S*-(2-carboxypropyl)-L-cysteine, one of the main organosulfur compounds found in urine and a precursor of NACPC, via the N-acetylation reaction.

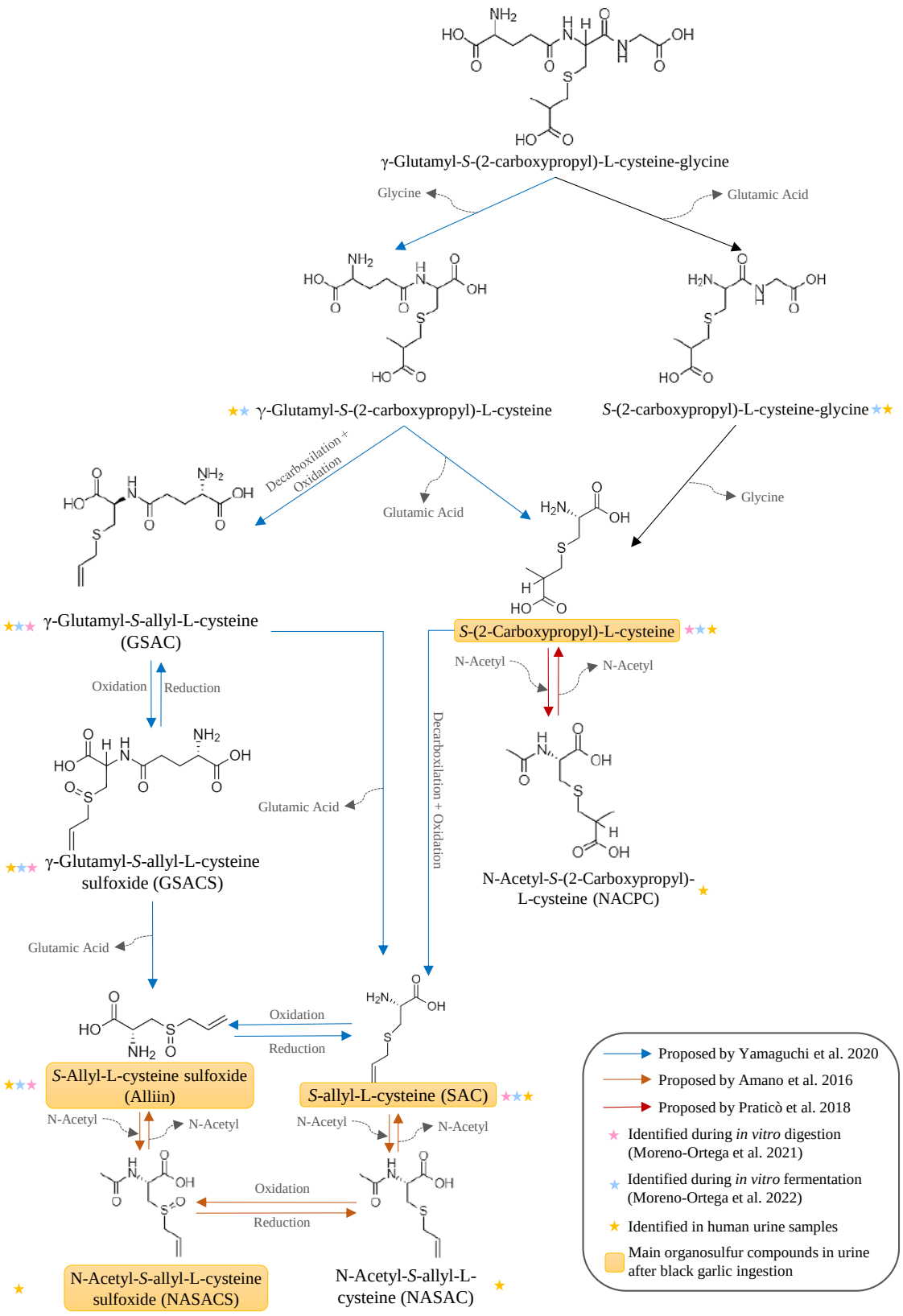

Figure 3.- Potential pathway for the metabolism of organosulfur compounds in the gastrointestinal tract after black garlic consumption. The proposed metabolism is in keeping with the findings of Yamaguchi et al. 2020, Amano et al. 2015 and Praticò et al. 2018 who monitored the metabolism of SAC in animal species. Boxed compounds indicate main components excreted in urine after black garlic intake (Amano et al., 2015; Praticò et al., 2018; Yamaguchi & Kumagai, 2020).

Similarly, potential degradation pathways for other organosulfur compounds have been proposed that lead to the formation of major compounds excreted in urine such as methiin and deoxypropiin (Figure 4 and 5). Methiin can be formed from the oxidation of GSMC to GSMCS and subsequent hydrolysis releasing glutamic acid or via elimination of glutamic acid from GSMC to obtain deoxymethiin and subsequent oxidation (Figure 4). Furthermore, methiin can also be N-acetylated resulting in N-acetyl-*S*-methyl-L-cysteine sulfoxide (NASMCS), previously determined in human urine samples after consumption of cruciferous vegetables (Edmands et al., 2011), although this has not been detected in our analysis. Indeed, methiin and NASCMCS can also be considered biomarkers of allium and cruciferous vegetable consumption by their presence in urine (Edmands et al., 2011).

Regarding the significant presence of deoxypropiin in urine, it can be formed after hydrolysis of γ-glutamyl-*S*-propyl-L-cysteine-glycine, which releases glycine and γ-glutamyl-*S*-propyl cysteine (GSPC). GSPC can be oxidised to γ-glutamyl-*S*-propyl-L-cysteine sulfoxide (GSPCS) or hydrolysed to *S*-propyl-L-cysteine (deoxypropiin). Both compounds are precursors of *S*-propyl-L-cysteine sulfoxide (propiin), from hydrolysis of GSPCS and oxidation of deoxypropiin (Figure 5). It is possible that due to the nature of these molecules and their chemical structures, N-acetylation reactions of propiin and deoxypropiin could take place, however they have not been described to date and have not been found in our samples.

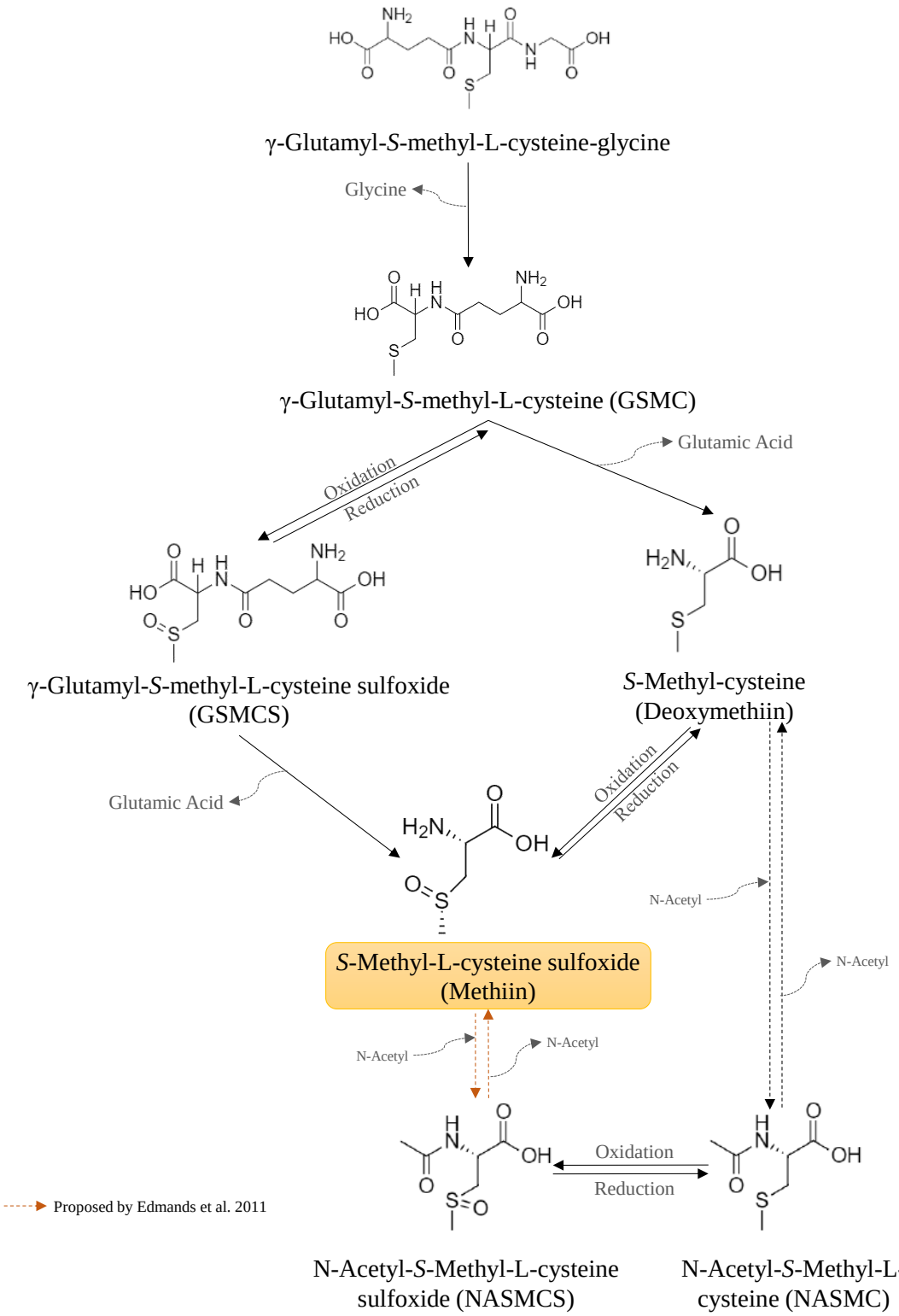

Figure 4.- Degradation pathway proposed of γ-Glutamyl-S-methyl-L-cysteine-glycine based on the reports of Edmands et al. 2011 (Edmands et al., 2011). *Boxes compounds indicate main components excreted in urine after black garlic intake.*

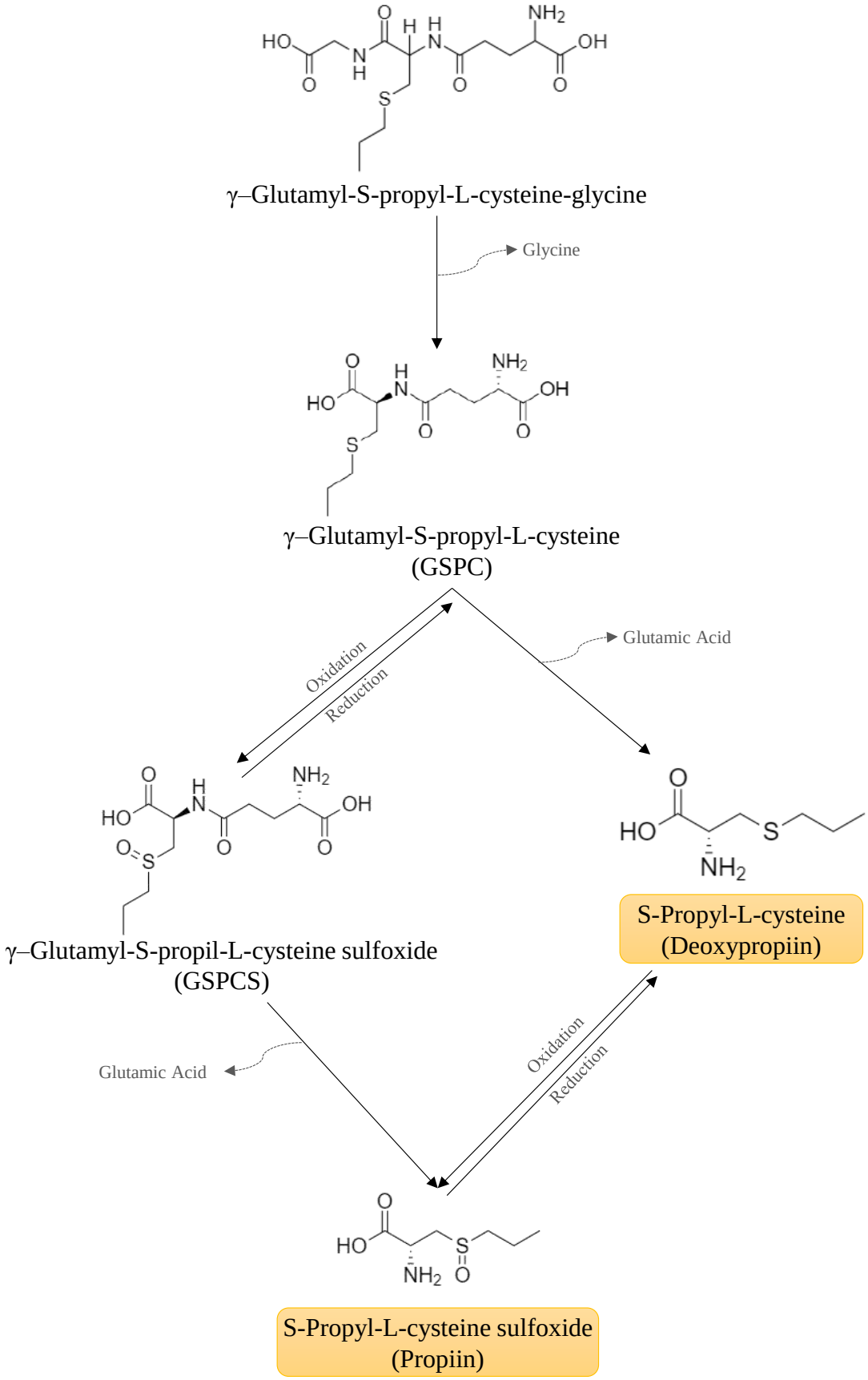

Figure 5.- Degradation pathway proposed for γ–Glutamyl-S-propyl cysteine-glycine. Boxes compounds indicate main components excreted in urine after black garlic intake.

However, the information available on the physiological transformations of these compounds is yet very limited. Fortunately, the identification of these urinary metabolites of organosulfur compounds after ingestion of black garlic can promote the study of the role of this product in human health and further the comprehension of the metabolism, distribution and excretion of these compounds.

4. Conclusions

Considering the limited information available, this study aims to identify and quantify the organosulfur compounds excreted in human urine 24 h after the acute intake of black garlic. In total, the excretion of organosulfur compounds 24 hours after the ingestion of black garlic was 13197±6657 nmol, equivalent to 2.65% of the 498.8 µmol intake of organosulfur compounds, being the 97.5% SAk derivatives and 2.5% GSAk derivatives. Thirty-three organosulfur compounds were identified and quantified in urine 24 hours after ingestion, being the major ones methiin (3433±1150 nmol), isoalliin (3027±2133 nmol), S-(2-carboxypropyl)-L-cysteine (2199±1913 nmol) and S-propyl-L-cysteine (deoxypropiin) (1294±253 nmol). Additionally, N-acetyl-S-allyl-L-cysteine (NASAC), N-acetyl-S-allyl-L-cysteine sulfoxide (NASACS) and N-acetyl-S-(2-carboxypropyl)-L-cysteine (NACPC) were detected in urine samples, which are recognised metabolites of S-allyl-L-cysteine (SAC), alliin and S-(2-carboxypropyl)-L-cysteine, respectively. These compounds are potentially N-acetylated in liver and kidney and metabolic degradation pathways have been proposed in order to explain the presence of the major compounds in urine. The identification of these organosulfur compounds as urinary metabolites of black garlic ingestion provides opportunities to study the role of black garlic in human health, and also the basis for further evaluation of the biological role and health potential of these secondary plant metabolites in humans.

Ethics statement

The protocol of the study was approved by the Ethical Committee of the Human Clinical Research Unit at the Arnau Vilanova University Hospital, Lleida, Spain (Approval Number: CEiC-1790)

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Conflict of interest: There is no conflict of interest.

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Artículo 8

Urinary Organosulfur Compounds Excretion After Acute Ingestion of Black Onion by Humans

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Urinary Organosulfur Compounds Excretion After Acute Ingestion of Black Onion by Humans

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Abstract

Onion (*Allium cepa* L.) and its derivative, black onion, are characterised by the presence of compounds with potential bioactivity, particularly polyphenols and organosulfur compounds. Nevertheless, the information about metabolization, distribution and excretion of organosulfur compounds is still limited. This study aims to monitor the human urinary excretion of organosulfur compounds after the acute intake of black onion by ultra-high-performance liquid chromatography coupled to a high-resolution mass spectrometry (UHPLC-HRMS) analysis. The excretion of total organosulfur compounds during 24 hours after the ingestion of black onion was 6572 ± 1835 nmol, equivalent to 0.49% of the 1329 µmol intake of organosulfur compounds. Thirty organosulfur compounds were detected in human urine after the acute ingestion of black onion, being the main ones isoalliin (1891 ± 681 nmol), *S*-methyl-L-cysteine sulfoxide (methiin) (1657 ± 340 nmol) and *S*-propyl-L-cysteine (deoxypropiin) (680 ± 169 nmol). Moreover, the potential N-acetylated metabolites of the major compounds in black onion, N-acetyl-*S*-(1-propenyl)-L-cysteine sulfoxide (NAS1PCS), N-acetyl-*S*-(1-propenyl)-L-cysteine (NAS1PC), have been found in urine. The N-acetylation reaction takes place in the kidney and liver, and metabolization pathways have been proposed to explain the excretion of some of these organosulfur compounds. The identification of these organosulfur compounds as urinary metabolites of black onion ingestion provides opportunities to assess the role of this product in human health.

Keywords: black onion, bioavailability, human urine, organosulfur compounds.

1. Introduction

Black onion is a product derived from onion, which is obtained by subjecting fresh onion to controlled temperature and humidity conditions. During this process, transformations in the product take place, resulting in physico-chemical and sensory characteristics that are significantly different from the original product. In this sense, Moreno-Ortega et al. (2020) monitored the evolution of the selected compounds throughout the black onion production process, reporting an up to 8-fold increase in the fructose content, a decrease in the total polyphenol content and a significant increase in the content of *S*-alk(en)yl-L-cysteine derivatives [1]. In terms of organoleptic properties, black onion is characterised by a sweet taste and low pungency, a soft consistency and a dark colour. Previous studies have related the regular consumption of organosulfur compounds to a protective effect against several diseases such as some types of cancer [2, 3], cardiovascular and neurodegenerative diseases [4, 5] and metabolic syndrome [6]. Nevertheless, the health potential of regular consumption of black onion, and thus of the phytochemicals present in it, has not yet been evaluated.

Previous *in vitro* studies have provided information on the bioaccessibility of phytochemical compounds in black onion, as well as on the evolution of these compounds during fermentation at the colonic level. In this sense, Moreno-Ortega et al. 2021 [7] reported higher bioaccessibility indexes of phenolic (41%) and organosulfur compounds (83%) in black onion than in fresh onion. In fact, organosulfur compounds in black onion showed higher stability throughout *in vitro* gastrointestinal digestion, with γ-glutamyl-*S*-methyl cysteine sulfoxide (GSMCS) (160%), methionine sulfoxide (169%) and isoalliin (83%) showing the highest bioaccessibility indexes. Additionally, Moreno-Ortega et al. (2022) reported that 3,4-dihydroxybenzoic acid, 4-hydroxyphenylacetic acid, methionine sulfoxide and *S*-propylmercapto-L-cysteine (SPMC) were the main phenolic and organosulfur compounds that remained stable after 24h of colonic fermentation and could be ready for absorption, reaching the target tissues via systemic circulation. Regarding this, studies have been conducted in which the bioavailability of fresh onion polyphenols was assessed, with glucuronide and sulphated conjugates of quercetin being among the main metabolites in plasma and urine after ingestion of fried onion [8, 9]. Moreover, onion quercetin has been shown to be more bioavailable than quercetin from supplementation [10, 11]. Nevertheless, to the best of our knowledge, there are no studies evaluating the bioavailability of organosulfur compounds after ingestion of black onion.

The aim of the present study was to evaluate the bioavailability via monitoring the 0-24 h urinary excretion of organosulfur compounds in urine after ingestion of black onion by ultra-high performance liquid chromatography analysis coupled to a high-performance mass spectrometer (UHPLC-HRMS).

2. Materials and methods

2.1. Chemicals

The reference standard compounds including *S*-allyl-L-cysteine sulfoxide (aka alliin) and *S*-allyl-L-cysteine (SAC) were purchased from Sigma-Aldrich (Madrid, Spain). Ammonium acetate, ammonium formate and ethanol were obtained from Sigma-Aldrich. Acetonitrile, methanol and water were of LC-MS grade.

2.2. Materials

Fresh and black onion were kindly provided by a local supplier Abuela Carmen®, Córdoba, Spain.

2.3. Study design

Twelve healthy volunteers (seven females and five males, mean age 29.0 ± 8.9 years) with a body mass index (BMI) of 23.5 ± 2.2 kg/m^2 (mean ± SD) participated in this study. Exclusion criteria were pregnancy or lactation, drug allergies, any chronic medication and any antibiotic treatment during the 6 months prior to the study. Written informed consent was provided by all participants. The study protocol was approved by the Ethical Committee of the Human Clinical Research Unit at the Arnau Vilanova University Hospital, Lleida, Spain (Approval Number: CEiC-1790).

Before attending the clinic, participants were asked to follow a diet absent of organosulfur compounds for two days by avoiding *Allium* vegetables. On the morning of the feeding trial, the volunteers brought their 12 h overnight urine (basal) and were invited to eat a portion of 20 g of black onion after fasting overnight and to continue with the organosulfur-free diet that day and until the next morning, when the last urine sample was collected. Human urine samples were obtained at the interval times of 0-2, 2-4, 4-8, 8-24 h after the black onion intake. The total volume of each sample was recorded before storing the aliquots (2 mL) at -80ºC until the chromatographic analysis.

2.4. Processing of urine samples

Urine samples were defrosted, vortexed, centrifuges at 15000 rpm for 10 min at 4ºC and the supernatant was collected and stored at -80ºC until UHPLC-HRMS analysis. Each sample was prepared in duplicate.

2.5. Analysis of phenolic and organosulfur compounds in urine samples

Aliquots of human urine samples were analysed using a UHPLC-PDA-MS mass spectrometer system (Thermo Scientific, San José, CA, USA) comprising of a UHPLC pump, a PDA detector scanning from 200 to 600 nm, and an autosampler operating at 4 °C (Dionex Ultimate 3000 RS, Thermo Corporation). The chromatographic conditions were previously described by Moreno-Rojas et al. (2018) [12]. Briefly, separation of organosulfur compounds was performed on a 2.1 × 150 mm ACQUITY UPLC 1.7 µm BEH amide column (equipped with an ACQUITY UPLC BEH amide 1.7 µm van-guard pre-column) (Waters, Spain), which was maintained at 35 °C and eluted at a flow rate of 0.4 ml/min with a 20 min using two mobile phases: A: deionized water with 5 mM of ammonium acetate, 5 mM ammonium formate and 1% formic acid and B: acetonitrile. The gradient started with 5% of A rising 46% in 13 min and finally return to 5% of A and was maintained 4 min to equilibrate the column.

After passing through the flow cell of the PDA detector, the column eluate was directly to an Exactive Orbitrap mass spectrometer (Thermo Scientific, San José, CA, USA) fitted with a Heated Electrospray Ionization Probe (HESI) operating in positive ionization mode for the determination of organosulfur compounds [12]. Data acquisition and processing were carried out using Xcalibur 3.0 software.

Identification of organosulfur compounds in human urine samples was achieved by comparing the exact mass and the retention time with available standards. In the absence of standards, compounds were tentatively identified by comparing the exact theoretical mass of the molecular ion with the measured accurate mass of the molecular ion and searched against metabolite databases, including Metlin, Human Metabolome DataBase (HMDB), and more general chemical databases, such as PubChem and ChemSpider. The quantification of phenolic compounds and organosulfur compounds was carried out by selecting the exact theoretical mass of the molecular ion by reference to standard curves, obtaining a linear regression analysis with R^2 values of >0.998 (n = 6). In the absence of reference compounds, they were quantified by reference to the calibration curve of a closely related parent compound.

2.6. Statistical analysis

A one-way ANOVA was carried out using R software (v.3.6.3., R Core Team, Vienna, Austria) to determine significant differences between the human urine samples at different interval times after intake of black onion. The significance being accepted for a *p*-value < 0.05. Next, Fisher's LSD pairwise comparison was performed on the data.

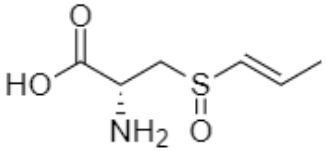
3. Results and discussion

3.1. Organosulfur Compound Analysis in Black Onion

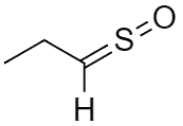
A total of nine organosulfur compounds were identified and quantified in black onion (Table 1). The details for their identification and quantification have been reported before by our group [12]. The main organosulfur compounds were isoalliin (1062 µmol), accounting for the 79.9% of the total organosulfur compounds, followed by propanethial sulfoxide (213 µmol) (Table 1). Small quantities of others S-alk(en)yl-L-cysteine derivatives, such as methionine sulfoxide (21.5 µmol), methiin (3.62 µmol), S-propylmercapto-L-cysteine (SPMC) (3.56 µmol) and propiin (1.66 µmol) were also present along with other γ-glutamyl-S-alk(en)yl-L-cysteine derivatives including γ-glutamyl-S-(1-propenyl)-L-cysteine sulfoxide (G1PCS) (17.2 µmol), γ-glutamyl-S-methyl-L-cysteine sulfoxide (GSMCS) (2.30 µmol) and γ-glutamyl-S-propyl-L-cysteine sulfoxide (GSPCS) (1.2 µmol). The 20 g of black onion consumed by the volunteers contained in total 1329 µmol of organosulfur compounds, of which 20.8 µmol corresponded to the total amount of γ-glutamyl-S-alk(en)yl-L-cysteine derivatives (≈ 2% of the total organosulfur content) and 1308 µmol to S-alk(en)yl-L-cysteine derivatives (≈ 98% of the total organosulfur content). Figure 1 shows the chemical structures of the main organosulfur compounds found in black onion.

Table 1.- Organosulfur compounds identified and quantified in black onion samples

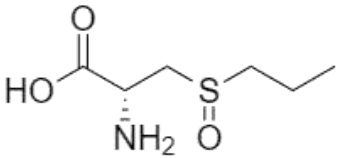
Black Onion	µmol per 20 g Black Onion	% in Black Onion
γ-Glutamyl-*S*-alk(en)yl-L-cysteine derivatives (GSAk)		
γ-Glutamyl-S-methyl-L-cysteine sulfoxide (GSMCS)	2.30±0.00	0.2
γ-Glutamyl-S-(1-propenyl)-L-cysteine sulfoxide (G1PCS)	17.22±0.08	1.3
γ–Glutamyl-S-propyl-L-cysteine sulfoxide (GSPCS)	1.24±0.01	0.1
Total GSAk	20.76±0.09	1.6
***S*-alk(en)yl-L-cysteine derivatives (SAk)**		0.0
S-Propylmercapto-L-cysteine (SPMC)	3.56±0.01	0.3
S-(1-propenyl)-L-cysteine sulfoxide (Isoalliin)	1,062.34±6.56	79.9
Propanethial sulfoxide (Lacrimatory factor)	213.26±1.17	16.0
S-Methyl-L-cysteine sulfoxide (Methiin)	3.62±0.02	0.3
S-Propyl-L-cysteine sulfoxide (Propiin)	1.66±0.00	0.1
Methionine sulfoxide	21.46±0.13	1.6
Total SAk	1,305.90±7.80	98.3
Total Organosulfur Compounds	1,329.04±7.89	100.0



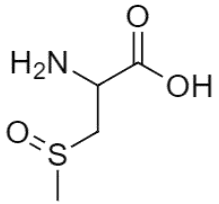
S-(1-Propenyl)-L-cysteine sulfoxide (Isoalliin)



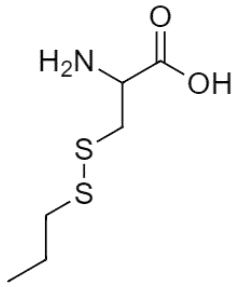
Propanethial sulfoxide



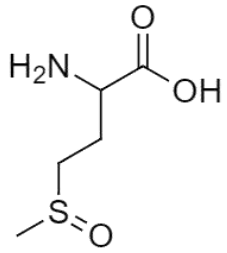
S-Propyl-L-cysteine sulfoxide (Propiin)



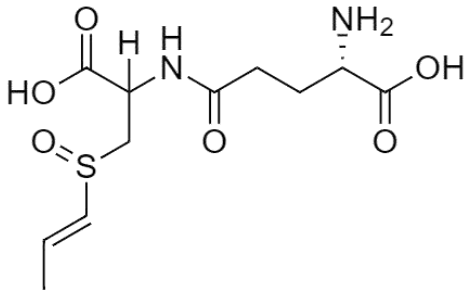
S-Methyl-L-cysteine sulfoxide (Methiin)



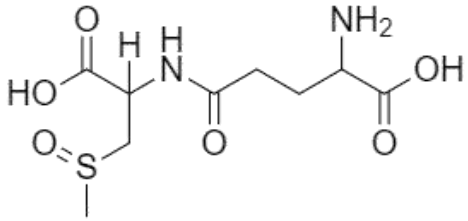
S-Propylmercapto-L-cysteine (SPMC)



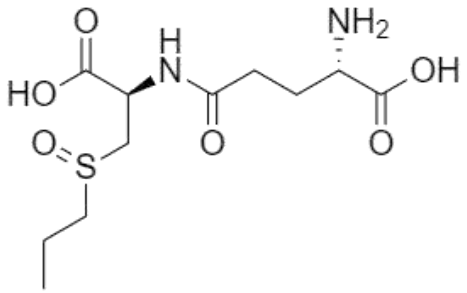
Methionine Sulfoxide



γ-Glutamyl-*S*-(1-propenyl)-L-cysteine sulfoxide (GS1PCS)



γ-Glutamyl-*S*-methyl-L-cysteine sulfoxide (GSMCS)



γ–Glutamyl-*S*-propy-L-cysteine sulfoxide (GSPCS)

Figure 1.- Chemical structures of the main organosulfur compounds identified in black onion

3.2. Excretion of organosulfur compounds in urine after black garlic consumption

In this study the bioavailability of organosulfur compounds was investigated by using UHPLC-HRMS to analyse urine collected after 24 h of the acute ingestion of 20 g of black onion. The analysis of the urine led to the identification of 31 organosulfur metabolites, being 12 γ-glutamyl-S-alk(en)yl-L-cysteine derivatives (GSAk) and 19 S-alk(en)yl-L-cysteine derivatives (SAk) (Table 2). The details of organosulfur compounds identification and quantification are presented in the Table 2.

Table 3 summarizes quantitative aspects of the urinary excretion of organosulfur compounds. No organosulfur compounds were detected in urine collected over a 12 h period before black onion consumption. After the ingestion of black onion, isoalliin (1891 ± 681 nmol), *S*-methyl-L-cysteine sulfoxide (methiin) (1657 ± 340 nmol) and *S*-propyl-L-cysteine (deoxypropiin) (680 ± 169 nmol) were the principal urinary metabolites, followed by *S*-allyl-L-cysteine (SAC) (381 ± 101 nmol), trans-*S*-(1-propenyl)-L-cysteine (S1PC) (366 ± 110 nmol) and methionine sulfoxide (351 ± 65 nmol), *S*-(2-carboxypropyl)-L-cysteine (233 ± 48 nmol), *S*-propyl-L-cysteine sulfoxide (propiin) (199 ± 65 nmol), cycloalliin (133 ± 38 nmol), N-acetyl-*S*-(1-propenyl)-L-cysteine sulfoxide (NAS1PCS) (126 ± 42 nmol) and γ −glutamyl-*S*-methyl-L-cysteine (GSMC) (123 ± 42 nmol) (Table 3). Some of these metabolites including isoalliin, methionine sulfoxide and SAC were found when digested black garlic were incubated with human faeces during 24 h (Moreno-Ortega et al. 2022). Overall, the total organosulfur compounds excreted in urine within 24 hours after ingestion of black onion were 6572±1835 nmol, accounting for the 0.49% of intake from the 1329 µmol of ingested organosulfur compounds (Table 3).

Table 2.- UHPLC-HRMS-based identification of phenolic and organosulfur compounds detected in human urine samples after black onion ingestion

Peak	Retention Time (min)	Compound	Chemical Formula $[m/z]^-$	Experimental Mass $[m/z]^-$	δ (ppm)	MSI MI level[a]
		γ-Glutamyl-*S*-alk(en)yl-L-cysteine derivatives (GSAk)				
1	4.34	γ −Glutamyl-*S*-(propyl)-L-cysteine sulfoxide	C11H21N2O6S	309.1115	-2.1191	2
2	6.34	γ −Glutamyl-*S*-(*S*-1-propenyl)-L-cysteine-glycine	C13H22N3O6S2	380.0944	0.8362	2
3	6.43	γ −Glutamyl-*S*-(2-carboxypropyl)-L-cysteine	$C12H19N2O7S^{-2}$	335.0907	-0.0579	2
4	7.86	γ −Glutamyl-*S*-(*S*-methyl)-L-cysteine-glycine	C11H20N3O6S2	354.0788	0.6916	2
5	7.88	γ −Glutamyl-*S*-methyl-L-cysteine (GSMC)	C9H17N2O5S	265.0853	0.1777	2
6	8.53	Glutathione	C10H18N3O6S	308.0911	0.7967	2
7	8.55	γ −Glutamyl-*S*-allyl-L-cysteine (GSAC)	C11H19N2O5S	291.1009	0.4285	2
8	8.71	γ −Glutamyl-L-cysteine	C8H15N2O5S	251.0696	-0.9444	2
9	9.20	γ −Glutamyl-*S*-(1-propenyl)-L-cysteine sulfoxide (G1PCS)	C11H19N2O6S	307.0958	-0.4247	2
10	9.60	γ −Glutamyl-*S*-methyl-L-cysteine sulfoxide (GSMCS)	C9H17N2O6S	281.0801	-0.8092	2
11	9.60	γ −Glutamyl-*S*-(propyl)-L-cysteine	C11H21N2O5S	293.1165	-0.3883	2
12	10.00	γ −Glutamyl-*S*-allyl-L-cysteine sulfoxide	C11H19N2O6S	307.0958	-1.0128	2
		***S*-alk(en)yl-L-cysteine derivatives (SAk)**				
13	1.39	*S*-(2-Propenyl)-allyl-L-cysteine sulfoxide	C9H16NO3S	218.0845	-1.8770	2
14	4.34	N-Acetyl-*S*-(1-propenyl)-L-cysteine sulfoxide (NAS1PCS)	C8H14NO4S	220.0638	-1.2877	2
15	3.96	*S*-Butanoyl-L-cysteine sulfoxide (Butiin)	C7H16NO3S	194.0845	-0.8589	2
16	4.53	N-Acetyl-*S*-(1-propenyl)-L-cysteine (NAS1PC)	C8H14NO3S	204.0689	-1.9576	2
17	4.87	*S*-Allyl-L-cysteine (SAC)	C6H12NO2S	162.0583	-1.0605	1
18	5.03	trans-*S*-(1-Propenyl)-L-cysteine (S1PC)	C6H12NO2S	162.0583	-0.5898	2
19	5.60	*S*-Propyl-L-cysteine (Deoxypropiin)	C6H14NO2S	164.0740	-4.2089	2
20	6.18	*S*-Allyl-L-cysteine sulfoxide (Alliin)	C6H12NO3S	178.0532	1.5374	1
21	6.70	*S*-(1-Propenyl)-L-cysteine sulfoxide (Isoalliin)	C6H12NO3S	178.0532	2.5793	2
22	7.10	Cycloalliin	C6H12NO3S	178.0532	2.3222	2
23	7.13	*S*-(2-Carboxypropyl) cysteine	C7H14NO4S	208.0638	1.7404	2

24	7.26	*S*-Methyl-L-cysteine sulfoxide (Methiin)	C4H10NO3S	152.0376	3.5869	2
25	7.56	Methionine sulfoxide	C5H12NO3S	166.0532	-0.6264	2
26	7.87	*S*-Propyl-L-cysteine sulfoxide (Propiin)	C6H14NO3S	180.0689	2.4960	2
27	8.09	*S*-Carboxymethyl-L-cysteine (Carbocysteine)	C5H10NO4S	180.0325	2.5441	2
28	8.43	*S*-(2-Carboxypropyl)-L-cysteine-glycine	C9H17N2O5S	265.0853	0.6382	2
29	8.55	*S*-Ethyl-L-cysteine sulfoxide (Ethiin)	C5H12NO3S	166.0532	-0.0752	2
30	9.58	N-Acetyl-*S*-(2-carboxypropyl)-L-cysteine (NACPC)	C9H16NO5S	250.0744	2.5686	2
31	9.74	S-Allylmercaptoglutathione	C13H22N3O6S2	380.0945	0.2443	2

**Metabolite standards initiative (MSI) metabolite identification (MI) levels* [13]. *Reference compounds were available for all compounds identified at MSI MI level 1.*

Table 3.- Quantification of organosulfur compounds found in human urine samples after acute intake of black garlic

Black Onion (nmol)	Basal 24h	0-2h	2-4h	4-8h	8-24h	0-24h	p-value	% Excretion
γ-Glutamyl-*S*-alk(en)yl-L-cysteine derivatives (GSAk)								
Glutathione	<LOD	4.42±1.85ab	2.22±1.72b	1.28±0.53b	7.73±2.9ab	15.64±6.99a	**	0.00
γ −Glutamyl-L-cysteine	<LOD	2.21±0.54bc	0.39±0.18c	1.18±0.6c	6.42±1.87ab	10.21±3.19a	***	0.00
γ −Glutamyl-S-methyl-L-cysteine (GSMC)	<LOD	14.6±5.4bc	2.4±0.3c	9.2±2.3c	96.5±33.8ab	123±42a	***	0.01
γ −Glutamyl-S-methyl-L-cysteine sulfoxide (GSMCS)	<LOD	4.55±0.79b	1.24±0.47b	2.91±1.3b	28.52±7.85a	37.21±10.42a	***	0.00
γ −Glutamyl-S-allyl-L-cysteine (GSAC)	<LOD	1.54±0.45bc	0.64±0.23c	0.92±0.4bc	3.28±0.91b	6.37±1.99a	***	0.00
γ −Glutamyl-S-(propyl)-L-cysteine	<LOD	2.09±0.8b	0.47±0.1b	1.42±0.58b	7.55±2.89ab	11.53±4.38a	***	0.00
γ −Glutamyl-S-(1-propenyl)-L-cysteine sulfoxide (G1PCS)	<LOD	1.43±0.4b	0.27±0.1b	0.52±0.21b	3.34±1.3ab	5.55±2.02a	***	0.00
γ −Glutamyl S-allyl L-cysteine sulfoxide (GSACS)	<LOD	0.16±0.1b	0.02±0.01b	0.06±0.05b	0.35±0.12ab	0.59±0.28a	***	0.00
γ −Glutamyl-S-(propyl)-L-cysteine sulfoxide	<LOD	5.53±4.11	1.17±1.02	2.44±1.94	3.56±1.67	12.7±8.73	ns	0.00
γ −Glutamyl-S-(2-carboxypropyl)-L-cysteine	<LOD	0.73±0.15b	0.18±0.05c	0.22±0.08bc	0.33±0.18bc	1.46±0.46a	***	0.00
γ −Glutamyl-S-(S-methyl)-L-cysteine-glycine	<LOD	10.27±1.99bc	3.48±1.18c	9.45±1.56bc	21.89±3.96b	45.08±8.69a	***	0.00
γ −Glutamyl-S-(S-1-propenyl)-L-cysteine-glycine	<LOD	0±0	0.02±0.02	0±0	0.05±0.05	0.07±0.07	ns	0.00
Total GSAk	**<LOD**	**47.5±16.6b**	**12.5±5.4b**	**29.6±9.6b**	**180±56a**	**269±89a**	*******	**0.02**
***S*-alk(en)yl-L-cysteine derivatives (SAk)**								
S-Methyl-L-cysteine sulfoxide (Methiin)	<LOD	299.1±45bc	214.6±45.6c	318.2±68.5bc	825.3±180.7b	1657±340a	***	0.12

S-Ethyl-L-cysteine sulfoxide (Ethiin)	<LOD	6.44±1.08b	4.67±1.47b	5.54±2.03b	29.76±6.78a	46.41±11.37a	***	0.00
S-Propyl-L-cysteine sulfoxide (Propiin)	<LOD	31.3±7.9b	18±5.9b	30.9±8.2b	118.2±43.4ab	199±65a	***	0.01
S-Butanoyl-L-cysteine sulfoxide (Butiin)	<LOD	2.13±0.54bc	2.42±1.13bc	5.84±2bc	10.8±2.99b	21.19±6.67a	***	0.00
S-Propyl-L-cysteine (Deoxypropiin)	<LOD	191.3±47.5bc	35.9±9.8c	84.8±24.9bc	368±87b	680±169a	***	0.05
S-Allyl-L-cysteine sulfoxide (Alliin)	<LOD	3.62±0.7b	3.21±1.9b	2.81±0.96b	4.1±0.57b	13.74±4.13a	***	0.00
S-(1-propenyl)-L-cysteine sulfoxide (Isoalliin)	<LOD	341±92b	578±110b	536±224b	436±254b	1891±681a	***	0.14
Cycloalliin	<LOD	38.3±9.5b	39.6±8.5b	40.6±17.3b	14.3±3b	133±38a	***	0.01
S-Allyl-L-cysteine (SAC)	<LOD	18.7±5.1b	23.9±12.1b	49.5±18.8b	289±65a	381±101a	***	0.03
trans-S-(1-Propenyl)-L-cysteine (S1PC)	<LOD	13.9±6b	22.8±10.3b	50.7±23b	278±71a	366±110a	***	0.03
S-(2-Carboxypropyl)-L-cysteine	<LOD	43.7±6.7c	22.9±5.5c	41.1±7.5c	125±28b	233±48a	***	0.02
S-(2-Carboxypropyl)-L-cysteine-glycine	<LOD	16.5±3.9b	9.1±3.5b	12.2±4.8b	58.8±21.1ab	96.7±33.3a	***	0.01
S-Carboxymethyl-L-cysteine (Carbocysteine)	<LOD	8.5±1.7c	6.7±2.2c	10.7±2.8c	34.8±7.1b	60.7±13.7a	***	0.00
S-Allylmercaptoglutathione	<LOD	0.33±0.11b	0.06±0.03b	0.05±0.03b	0.52±0.18ab	0.95±0.35a	***	0.00
S-(2-Propenyl)-allyl-L-cysteine sulfoxide	<LOD	4.21±1.1a	1.29±0.78a	3.11±2.48a	13.3±7.69a	21.92±12.05a	*	0.00
N-Acetyl-S-(1-propenyl)-L-cysteine (NAS1PC)	<LOD	0.31±0.21b	0.5±0.3b	0.45±0.19b	1.11±0.47ab	2.37±1.17a	***	0.00
N-Acetyl-S-(1-propenyl)-L-cysteine sulfoxide (NAS1PCS)	<LOD	27.1±9.3b	33.5±15.2b	28.2±6.6b	37.2±10.9b	126±42a	***	0.01
N-Acetyl-S-(2-carboxypropyl)-L-cysteine (NACPC)	<LOD	4.92±0.82c	1.54±0.5c	3.06±0.9c	13.96±2.54b	23.48±4.76a	***	0.00
Methionine sulfoxide	<LOD	44.8±5.5c	60.4±19.2c	92.7±17bc	153±23b	351±65a	***	0.03
Total SAk	**<LOD**	**1096±245bc**	**1079±254c**	**1317±432bc**	**2810±815b**	**6303±1746a**	*******	**0.47**
Total Organosulfur Compounds	**<LOD**	**1144±261bc**	**1092±259c**	**1346±442bc**	**2990±873b**	**6572±1835a**	*******	**0.49**

*Results are expressed as mean ± SE (standard error). LOD: Limit of Detection; LOQ: Limit of Quantification. Different letters (one-way ANOVA) denote significant differences (p < 0.05) among the stages for the same compound. Ns, non-significant; * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001.*

The time-course profiles of the organosulfur compounds identified during the urinary excretion after the intake of black onion are shown in Figure 2. Regarding the total content of organosulfur compounds, 45% were excreted between 8 and 24 hours after ingestion of black onion, which means that the absorption of these compounds mainly occurs at the intestinal level. However, a significant percentage of organosulfur compounds can also be absorbed at the gastric level since 22% of GSAk derivatives and 34% of SAk derivatives have been excreted from 0 to 4 h. (Figure 2).

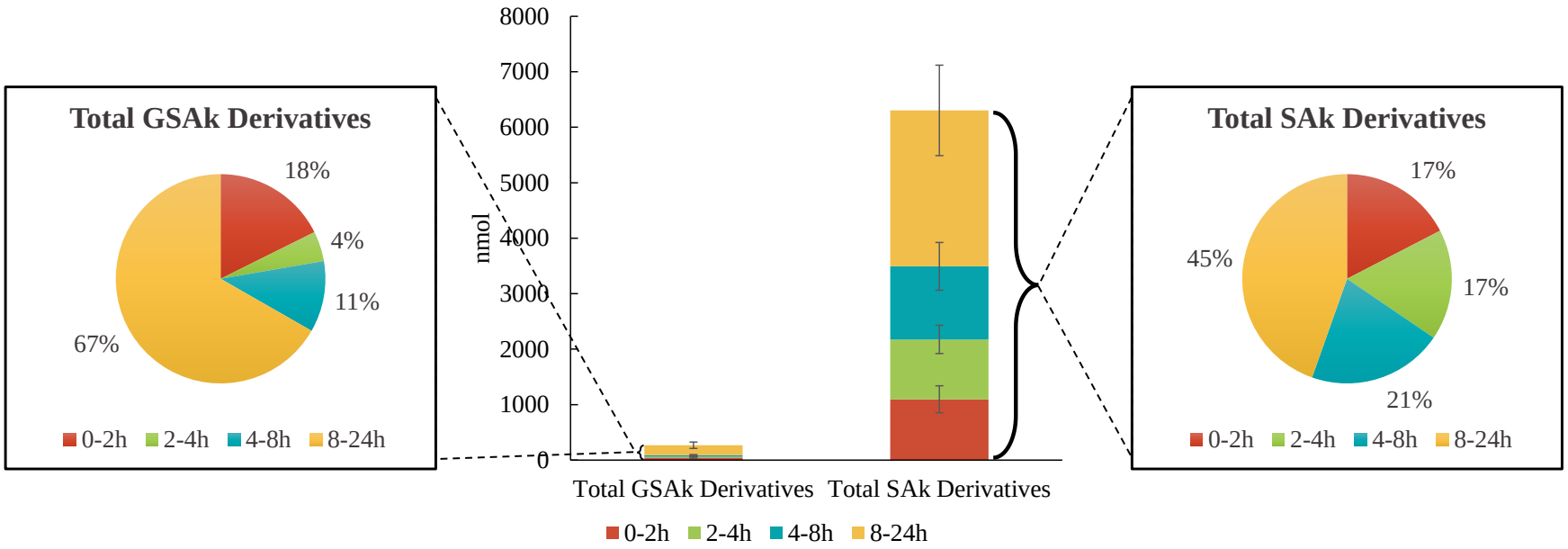


Figure 2.- Time-course profiles of the groups of organosulfur compounds identified during the urinary excretion after the intake of black onion. GSAk: γ-glutamyl-S-alk(en)yl-L-cysteine; SAk: S-alk(en)yl-L-cysteine.

It is interesting to note that compounds including N-acetyl-*S*-(1-propenyl)-L-cysteine (NAS1PC) and N-acetyl-*S*-(1-propenyl)-L-cysteine sulfoxide (NAS1PCS) have been identified for the first time in our study. They are formed tentatively from a N-acetylation of isoalliin and trans-*S*-(1-propenyl)-L-cysteine (S1PC), the main organosulfur compounds present in black onion. This acetylation reaction, which has been described to occur in the liver and kidney by amino acid transported located in the intestinal lumen of mammalian species [14, 15], has been previously described for SAC and alliin leading N-acetyl-*S*-allyl-L-cysteine (NASAC) and N-acetyl-*S*-allyl-L-cysteine sulfoxide (NASACS) being considered biomarkers of garlic consumption [16–18].

Based in our findings, a possible pathway for the *in vivo* metabolism of organosulfur compounds in humans that could potentially result in the formation of these biomarkers after consumption of black onion is depicted in Figure 3. Briefly, the precursor compound γ-glutamyl-*S*-(2-carboxypropyl)-L-cysteine-glycine of these biomarkers, described as one of the major compounds in fresh onion and present in black onion, suffer a hydrolysis that would result in the release of glycine or glutamic acid and the formation of γ-glutamyl-*S*-(2-carboxypropyl)-L-cysteine or *S*-(2-carboxypropyl)-L-cysteine-glycine, respectively. Both compounds, after hydrolysis releasing again a glycine or a glutamic acid and obtaining *S*-(2-carboxypropyl)-L-cysteine, the precursor of NACPC, as was described by Praticò et al. (2018) [19].

Alternatively, γ-glutamyl-*S*-(2-carboxypropyl)-L-cysteine can undergo decarboxylation and oxidation, releasing γ-glutamyl-*S*-(1-propenyl)-L-cysteine (GS1PC), which after oxidation can give rise to γ-glutamyl-*S*-(1-propenyl)-L-cysteine sulfoxide (GS1PCS), the precursor of the main organosulfur compounds in onion, isoalliin, which will be formed when glutamic acid is released by hydrolysis. N-acetylation of isoalliin could potentially cause NAS1PS formation. Simultaneously, S1PC can be formed by the hydrolysis of GS1PC, which releases the glutamic acid from the molecule. This compound, after oxidation, can also produce isoalliin, in the same way that isoalliin, when reduced, would form S1PC. Finally, when S1PC is N-acetylated, it is transformed into NAS1PC.

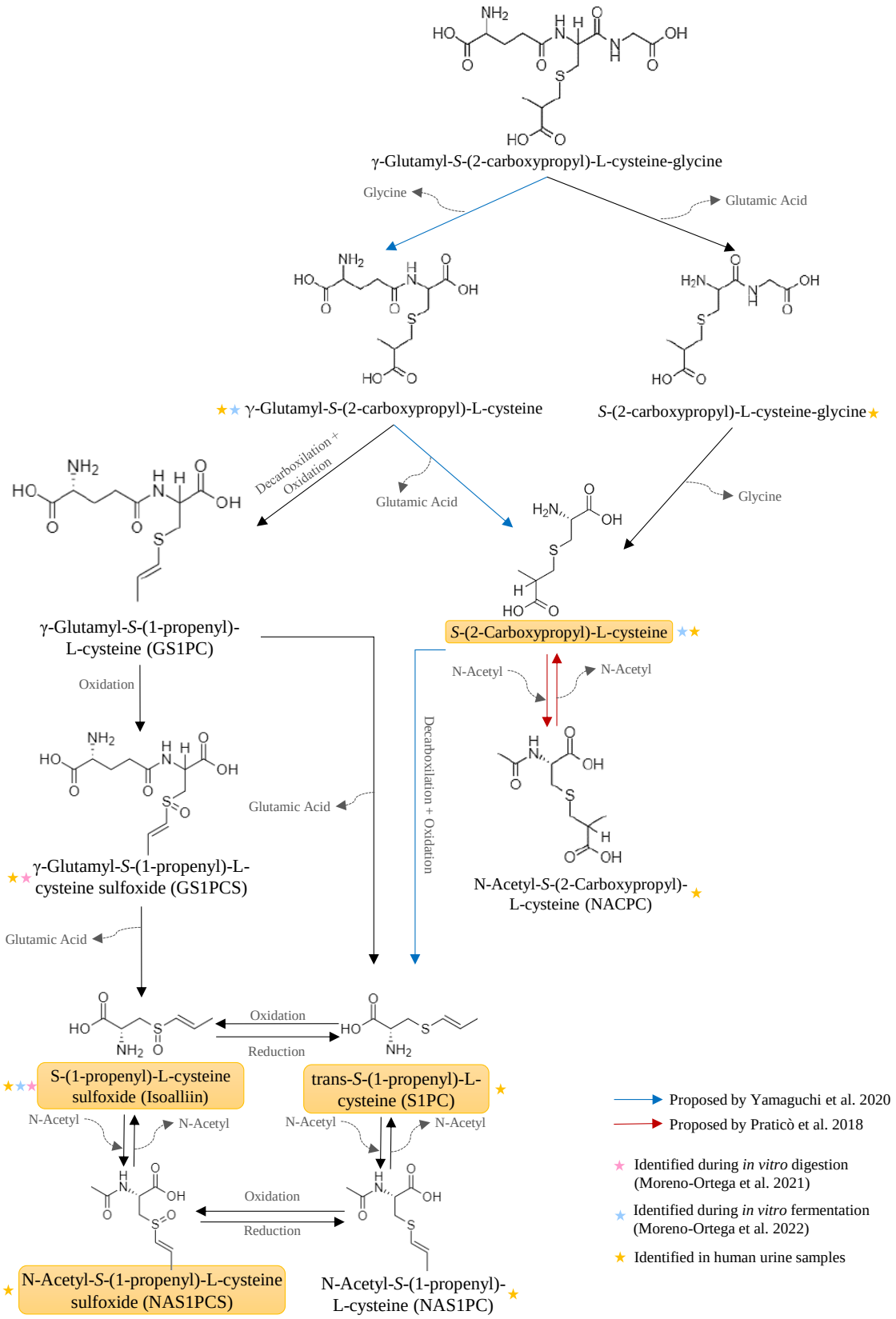


Figure 3.- Potential pathway for the metabolism of organosulfur compounds in the gastrointestinal tract after black onion consumption based in degradation pathways reported by Yamaguchi et al. 2020 and Praticò et al. 2018 [19, 20]. *Compound in boxes names indicate main components excreted in urine after black onion intake.*

Similarly, potential degradation pathways for other organosulfur compounds have been proposed that lead to the formation of major compounds excreted in urine after black onion intake including methiin and deoxypropiin (Figure 4 and 5). Methiin can be obtained from the degradation of more complex molecules such as γ-glutamyl-*S*-methyl-L-cysteine-glycine, present in black onion (Figure 4). Tentatively, hydrolysis of this compound may release glycine and γ-glutamyl-*S*-methyl-L-cysteine (GSMC), a compound that has been found after colonic fermentation of black onion (Moreno-Ortega et al., 2022). GSMC can be oxidised leading γ-glutamyl-*S*-methyl-L-cysteine sulfoxide (GSMCS) but if it is subjected to hydrolysis, it produces glutamic acid and *S*-methyl-L-cysteine (deoxymethiin). Methiin could finally be obtained from the oxidation of deoxymethiin or from the hydrolysis of GSMCS. In addition, at the physiological level, N-acetylation of methiin and deoxymethiin can also be produced, resulting in N-acetyl-*S*-methyl-L-cysteine (NASMC) and N-acetyl-*S*-methyl-L-cysteine sulfoxide (NASMCS), respectively. NASMCS was previously described by Edmands et al. (2011) as a metabolite of methiin in human urine samples and as a biomarker of cruciferous vegetable consumption [21]. However, these compounds have not been detected in our study.

Similarly, deoxypropiin and propiin are also found as major organosulfur compounds in urine after ingestion of black onion. These compounds can be formed in reactions similar to those mentioned above with deoxymethiin and methiin. In this sense, the parent compound, γ-glutamyl-*S*-propyl-L-cysteine-glycine, which has been found in black onion (Figure 5) suffer a hydrolysis by which glycine is released, obtaining γ-glutamyl-*S*-propyl-L-cysteine (GSPC). This compound releases glutamic acid and *S*-propyl-L-cysteine (deoxypropiin), while if it is oxidised, it forms γ-glutamyl-*S*-propyl-L-cysteine sulfoxide (GSPCS). Both compounds can be transformed into *S*-propyl-L-cysteine sulfoxide (propiin), for which GSPCS must be hydrolysed and deoxypropiin oxidised.

There is no evidence of N-acetylation of deoxypropiin and propiin, but given the nature of these molecules and the reactions produced with other structurally similar molecules, it is possible that these conjugates could be formed, however, they have not been detected in our analyses either.

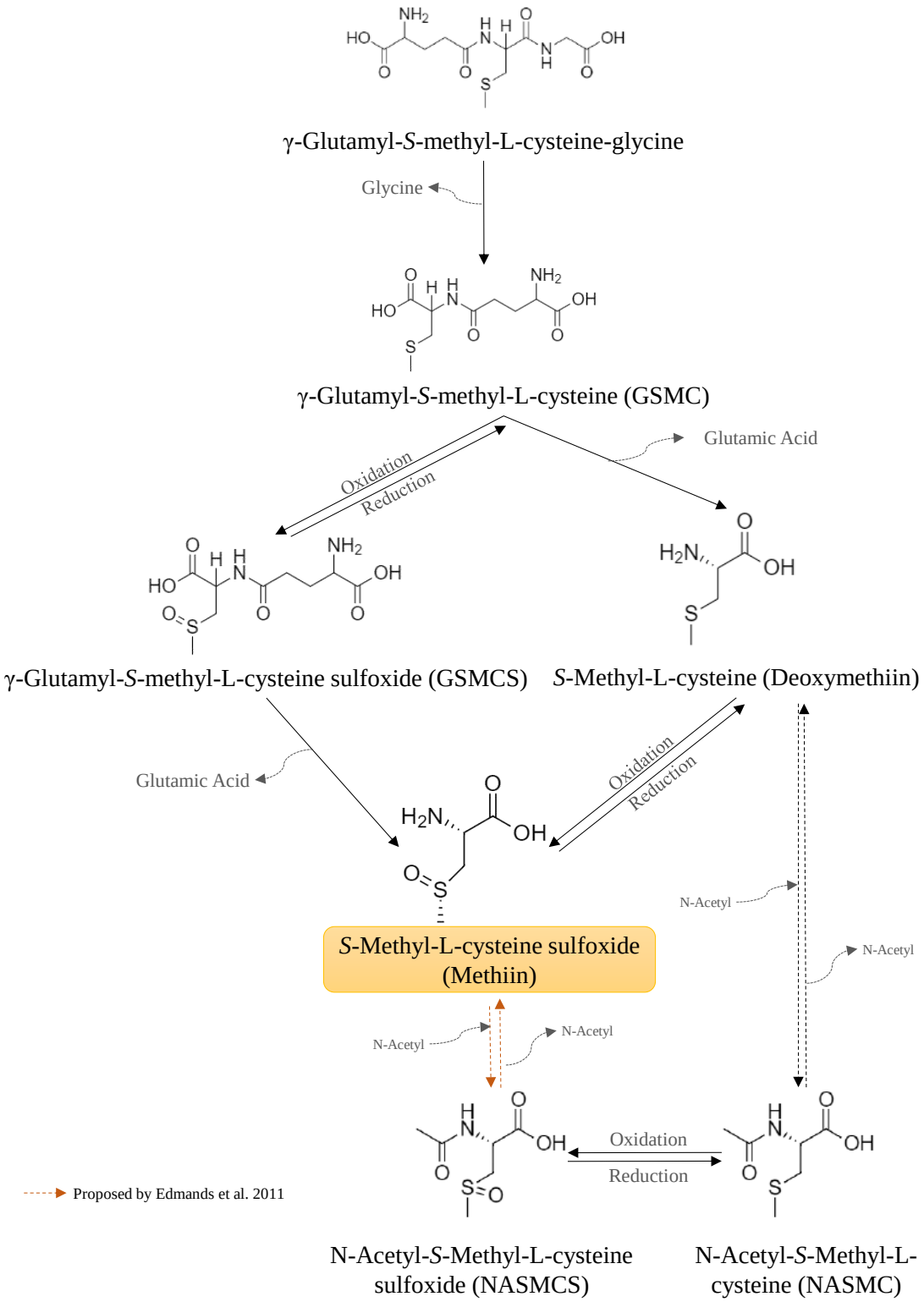


Figure 4.- Tentative degradation pathways of γ-Glutamyl-S-methyl-L-cysteine-glycine based in previous report of Edmands et al. 2011 [21]*. Compound in boxes names indicate main components excreted in urine after black onion intake.*

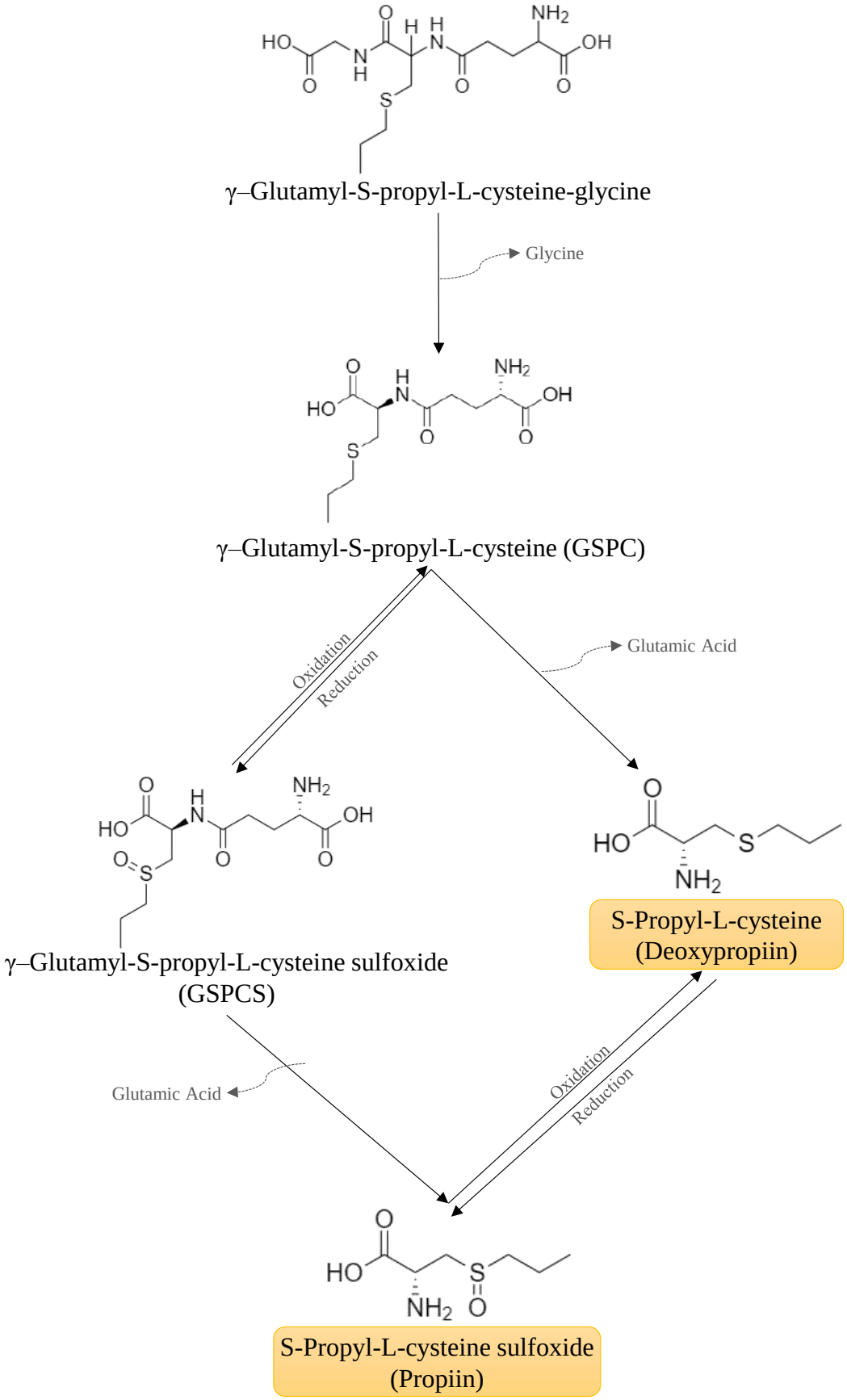


Figure 5.- Tentative degradation pathways of γ–Glutamyl-S-propyl cysteine-glycine. Compound in boxes names indicate main components excreted in urine after black onion intake.

4. Conclusions

Considering the limited information available, this study aims to identify and quantify the organosulfur compounds profile in human urine after the acute intake of black onion. Regarding the total content of organosulfur compounds, 45% were excreted between 8 and 24 hours after ingestion of black onion, which means that the absorption of these compounds mainly occurs at the intestinal level. However, a significant percentage of organosulfur compounds can also be absorbed at the gastric level since 22% of GSAk derivatives and 34% of SAk derivatives have been excreted from 0 to 4 h. Isoalliin (1891 ± 681 nmol), *S*-methyl-L-cysteine sulfoxide (methiin) (1657 ± 340 nmol) and *S*-propyl-L-cysteine (deoxypropiin) (680 ± 169 nmol) were the main urinary metabolites found in human urine samples. Moreover, the potential N-acetylated metabolites of the major compounds in black onion, N-acetyl-*S*-(1-propenyl)-L-cysteine sulfoxide (NAS1PCS), N-acetyl-*S*-(1-propenyl)-L-cysteine (NAS1PC), have been found in urine. Based in our findings, a possible pathway for the *in vivo* metabolism of organosulfur compounds in humans that could potentially result in the formation of these biomarkers after consumption of black onion has been proposed. The identification of these organosulfur compounds as urinary metabolites of black onion consumption provides opportunities to assess the role of this product in human health and also the basis for further evaluation of the biological role and health potential of these secondary plant metabolites in humans.

5. Ethics statement

The protocol of the study was approved by the Ethical Committee of the Human Clinical Research Unit at the Arnau Vilanova University Hospital, Lleida, Spain (Approval Number: CEiC-1790)

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Conflict of interest: There is no conflict of interest.

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CAPÍTULO 6

CAPÍTULO 6: PERSPECTIVAS FUTURAS

Durante mi estancia en la Universidad de Parma, dediqué parte del tiempo al aprendizaje y manejo de nuevas técnicas de co-cultivos celulares, para lo que estuve colaborando con un proyecto en el que se trabajaba con fluido ileofecal de sujetos que han consumido mango, con el objetivo de observar si se puede relacionar el consumo de mango con algún efecto preventivo o de tratamiento frente a una respuesta inflamatoria. Contábamos con las muestras de fluido ileofecal de seis sujetos previo consumo de mango y tras el consumo de este.

Nunca había trabajado con cultivos celulares por lo que mi aprendizaje y manejo ha sido desde cero. En primer lugar, establecimos el tipo de cultivo celular con el que se iba a trabajar, que sería un co-cultivo de células Caco-2 y HT29-MTX, en una relación 7:3, respectivamente. Para ello, en primer lugar, tendríamos que cultivar cada uno de los tipos de células de forma individual, las Caco-2 en medio MEM y las HT29-MTX en DMEM hasta que alcancen una determinada densidad celular y hayan retomado su actividad. Normalmente se hacen unos 7 u 8 pasajes hasta que las células se encuentran preparadas para sembrar el co-cultivo, haciéndose estos pasajes cada 3 días. Una vez las células se encuentran preparadas, calculamos el número de células de cada una que necesitaremos para sembrar las placas que tenemos. Sabiendo esto, con un contador de células automático, vemos cuántas células tenemos de Caco-2 y HT29-MTX en nuestros cultivos y establecemos la cantidad de medio cultivado que necesitamos de cada uno de ellos. Unimos ambos en un tubo de ensayo, centrifugamos tres minutos para que sedimenten las células en el fondo y extraemos el medio antiguo para resuspender las células en la cantidad de medio nuevo necesario para realizar la siembra. Tras esto, añadimos 250µL en cada trans-wells, que cuenta con una membrana que será la base donde crecerán nuestras células para simular la barrera intestinal, esto constituirá el medio apical del cultivo, mientras que, para el medio basolateral, añadiremos 750µL de medio nuevo, sin células.

Este cocultivo se mantiene durante 21 días, cambiando el medio apical y basolateral cada 3 días. Una vez trascurrido este tiempo, se puede comenzar con el tratamiento de las células.

Hemos hecho diferentes experimentos durante este tiempo, en primer lugar, añadimos una concentración alta de la muestra de uno de los sujetos tras el consumo de mango para comprobar si resultaba tóxico para las células, y comprobamos que la vitalidad celular no se veía afectada por esto. Por otro lado, hicimos un experimento en el que añadimos concentraciones conocidas de una mezcla de citoquinas, responsables de la

respuesta inflamatoria, para comprobar cuál era la dosis mínima efectiva, ya que el objetivo es el de simular en la mayor medida posible las condiciones fisiológicas. Además, se realizó la cuantificación del contenido total de polifenoles de las muestras por medio de Folin-Ciocalteau para una cantidad de 200 mg de muestra extraída. Tras esto, se decidió trabajar con 50 µg de contenido total de polifenoles para tratar de mantener las condiciones fisiológicas naturales.

Tras esto, se hizo un primer tratamiento de las células en el que solo se añadió la muestra de los sujetos previo al consumo del mango y tras el consumo de este, manteniendo el tratamiento durante 96h y controlando la resistencia eléctrica transepitelial (TEER) cada 24h. Además, determinamos la resazurina (para estudiar la viabilidad celular) y posteriormente realizamos un Western-Blot, técnica empleada para detectar proteínas específicas, en nuestro caso, estructurales de la barrera celular formada por nuestro cultivo, por su reacción con anticuerpos primarios y secundarios.

Por último, realizamos dos experimentos más en los que añadimos las muestras de los sujetos al cultivo, tanto previo el consumo de mango como tras el consumo de este, durante 24h, para posteriormente añadirles el mix de citoquinas y observar si el consumo de mango tiene algún efecto.

Este es un proyecto que aún no ha finalizado por lo que no se han obtenido los resultados finales, de igual forma, me mantengo en comunicación con el laboratorio donde he estado trabajando para continuar con el seguimiento del proyecto y estar al tanto de los resultados que finalmente se obtengan.

Aunque para la consecución de este objetivo no voy a obtener resultados analíticos relacionados directamente con mi tesis, he aprendido y me encuentro resuelta en el manejo de nuevas técnicas en sistemas de co-cultivo, así como en el post-tratamiento y la obtención de resultados de los experimentos celulares en líneas celulares del epitelio intestinal humano, desde el planteamiento del experimento hasta la evaluación de las propiedades antiinflamatorias que puedan presentar los compuestos bioactivos presentes en diferentes matrices alimentarias. Estos conocimientos servirán de base para investigaciones futuras en líneas celulares con el objetivo de evaluar la biodisponibilidad y el metabolismo de los fitoquímicos del ajo negro y la cebolla negra en líneas celulares Caco-2 como modelo de absorción intestinal humano, así como permitirán estudiar el efecto quimiopreventivo de estos compuestos en líneas celulares Caco-2 y HT29.

CAPÍTULO 7

CAPÍTULO 7: DISCUSIÓN GENERAL

En este capítulo se presenta una discusión general de los resultados obtenidos en los capítulos que conforman la Tesis Doctoral. Tal y como contemplan los objetivos de la tesis, las investigaciones se han centrado en la caracterización fitoquímica de un nuevo producto transformado derivado de la cebolla fresca (*Allium cepa* L.), denominado cebolla negra. Así como la evaluación de la bioaccesibilidad *in vitro* y biodisponibilidad *in vivo* de los compuestos bioactivos identificados en la cebolla negra y su análogo, el ajo negro, en comparación con sus matrices originales, la cebolla y el ajo frescos (*Allium sativum* L.). Estos trabajos han sido divididos en varios capítulos, encontrándose en primer lugar la validación de métodos de análisis para un nuevo producto, la cebolla negra, y la caracterización fitoquímica de ésta. En segundo lugar, se desarrollaron técnicas *in vitro* que permitían evaluar la bioaccesibilidad y la estabilidad a nivel colónico de los fitoquímicos identificados en cebolla negra y ajo negro, junto con sus matrices originales, cebolla fresca y ajo fresco. Y, por último, la evaluación *in vivo* de la biodisponibilidad de los compuestos bioactivos de estos productos mediante un estudio de intervención y la toma de muestras de orina tras su ingesta.

Como se ha mencionado anteriormente, la cebolla negra es un producto análogo al ajo negro que se obtiene mediante un proceso de elaboración similar que consiste en un envejecimiento del producto. Para ello, se somete la cebolla fresca (*Allium cepa* L.) a condiciones de temperatura y humedad controladas durante un periodo de tiempo determinado. Este proceso ocasiona una serie de cambios físico-químicos en el producto que permiten obtener un producto con una cualidades sensoriales radicalmente diferentes a las del producto inicial y con cambios sustanciales en su composición fitoquímica. Hasta la fecha, la cebolla negra no ha sido objeto de investigaciones a pesar del éxito que ha supuesto la comercialización del ajo negro y las propiedades beneficiosas para la salud con las que se ha relacionado. Esto hace necesaria la caracterización fitoquímica de este nuevo producto, para lo que se deben validar y optimizar los métodos de extracción y análisis, así como evaluar in vitro e in vivo los posibles cambios producidos en el perfil de los compuestos fitoquímicos durante la digestión gastrointestinal, la fermentación colónica y la metabolización y excreción de estos.

Validación de los métodos de extracción y análisis cromatográficos de los fitoquímicos de la cebolla negra

El primer objetivo de esta tesis fue el de optimizar los métodos de extracción del perfil de nutrientes, como los aminoácidos, así como de fitoquímicos de la cebolla negra, y la de validar los métodos cromatográficos que permitieran identificar y cuantificar estos compuestos de interés nutricional y funcional.

La optimización del método de extracción se realizó para un total de 10 flavonoides, 27 compuestos organosulfurados y 21 aminoácidos, seleccionados en base a su presencia en la matriz y a la disponibilidad comercial. Para optimizar el método de extracción se determinó su rendimiento con diferentes solventes de extracción para los diferentes compuestos evaluados. Se observó que el solvente que ofrecía un mejor rendimiento de la extracción para la mayoría de los compuestos estudiados fue agua desionizada y metanol (20:80, v/v) acidificada con 1% de ácido fórmico, con valores que fueron desde el 80 al 111% para todos los compuestos en cebolla fresca y desde el 71 al 111% para todos los compuestos en cebolla negra.

Por otro lado, se realizó la validación de dos métodos de análisis mediante cromatografía de líquidos acoplada a espectrometría de masas de alta resolución (UHPLC-HRMS); uno para flavonoides y otros para compuestos organosulfurados y aminoácidos, utilizando los mismos compuestos utilizados para la optimización de la extracción. Los nuevos métodos se validaron en términos de linealidad, sensibilidad, precisión y efecto matriz de acuerdo a los criterios establecidos por la *Association of Analytical Communities* (AOAC) (AOAC International, 2002). Todos los compuestos presentaron un coeficiente de correlación (R^2) superior a 0.9907, obteniéndose una buena linealidad entre las concentraciones de los compuestos evaluados y la respuesta del detector del equipo de cromatografía. La sensibilidad de los métodos se evaluó determinando los límites de detección (LDD) y de cuantificación (LDC) para cada uno de los compuestos. Para el método de flavonoides se obtuvieron LDDs que oscilaron entre 0.0036 y 0.007 ng/µL y LDCs entre 0.012 y 0.024 ng/µL para ambas matrices, mientras que para el método de compuestos organosulfurados y aminoácidos se obtuvieron un LDDs entre 0.012 a 3.12 ng/µL y LDCs entre 0.039 y 6.25 ng/µL. Por último, la precisión se evaluó mediante la repetibilidad y la precisión intermedia, obteniéndose una desviación estándar relativa (RSD) inferior al 14% para el método de flavonoides e inferior al 18% para el método de organosulfurados y aminoácidos. Por último, se obtuvo que el efecto matriz tuvo un valor de ± 20% en la cuantificación de los diferentes compuestos evaluados en ambas matrices, lo que se considera aceptable

para la identificación y cuantificación de estos compuestos mediante UHPLC-HRMS (Feliciano et al., 2016; Gasperotti et al., 2014).

Una vez validado, el método fue aplicado para observar las diferencias composicionales provocados por el proceso de elaboración en las diferentes familias de compuestos estudiados: flavonoides, aminoácidos y compuestos organosulfurados tanto en muestras de cebolla fresca y cebolla negra para . Se identificaron 10 flavonoides, 21 aminoácidos libres y 22 compuestos organosulfurados en las muestras de cebolla fresca y cebolla negra mediante la masa exacta del compuesto y la comparación con el compuesto estándar, con las bases de datos y/o con publicaciones anteriores.

El contenido total de flavonoides identificados en cebolla fresca y cebolla negra varió significativamente entre ambas matrices, mostrando una concentración de 153.3 y 199.7 µg/g de peso fresco (PF), respectivamente. Esto indica que el proceso de elaboración de la cebolla negra podría suponer un descenso en el contenido fenólico total de la cebolla. Sin embargo, la concentración de quercetina libre en cebolla negra (144 µg/g de PF) fue significativamente superior a la encontrada en cebolla fresca (87 µg/g de PF). Esta diferencia en el contenido de la quercetina aglicona puede estar relacionada con la diferencia en la concentración de los glucósidos de quercetina entre ambas matrices, encontrando quercetina-7,4'-diglucósido, quercetina-3,4'-diglucósido y quercetina-4-glucósido como principales compuestos en cebolla fresca que no fueron identificados en cebolla negra. Esto puede ser debido a que durante el proceso térmico de elaboración de la cebolla negra, se produce una hidrólisis de estos compuestos glicosilados con la correspondiente liberación de quercetina (Juániz et al., 2016; Rohn et al., 2007).

Por otra parte, arginina, glutamina y ácido glutámico fueron los principales aminoácidos libres encontrados en las muestras de cebolla fresca, mientras que arginina, leucina e isoleucina fueron los predominantes en las muestras de cebolla negra. Estos resultados coincidieron con lo encontrado por Hansen (2001), quién observó que los aminoácidos libres mayoritarios en cebolla eran arginina y glutamina (Hansen, 2001). Además, en cuanto al contenido de compuestos organosulfurados, en cebolla fresca se identificaron como mayoritarios γ-glutamil-*S*-(1-propenil) cisteína sulfóxido con 1387.2 µg/g de PF, seguido de γ–glutamil-*S*-(1-propenil) cisteína y γ –glutamil-*S*-(2-carboxipropil) cisteína-glicina con 311.1 y 221.1 µg/g de PF, respectivamente. Esto coincide con lo que mostraron Kubec y col. 2009, quienes identificaron el γ -glutamil-*S*-(1-propenil) cisteína sulfóxido como el dipéptido más abundante en las muestras de cebolla fresca (Kubec & Dadáková, 2009). Mientras que, en cebolla negra, la isoaliína fue identificada como el

compuesto organosulfurado mayoritario con 1584 µg/g de PF. Por otro lado, el hecho de que la concentración de isoaliína sea mayor en la muestra de cebolla negra que en la de cebolla fresca puede deberse a que durante los tratamientos térmicos se produzca la degradación de compuestos como el γ -glutamil-*S*-(1-propenil) cisteína sulfóxido por medio de la acción de la enzima γ -glutamil peptidasa, dando lugar a la formación de derivados de la *S*-alqu(en)il-L-cisteína, como la isoaliína, como explican Kim y col. (2016) (Kim et al., 2016).

Finalmente, también se evaluó la actividad antioxidante mediante los métodos de medida de capacidad antioxidante ABTS y ORAC en cebolla fresca y cebolla negra, obteniendo valores significativamente mayores la cebolla negra para ambos métodos.

Por todo ello, se pudo concluir que se han desarrollado y validado dos metodologías efectivas para la identificación y cuantificación de flavonoides, compuestos organosulfurados y aminoácidos en cebolla fresca y cebolla negra, que no requieren pretratamientos, derivatizaciones o complejas acciones previas al análisis. Además, se han podido realizar unas primeras aproximaciones al perfil de compuestos fitoquímicos del nuevo producto, cebolla negra, y su matriz de origen, la cebolla fresca.

Caracterización de la composición fitoquímica de la cebolla negra durante el proceso de elaboración a partir de 3 variedades de cebolla fresca

El siguiente objetivo de la tesis fue el de estudiar la influencia del proceso de elaboración de cebolla negra sobre el perfil de compuestos nutricionales y bioactivos de tres variedades de cebolla fresca: Chalota, Chata y Echalion. Para ello, se evaluó el perfil de compuestos fenólicos, aminoácidos, compuestos organosulfurados, azúcares, ácidos orgánicos y potencial antioxidante durante el proceso de elaboración de la cebolla negra a diferentes tiempos a partir de tres variedades iniciales de cebolla fresca.

Se identificaron y cuantificaron un total de 7 flavonoides diferentes, siendo las variedades Chalota y Echalion las que presentaron un contenido total mayor. Este contenido se vio significativamente afectado por el proceso de elaboración de la cebolla negra, disminuyendo 5, 8 y 6 veces para Chalota, Chata y Echalion, respectivamente. El flavonoide mayoritario presente en las muestras de cebolla negra fue la quercetina libre, con una concentración que suponía el 94% del contenido total en Chalota, el 97% en Chata y el 99% en Echalion. La disminución del contenido total de flavonoides observado durante el proceso de elaboración de la cebolla negra para todas las variedades puede deberse, como ya se ha comentado, al proceso térmico seguido para

su obtención que, a pesar de ser menos severo que otros tratamientos, se mantiene durante un periodo prolongado, lo que puede ocasionar la degradación de estos compuestos (Sharma et al., 2015). Además, estos compuestos pueden oxidarse dando lugar, en último término, a quinonas capaces de reaccionar entre ellas para dar lugar a pigmentos oscuros, característicos de este nuevo producto (Friedman, 1996; Kroll et al., 2003).

Respecto al contenido de aminoácidos, se identificaron y cuantificaron un total de 21 aminoácidos en las muestras de cebolla. La arginina fue el aminoácido mayoritario en las muestras de cebolla de las 3 variedades. Además, se observó un considerable descenso del contenido de glutamina (más del 99%) para las tres variedades, esto puede deberse a que la glutamina, la asparagina y el ácido glutámico son los principales precursores de los productos obtenidos durante la reacción de Maillard, reacción de pardeamiento no enzimático, responsable del color oscuro característico del producto final (Niquet & Tessier, 2007). Por otro lado, se observó un descenso en el contenido de arginina, histidina y triptófano, principales aminoácidos relacionados con el sabor amargo, mientras que aumentó el contenido de alanina, glicina y prolina, relacionados con el sabor dulce (Bachmanov et al., 2016).

En cuanto al perfil de compuestos organosulfurados, se pudieron identificar y cuantificar un total de 27. La isoaliína fue el principal compuesto organosulfurado encontrado en el producto final para las 3 variedades, suponiendo más del 80% del contenido total de compuestos organosulfurados determinados. Esto coincide que lo reportado anteriormente por otros autores, que establecen que la isoaliína es el principal derivado de la *S*-alqu(en)il-cisteína identificado en cebolla (Batchu et al., 2013; Wiczkowski, 2011). Por otro lado, el proceso tiene un gran impacto en el contenido de γ-glutamil-*S*-(1-propenil)-L-cisteína sulfóxido, el cuál se encuentra en el producto final en una concentración hasta 10 veces menor en la variedad Chalota, 3 veces menor en la variedad Chata y 4 veces menor en la variedad Echalion. Molina-Calle y col. 2017 observaron un descenso similar en el contenido de cicloaliína y γ-glutamil-*S*-(1-propenil)-L-cisteína durante el proceso de elaboración del ajo negro (Molina-Calle, de Medina, et al., 2017). Estos cambios en el contenido total de compuestos organosulfurados durante el proceso de elaboración de la cebolla negra se deben fundamentalmente a la degradación de los compuestos identificados a compuestos intermedios, como tiosulfinatos, y compuestos volátiles que en la literatura se han encontrado determinados mediante cromatografía de gases (Molina-Calle, Priego-Capote, et al., 2017; Yu et al., 1994).

Los ácidos orgánicos más abundantes en la cebolla fresca fueron el ácido málico, el tartárico y el oxálico. La concentración de éstos aumentó progresivamente durante todo el proceso, mientras que el resto de ácidos orgánicos no mostraron una tendencia clara a lo largo del proceso de elaboración de la cebolla negra. Estos resultados coinciden con los observados por Colina-Coca y col. (2014), quienes encontraron que el ácido málico y el oxálico fueron los principales ácidos orgánicos de las muestras de cebolla sin tratar (Colina-Coca et al., 2014). En el producto final, el ácido tartárico fue el mayoritario seguido del ácido málico. Por otro lado, en base a la concentración de ácido pirúvico de las muestras de cebolla negra de las 3 variedades de cebolla, este producto es clasificado como de baja pungencia, según los criterios establecidos por Dhumal y col. (2007) (Dhumal et al., 2007)

En cuanto a los azúcares, se determinó el contenido de glucosa, fructosa y sacarosa. Se observó que, durante el proceso de elaboración de la cebolla negra, el contenido total de azúcares aumentó significativamente, mostrando hasta 10, 2 y 5 veces mayor concentración en las variedades Chalota, Chata y Echalion, respectivamente. El contenido de fructosa y glucosa aumentó durante todo el proceso, con el correspondiente descenso en el contenido de sacarosa. Resultados similares obtuvo Martínez-Casas (2017) durante el estudio de la evolución del perfil de azúcares en el proceso de elaboración del ajo negro, donde observó también un aumento del contenido de fructosa de hasta 10 veces (Martinez-Casas et al., 2017). Además de la evidente proporción que vendría de la descomposición de la sacarosa, se observan incrementos de la fructosa y de la glucosa no compatibles solo con esta explicación por lo que además, se postula que existe una contribución muy importante a la concentración de glucosa y fructosa debida a la hidrólisis de los fructooligosacáridos, hidratos de carbonos característicos de la cebolla que forman parte de la fibra dietética, durante el tratamiento térmico de elaboración de la cebolla negra (Yuan et al., 2016). El aumento del contenido de la fructosa se traduce en un aumento del sabor dulce, característico de la cebolla negra, esto se debe, fundamentalmente, a que la fructosa presenta un poder endulzante hasta dos veces mayor que la glucosa y 1.7 veces mayor que la sacarosa (Yuan et al., 2018; Zhang et al., 2016).

También se determinó la actividad antioxidante mediante ABTS, DPPH y ORAC. Se observó un aumento de la actividad antioxidante de las muestras determinadas con los métodos ABTS y DPPH, mientras que en la determinación mediante ORAC se observó un ligero descenso de ésta a lo largo del proceso de elaboración de la cebolla negra. La diferencia de las tendencias en función de las técnicas empleadas para la determinación

de la actividad antioxidante se debe a los principios químicos en los que se encuentra basada cada metodología (Craft et al., 2012; Ou et al., 2002).

Por todo ello, se puede concluir que el proceso de elaboración de la cebolla negra influye significativamente sobre los compuestos nutricionales y funcionales de la misma, suponiendo una serie de cambios fitoquímicos en el producto que han sido caracterizados mediante la identificación y cuantificación de 62 compuestos, incluyendo flavonoides, aminoácidos, compuestos organosulfurados, ácidos orgánicos y azúcares en tres variedades de cebolla sujetos de trabajo para la elaboración de cebolla negra. Estos cambios estarían influidos por la sensibilidad del compuesto al tratamiento térmico, a cambios en el pH, la presencia de oxígeno e, incluso, a la presencia de otros fitoquímicos. La variedad Chalota fue la que presentó mayores diferencias en cuanto al perfil fitoquímico, ya que, por ejemplo, muestra un mayor incremento del contenido total de azúcares, lo que tendrá una repercusión positiva en el sabor final y en la aceptación del producto final por parte del consumidor.

Evaluación de la bioaccesibilidad y la estabilidad gastrointestinal *in vitro* del perfil fitoquímico del ajo fresco y el ajo negro; y de la cebolla fresca y la cebolla negra

Como se ha descrito previamente, es necesario determinar la bioaccesibilidad de los compuestos presentes en los alimentos, más aún cuando estos se asocian con una potencial bioactividad. El índice de bioaccesibilidad indica el porcentaje del compuesto inicial que es liberado de la matriz durante la digestión gastrointestinal y que, por tanto, está preparado para ser absorbido. Este fue el objetivo de dos investigaciones, en las que se pretendió evaluar los cambios que se producían en el perfil de compuestos fenólicos y organosulfurados durante una digestión gastrointestinal *in vitro* de ajo negro y cebolla negra, en comparación con sus matrices originales, ajo fresco y cebolla fresca.

La cantidad total de compuestos fenólicos en ajo fresco aumentó significativamente después de la digestión oral, principalmente debido a la notable aparición del ácido benzoico y al aumento significativo del contenido de ácido cafeico y ácido ferúlico. Posteriormente, el contenido total disminuyó en las siguientes etapas de la digestión, siendo esta disminución más acusada durante la digestión intestinal. Esto puede deberse a que el contenido fenólico en el ajo está principalmente representado por ácidos fenólicos, los cuáles presentan una mayor inestabilidad en las condiciones de la digestión intestinal, es decir, a pH básico, mientras que, a pH ácido, estos compuestos se liberan fácilmente de la matriz (Ma et al., 2020). Por el contrario, en el ajo negro, aunque la disminución del contenido fenólico durante la digestión gastrointestinal fue

muy significativa, el efecto de las condiciones gástricas e intestinales no fue tan marcado como en el ajo fresco, sin embargo, solo el ácido cafeico fue bioaccesible tras el proceso digestivo. No obstante, ambas matrices se vieron significativamente afectadas por la digestión gastrointestinal *in vitro*, mostrando unos índices de bioaccesibilidad total del 58.6 y el 42.7% para ajo fresco y ajo negro, respectivamente.

En el caso de la cebolla fresca y negra, durante la digestión gastrointestinal, se observó una disminución gradual en el contenido total de polifenoles desde la fase oral hasta la fase intestinal en ambas matrices. En cebolla fresca, el contenido en quercetina e isorhamnetinas libres aumentó durante todo el proceso, fundamentalmente debido a la hidrólisis de sus grupos glucosídicos, dando lugar a la liberación de las agliconas. La disminución del contenido total de flavonoides durante el proceso digestivo puede ser debido a la interacción de estos compuestos con otros componentes de la matriz, como carbohidratos o proteínas, o por la formación de complejos enzimáticos como han indicado otros autores en estudios anteriores (De Santiago et al., 2018; Gonzales et al., 2015; Su et al., 2018). En cebolla negra, el proceso digestivo estuvo marcado por la estabilidad de la quercetina, compuesto mayoritario de este producto, que fue disminuyendo su concentración durante todo el proceso digestivo, mostrando un índice de bioaccesibilidad del 39%. Observando el proceso digestivo llevado a cabo en ambos productos cabe destacar la importancia de la matriz alimentaria en este proceso, especialmente a la hora de la liberación e interacción de compuestos como los polifenoles (Mandalari et al., 2016; Tarko & Duda-Chodak, 2020; Wojtunik-Kulesza et al., 2020). Los compuestos más bioaccesibles en cebolla fresca fueron el ácido gálico, quercetina e isorhamnetina, los cuáles presentaron índices de bioaccesibilidad del 317, 165 y 211%, respectivamente. Respecto a la cebolla negra, la querecetina-4'-glucósido y la isorhamnetina mostraron los índices de bioaccesibilidad más altos, con un 95 y un 82%, respectivamente.

Por otro lado, la digestión gastrointestinal *in vitro* del ajo fresco afectó significativamente al contenido de compuestos organosulfurados, el cual disminuyó durante todo el proceso. Los derivados de SAk se vieron más afectados que los derivados de GSAk, especialmente durante la digestión oral. Sin embargo, el descenso del contenido de algunos derivados del GSAk se puede deber a la actividad hidrolítica enzimática que ocasiona la ruptura de los grupos γ-glutamil, dando lugar a la formación de otros compuestos organosulfurados (Verhagen, Hageman, Rauma, Versluis-de Haan, et al., 2001). Al final del proceso de digestión, los compuestos organosulfurados del ajo fresco mostraron una bioaccesibilidad media total del 15%, mostrando los derivados de GSAk (26%) un mayor índice de bioaccesibilidad que los derivados de SAk (12%). El

compuesto organosulfurado GSAC, seguido de γ-glutamil-*S*-metil-L-cisteína (GSMC) y G1PCS fueron los principales derivados del GSAk encontrados tras la digestión gastrointestinal del ajo fresco (39%), mientras que aliina, metiina, cicloaliina, SAC, *S*-alilmercaptocisteína (SAMC) y trans-*S*-(1-propenil)-L-cisteína (S1PC) fueron los principales derivados del SAk, representando el 57% del contenido total de compuestos organosulfurados. Los compuestos organosulfurados que presentaron los mayores índices de bioaccesibilidad fueron SAC, sulfóxido de metionina, γ-glutamil-*S*-metil-L-cisteína sulfóxido (GSMCS) y SAMC, con 61, 72, 77 y 329%, respectivamente. El aumento del SAMC se produjo durante todo el proceso digestivo, y se debe potencialmente a dos causas, por un lado, ya que se trata de un metabolito de la aliína que se produce al reaccionar con la cisteína tras la ingestión de ajo fresco, por otro, puede deberse a la hidrólisis de su precursor γ-glutamil-*S*-alilmercaptocisteína (GSAMC), cuya concentración disminuyó casi un 90% durante la digestión *in vitro* (Trio et al., 2014).

Además, la digestión gastrointestinal *in vitro* del ajo negro tuvo un menor impacto sobre el perfil de compuestos organosulfurados que en el ajo fresco. El contenido total disminuyó progresivamente durante todo el proceso digestivo, observándose un marcado descenso del contenido total de derivados del GSAk durante la etapa intestinal, esto se debe principalmente a la inestabilidad química de estos compuestos a pH alcalinos (Verhagen, Hageman, Rauma, Versluis-de Haan, et al., 2001). Al final del proceso de digestión, los compuestos organosulfurados del ajo negro mostraron una bioaccesibilidad media total del 55%, mostrando los derivados del GSAk (43%) un menor índice de bioaccesibilidad que los derivados del SAk (62%). Entre los derivados del GSAk, G1PCS y GSAC fueron los mayoritarios al final del proceso, representando un 23% del contenido total de organosulfurados, mientras que aliína, SAC y sulfóxido de metionina fueron los principales derivados del SAk tras la digestión intestinal (60%). Los compuestos organosulfurados que presentaron los mayores índices de bioaccesibilidad fueron el sulfóxido de metionina, S1PC, SAMC y GSMCS, con 262, 151, 106 y 89%, respectivamente. El sulfóxido de metionina aumenta casi 3 veces su contenido durante toda la digestión in vitro, probablemente debido a las reacciones de oxidación de la metionina durante este proceso (Lee & Gladyshev, 2011).

A pesar de que el contenido total inicial de compuestos organosulfurados era significativamente superior en ajo fresco que, en ajo negro, se ha demostrado que éstos presentan una mejor estabilidad a las condiciones de la digestión gastrointestinal *in vitro* en ajo negro que en ajo fresco, finalizándola con una mayor concentración total en ajo negro (13923 nmol/ g PF) que en ajo fresco (11518 nmol/g PF). Entre otros compuestos,

la aliína, al inicio de la digestión presentaba una concentración hasta 7 veces mayor en ajo fresco que en ajo negro, sin embargo, el contenido final fue dos veces más alto en ajo negro que en ajo fresco, lo que implica que la bioaccesibilidad de este compuesto es considerablemente mayor en ajo negro que en ajo fresco.

En cuanto al estudio en cebolla fresca y cebolla negra, la digestión oral afectó en mayor medida al contenido de organosulfurados de la cebolla fresca que al de cebolla negra, siendo más susceptibles los derivados del SAk que los del GSAk a las condiciones de esta etapa, tal y cómo ocurría en el estudio de ajo fresco y ajo negro. Esto puede deberse a la presencia de la enzima aliínasa que interactuará principalmente con compuestos como la metiína, propiína y aliína (Keusgen et al., 2002). Durante la digestión gástrica continúa el descenso del contenido en organosulfurados en ambas matrices hasta la digestión intestinal. Siendo esta última etapa la que tuvo un mayor impacto en el contenido total de compuestos organosulfurados de la cebolla negra, principalmente debido al aumento en el contenido de sulfóxido de metionina y GSMCS, probablemente a causa de las reacciones de oxidación de sus respectivos precursores, metionina y GSMC (Lee & Gladyshev, 2011; Moreno-Ortega et al., 2020; Moreno-Rojas et al., 2018). Durante la digestión intestinal también se produjo un descenso significativo del contenido de metiína, propiína y S(S-propil) cisteína, lo cual puede estar causado por la inestabilidad de estas pequeñas moléculas a las condiciones de pH (básico) de la digestión intestinal (Shen et al., 2002).

Tras la digestión *in vitro*, los compuestos G2PCS, isoaliína, γ-glutamil-*S*-(2-carboxipropil) cisteína-glicina y GSAC fueron los principales compuestos organosulfurados encontrados en cebolla fresca, suponiendo un 76% del total de compuestos organosulfurados, mientras que en cebolla negra los compuestos mayoritarios al final de la digestión fueron isoaliína, sulfóxido de metionina y G2PCS, representando el 85% del total. Además, en cebolla fresca G1PCS, S-(S-propil) cisteína y sulfóxido de metionina fueron identificados como los compuestos con mayores índices de bioaccesibilidad, con valores de 206, 67 y 66%, respectivamente. Sin embargo, en cebolla negra, los compuestos que presentaron mayores índices de bioaccesibilidad fueron el sulfóxido de metionina (169%), GSMCS (160%) e isoaliína (83%).

En resumen, la cebolla fresca se vio afectada en mayor medida por el proceso digestivo que la cebolla negra, con un contenido de compuestos organosulfurados que disminuyó hasta un 69%, desde 8432 a 2594 nmol/g PF. En cebolla negra, la disminución del contenido total de organosulfurados fue desde 66,452 a 55,153 nmol/g PF, suponiendo una pérdida total del 17%. La mayor estabilidad de los principales compuestos

organosulfurados en cebolla negra que en cebolla fresca puede ser debida a que durante el proceso de elaboración de la cebolla negra se produce la inactivación de la enzima aliinasa, ya que se alcanzan temperaturas alrededor de los 60ºC, lo que evita la interacción de esta enzima con sus sustratos (Méndez Lagunas & Castaigne, 2008).

Estos resultados indican que los compuestos que quedan después de la digestión intestinal pueden atravesar el intestino delgado y llegar al colon, donde se someten al metabolismo mediado por la microbiota colónica antes de su absorción. Además, se ha comprobado que el proceso de elaboración por el que se obtienen tanto el ajo negro como la cebolla negra podría tener un impacto positivo en la bioaccesibilidad del perfil de compuestos organosulfurados, aunque no se sucede de la misma forma con el perfil de compuestos fenólicos.

Evaluación del impacto de la fermentación colónica *in vitro* sobre el perfil fitoquímico del ajo fresco y el ajo negro; y de la cebolla fresca y la cebolla negra.

Siguiendo con el proceso de digestión, el siguiente paso lógico a la digestión gastrointestinal *in vitro* sería la llegada de esos compuestos al colon donde serían transformados por acción de la microbiota colónica previo a su absorción. Para ello, se han llevado a cabo dos estudios que nos permiten evaluar la evolución de los compuestos fenólicos y organosulfurados del ajo fresco y el ajo negro, así como de la cebolla fresca y la cebolla negra, tras un proceso de fermentación colónica *in vitro*.

Durante la fermentación colónica *in vitro* de ajo fresco y ajo negro se observaron tendencias similares. Los ácidos hidroxicinámicos, los derivados del ácido fenilpropiónico, del ácido fenilacético y el bencenotriol disminuyeron su concentración tras el proceso fermentativo, mientras que los flavonoles y los derivados del ácido benzoico mostraron un incremento de su contenido durante la fermentación. El aumento de la concentración de los flavonoles se debe principalmente a la liberación de las agliconas, quercetina e isorhamnetina de sus formas glucosiladas por las reacciones de hidrólisis derivadas de la fermentación (Amaretti et al., 2015; Hollman et al., 1995, 1997). Este hecho, también puede ser debido a la liberación de flavonoles producida por la fermentación de fracciones de la fibra dietética donde se encuentran retenidos (Edwards et al., 2017). Mientras que el aumento del contenido de los derivados del ácido benzoico puede ser debido a que este grupo está formado, principalmente, por catabolitos finales de las rutas de degradación de los compuestos fenólicos durante la fermentación colónica. De hecho, el aumento de la concentración del ácido 4-hidroxibenzoico puede producirse por la dehidroxilación del ácido 3,4-dihidoxibenzoico, el cuál ve aumentada

también su concentración ya que deriva de la oxidación del ácido 3-(3',4'-dihidroxifenil) propiónico (Di Pede et al., 2020; Ludwig et al., 2013). Finalmente, el compuesto 1,3,5-bencenotriol es un catabolito de la acción de la microbiota colónica sobre la quercetina, razón por la que potencialmente aumenta su concentración durante las primeras 8 horas del proceso fermentativo (Aura, 2008; Dueñas et al., 2015). Posteriormente, este compuesto se degrada dando lugar a ácidos grasos de cadena corta, sustrato energético de la microbiota colónica, viendo disminuida su concentración final (Braune & Blaut, 2016).

En general, los principales compuestos fenólicos encontrados tras la fermentación *in vitro* del ajo fresco fueron el ácido 4-hidroxibenzoico, el ácido benzoico, el ácido 4-hidroxifenilacético, el 1,3,5-bencenotriol y el ácido 3,4-dihidroxibenzoico, suponiendo el 85% del total de compuestos fenólicos, mientras que el ácido 4-hidroxifenilacético, el ácido 4-hidroxibenzoico, el 1,3,5-bencenotriol, el ácido 3,4-dihidroxibenzoico, el ácido benzoico y el ácido 4-hidroxi-3-metoxibenzoico fueron los principales productos finales en el ajo negro, comprendiendo el 91% del total de compuestos fenólicos.

Por otro lado, durante la fermentación colónica *in vitro*, la cebolla fresca y la cebolla negra mostraron tendencias distintas para algunas de las familias de compuestos fenólicos. En términos generales, el contenido total de fenólicos experimentó un aumento durante la fermentación colónica de la cebolla fresca, mientras que en cebolla negra se vio disminuido. Ambas matrices mostraron un descenso del contenido total de derivados del ácido benzoico, mientras que la concentración de derivados del fenilacético y del 1,3,5-bencenotriol aumentó durante el proceso fermentativo. Sin embargo, la cebolla fresca experimentó un aumento significativo en el contenido de flavonoles, tal y como ocurría en ajo fresco y ajo negro, probablemente debido a la acción microbiana que produce la hidrólisis de los glucósidos de quercetina e isorhamnetina, aumentando la concentración de sus agliconas más de 6 y 8 veces, respectivamente (Cassidy & Minihane, 2017; Kawabata et al., 2019). De hecho, un estudio reciente realizado por Fernández-Jalao y col. (2021) en cebolla mostró un aumento del contenido de quercetina libre en el colon ascendente durante una digestión gastrointestinal dinámica con un simulador de la fermentación colónica (Fernández-Jalao et al., 2021). Por otro lado, en cebolla negra se observó un aumento en la concentración de los derivados del ácido fenilpropiónico y del fenilacético. El significativo aumento de los ácidos 3-(4'-hidroxi-3'-metoxifenil) propanoico y 3-(3',4'-dihidroxifenil) propanoico, es potencialmente debido a la degradación microbiana de los ácidos cafeico y ferúlico, o por la rotura de los anillos de los derivados de la quercetina y la isorhamnetina, al igual que ocurre en cebolla fresca (De Santiago et al., 2019).

Asimismo, los derivados del ácido fenilpropiónico serán sustrato de la microbiota colónica, dando lugar a la formación de otros catabolitos como los derivados del ácido fenilacético y del ácido benzoico. Finalmente, respecto al 1,3,5-bencenotriol,, es un catabolito del metabolismo de la quercetina como se ha comentado previamente, el cual se convertirá en sustrato de la microbiota colónica para la formación de ácidos grasos de cadena corta (Schoefer et al., 2003).

En general, los principales productos finales que permanecieron en la cebolla fresca tras la fermentación *in vitro* fueron la isorhamnetina (141 μM), la quercetina (95,3 μM), el ácido 3,4-dihidroxibenzoico (53 μM) y el ácido 4-hidroxibenzoico (12 μM), suponiendo el 85% de los compuestos fenólicos totales, mientras que el ácido 3,4-dihidroxibenzoico (70 μM), el ácido 4-hidroxifenilacético (68 μM), el ácido 4-hidroxibenzoico (17 μM) y el 1,3,5-bencenotriol (15 μM), que comprenden el 90% de los compuestos fenólicos totales, fueron los compuestos fenólicos mayoritarios tras la fermentación fecal *in vitro* de la cebolla negra.

Estos compuestos fenólicos serían los responsables de los posibles efectos beneficiosos asociados al consumo de ajo fresco, ajo negro, cebolla fresca y cebolla negra, en particular, de la protección frente a la proliferación de células cancerosas a nivel colónico. En este sentido, Dobani y col. (2021) han demostrado que la presencia de ácido benzoico y ácido 4-hidroxibenzoico presentan un efecto protector frente a la carcinogénesis debido a los mecanismos de activación de enzimas detoxificantes (Dobani et al., 2021). También se ha visto que la presencia de 1,3,5-bencenotriol puede causar la apoptosis celular en líneas celulares de cáncer de colon, previniendo la proliferación de estas (Kang et al., 2014). Por otro lado, se ha demostrado la capacidad de la quercetina para inhibir el crecimiento de las células en cáncer de colon, así como a inducir su apoptosis (Raja et al., 2017; Ranelletti et al., 2000; Shan et al., 2009). La isorhamnetina también ha demostrado que es capaz de inhibir el crecimiento celular e inducir la apoptosis en líneas celulares de cáncer de colon (Jaramillo et al., 2010). Respecto al ácido 3,4-dihidroxibenzoico, se ha observado también un efecto quimiopreventivo frente al cáncer de colon químicamente inducido (Tanaka et al., 2011).

Por otro lado, el total de compuestos organosulfurados en ajo negro se vio fuertemente afectado por la fermentación colónica, mientras que los derivados del GSAk y del SAk, así como el total de compuestos organosulfurados en ajo fresco se mantuvieron estables durante todo el proceso, presentando al final una recuperación del 93, 112 y 110%, respectivamente. Además, tras la fermentación colónica, el contenido total de

compuestos organosulfurados en ajo fresco aumentó un 10%, mientras que en el ajo negro mostró una disminución del 23%.

En el ajo fresco se observa un aumento en el contenido de SAC y GSAC. El aumento del contenido de SAC puede deberse a la degradación de sus precursores como el GSAC, el cual también experimenta un incremento durante la fermentación colónica, lo que puede significar que se puede estar formando, a la vez que está siendo, en parte, degradado por la microbiota colónica, por la degradación de compuestos más complejos como el glutatión, el *S*-(2-carboxypropil) glutatión y el γ-glutamil-*S*-(2-carboxipropil) cisteína (Colín-González et al., 2012; Lawson & Hunsaker, 2018; Yamaguchi & Kumagai, 2020; Yoshimoto et al., 2015). Por otro lado, en ajo fresco y ajo negro se experimentó el aumento de la concentración de sulfóxido de metionina, probablemente debido a la interacción de la metionina liberada de los complejos proteicos con las especies reactivas de oxígeno (ROS), presentes debido a las reducciones incompletas del oxígeno durante la fermentación colónica, lo que puede dar lugar a la formación de sulfóxido de metionina (Campbell et al., 2016; R. Jones et al., 2012).

Los principales compuestos encontrados tras la fermentación colónica *in vitro* del ajo fresco y del ajo negro fueron el SAC y el sulfóxido de metionina, que representaron el 92 y el 94% del total de compuestos organosulfurados, respectivamente.

En relación a la cebolla fresca, el contenido total de derivados del GSAk disminuyó significativamente (59%), mientras que la concentración de derivados del SAk aumentó durante la fermentación colónica (121%), lo que hizo que el contenido total de compuestos organosulfurados permaneciera estable durante todo el proceso (106%). Sin embargo, en cebolla negra, los derivados del GSAk y del SAk mostraron una disminución significativa durante el proceso fermentativo, presentando un 26 y un 50% del contenido inicial, respectivamente. Es reseñable, que el contenido inicial de compuestos organosulfurados en cebolla negra era 1’6 veces más alto que en cebolla fresca, mientras que al final del proceso, el contenido total en cebolla fresca fue 1’4 veces más alto que en cebolla negra.

Durante la fermentación colónica *in vitro* de la cebolla fresca se observó el aumento de la concentración de GSAC, el cual se produce, como se ha comentado anteriormente, por la degradación de compuestos más complejos, como la γ -glutamil-*S*-(2-carboxipropil) cisteína-glicina (también conocida como S-(2-carboxipropil) glutatión) que a través de la eliminación de la glicina produce γ -glutamil-S-(2-carboxipropil) cisteína y tras la descarboxilación y oxidación del grupo carboxipropilo produce GSAC (Yamaguchi & Kumagai, 2020). También hubo un aumento significativo del contenido de GS2PC

(147,2%), probablemente causado por la reducción de G2PCS, que durante la fermentación colónica disminuyó hasta no ser detectado al final de la fermentación (Guo et al., 2020). Además, se produce la disminución del contenido de isoaliína y del *S*-(2-carboxipropil) cisteína-glicina y el aumento del SAC y el sulfóxido de metionina. La isoaliína tiende a ser metabolizada dando lugar a compuestos volátiles como el sulfóxido de tiopropanal (Keusgen et al., 2002). Por otro lado, la *S*-(2-carboxipropil) cisteína-glicina durante la fermentación colónica podría perder la glicina, convirtiéndose en un compuesto precursor del SAC, tras una reacción de descarboxilación y oxidación del grupo carboxipropilo, dando lugar a un grupo alilo (M. G. Jones et al., 2004). Además, el SAC también puede formarse por la acción de la γ-Glutamil transferasa sobre el GSAC (Colín-González et al., 2012; Lawson & Hunsaker, 2018). En este caso, la degradación de los precursores del GSAC puede ocurrir con un mayor ratio de lo que se origina el SAC a partir del GSAC, lo que se traduce en el aumento de ambos compuestos tras el proceso fermentativo. Y respecto al sulfóxido de metionina, tal y como ocurría en la fermentación colónica del ajo, la metionina liberada de proteínas y péptidos puede estar reaccionando con los ROS, oxidándose y dando lugar a la formación de sulfóxido de metionina (Campbell et al., 2016).

En cuanto a la cebolla negra, se observó el aumento del contenido de γ -glutamil-*S*-(2-carboxipropil) cisteína y GS2PC durante las primeras horas de la fermentación colónica *in vitro*, lo que puede deberse a la degradación de sus precursores γ -glutamil-*S*-(2-carboxipropil) cisteína-glicina y GS2PCS, respectivamente, no detectados en este estudio, pero identificados en cebolla negra en anteriores publicaciones (Moreno-Ortega et al., 2020; Moreno-Rojas et al., 2018). También se observa un descenso en el contenido final de isoaliína y un aumento en el contenido del sulfóxido de metionina. Sin embargo, el contenido de SAC disminuyó progresivamente durante todo el proceso fermentativo, lo que puede deberse a la ausencia de GSAC, precursor principal de este compuesto.

Finalmente, el sulfóxido de metionina, el SAC y el GSAC fueron los principales compuestos organosulfurados tras la fermentación colónica *in vitro* de la cebolla fresca, representando el 93% del total de compuestos organosulfurados, mientras que, para la cebolla negra, los principales fueron el sulfóxido de metionina y el *S*-propilmercapto-L-cisteína (SPMC) con el 93% del contenido total.

La presencia de estos compuestos a nivel colónico tiene un efecto potencialmente beneficioso para la salud, como en el caso del sulfóxido de metionina, que se forma por reacción con los ROS, evitando su contacto con las células colónicas y el daño celular

que provocan. Posteriormente, el sulfóxido de metionina sería metabolizado por las enzimas de la metionina sulfóxido transferasa presentes en las bacterias del colon (Luo & Levine, 2009; Pamplona & Barja, 2006). También se ha descrito el papel del SAC contra el cáncer de colon en ratones y ratas, ejerciendo una acción detoxificante contra algunos compuestos carcinogénicos y previniendo la aparición del cáncer (Agbana et al., 2020). En cuanto al SPMC, se sabe que es un compuesto potencialmente utilizado por la microbiota colónica como sustrato para la formación de compuestos volátiles, los cuales tienen la capacidad de inhibir la invasión tumoral en las células colónicas, por medio de la regulación de enzimas relacionadas con la capacidad de invasión de las células cancerosas (Lai et al., 2013; Starkenmann et al., 2011).

La mayoría de estos compuestos podrían ser los responsables de los posibles beneficios para la salud relacionados con el consumo de ajo fresco, ajo negro, cebolla fresca y cebolla negra, incluyendo la posible acción a nivel colónico contra los compuestos carcinogénicos y los mecanismos que impiden o retrasan la proliferación de las células cancerosas.

Evaluación de la biodisponibilidad *in vivo* de los compuestos organosulfurados del ajo negro y la cebolla negra.

Una vez realizados los estudios *in vitro* de digestión gastrointestinal y fermentación colónica, se llevó a cabo la evaluación de la biodisponibilidad *in vivo* de los compuestos organosulfurados del ajo negro y la cebolla negra, con el fin de obtener más información sobre la metabolización, distribución y excreción de estos compuestos en el organismo.

Para ello, se realizó un estudio de intervención de ingesta aguda con 12 participantes sanos de edades comprendidas entre los 22 y los 49 años. Estos participantes consumieron 20 gramos de ajo negro a primera hora de la mañana tras dos días siguiendo una dieta libre de compuestos organosulfurados, es decir, evitando el consumo de vegetales de los géneros *Allium* y *Cruciferous*. Después, se llevaron a cabo dos semanas de lavado antes de repetir el estudio con cebolla negra. Se tomaron muestras de orina durante las 24 horas del estudio de intervención y 12 horas antes del consumo del producto.

Durante las 24h tras la ingesta de ajo negro, se excretaron un total de 13,197±6,657 nmol, entre los cuáles se identificaron 33 compuestos organosulfurados. Aproximadamente el 70% de los compuestos organosulfurados fueron excretados entre las 8 y las 24 horas tras el consumo de ajo negro, lo que podría indicar que la absorción de estos compuestos se estaría produciendo principalmente a nivel intestinal. Entre los compuestos organosulfurados identificados en orina, los mayoritarios fueron metiína,

isoaliína, *S*-(2-carboxipropil) cisteína y deoxipropiína, suponiendo el 75% del total de compuestos excretados. También se encontraron cantidades significativas de SAC, S1PC, sulfóxido de metionina y alliína. Mientras que 24 horas después de la ingesta de cebolla negra, las muestras de orina mostraron altas concentraciones de isoaliína, metiína y deoxipropiína, seguidas por SAC, S1PC y sulfóxido de metionina. En total, se han identificado y cuantificado 31 compuestos organosulfurados, con un total de 6572±1835 nmol. Alrededor del 45% del total de compuestos organosulfurados fue excretado entre las 8 y 24 horas tras la ingesta de cebolla negra, coincidiendo con una mayor absorción de estos compuestos a nivel intestinal, como ocurría con el ajo negro. Aunque el 22% de los GSAk y el 34% de los SAk fueron excretados desde las 0 hasta las 4 horas tras la ingesta, lo que sería indicativo de una absorción a nivel gástrico.

En cuanto a los compuestos organosulfurados identificados en orina tras el consumo de ajo negro, se encontraron el N-acetil-*S*-alil-L-cisteína (NASAC), el N-acetil-*S*-alil-L-cisteína sulfóxido (NASACS) y el N-acetil-*S*-(2-carboxipropil)-L-cisteína (NACPC), que son las formas N-acetiladas de los compuestos mayoritarios del ajo negro. Estos compuestos se forman a nivel hepático y renal, por la acción de la enzima N-acetiltransferasa sobre el SAC, la aliína y la S-(2-carboxipropil)-L-cisteína (Colín-González et al., 2012). De hecho, NASAC y NASACS son considerados biomarcadores del consumo de ajo, ya que diferentes estudios han podido observar su aparición en orina tras la ingesta de ajo o la administración oral de SAC. Nagae y col. (1994) administraron SAC de forma oral e intravenosa a ratas, ratones y perros, para posteriormente determinar la concentración de este compuesto y sus metabolitos (NASAC y NASACS) en plasma, algunos órganos, orina y bilis (Nagae et al., 1994). Por otro lado, Verhagen y col. (2001) realizaron un estudio con humanos a los que se administraba una dieta suplementada con ajo, mientras que el grupo control llevaba a cabo la misma dieta, pero en ausencia de ajo, identificándose la presencia de NASAC en la orina de los voluntarios de la dieta con ajo y ausencia de este compuesto en el grupo control (Verhagen, Hageman, Rauma, Versluis-De Haan, et al., 2001).

No obstante, el metabolismo de estos compuestos es complejo y se ha observado que en hígado y riñón se pueden dar también reacciones de deacetilación. De hecho, Amano y col. (2015) llevaron a cabo la evaluación de la actividad de N-acetilación y deacetilación del hígado y riñón en ratas y en perros, observando que la rata mostraba una alta actividad de N-acetilación en hígado y riñón, mientras que el perro mostraba una actividad significativamente más alta de deacetilación del NASACS que de N-acetilación en el hígado. La alta concentración de SAC (622.8±242.3 nmol) en orina encontrada en nuestro estudio 24 horas después de la ingesta de ajo negro sugiere que

es posible que en el hígado y riñón humanos también se esté produciendo una elevada actividad de deacetilación. Esto, además, puede deberse a la reabsorción activa que se produce de los aminoácidos vía urinaria mediante diferentes tipos de transportadores presentes a nivel renal (Bröer, 2008; Silbernagl et al., 1975). Además, la presencia de SAC en orina también puede estar ocasionada por la degradación de moléculas más complejas como el GSAC, γ -glutamil-*S*-alil-L-cisteína sulfóxido (GSACS), aliín o S-(2-carboxipropil)-L-cisteína (Yamaguchi & Kumagai, 2020).

Por otro lado, en las muestras de orina tras el consumo de cebolla negra, se han podido identificar tentativamente los metabolitos N-acetilados de los compuestos organosulfurados principales de la cebolla negra, isoaliína y S1PC, denominados N-acetil-*S*-(1-propenil)-L-cisteína sulfóxido (NAS1PCS) y del N-acetil-*S*-(1-propenil)-L-cisteína (NAS1PC), respectivamente. De forma análoga a lo que ocurre con la aliína y el SAC del ajo negro, la isoaliína y el S1PC de la cebolla negra pueden servir de sustrato a la enzima N-acetiltransferasa en hígado y riñón, formando sus metabolitos N-acetilados.

De la misma forma, la metiína, la propiína o la deoxipropiína, se encuentran en altas concentraciones en orina tras la ingesta de ambos productos fundamentalmente debido a la degradación de compuestos más complejos como la γ-glutamil-*S*-metil-L-cisteína o la γ -glutamil-*S*-propil-L-cisteína.

Por otro lado, el NACPC ha sido identificado como un metabolito del *S*-(2-carboxipropil)-L-cisteína, encontrado en orina tras la ingesta de ajo y cebolla, por lo que también ha sido considerado un biomarcador del consumo de vegetales del género *Allium* (Praticò et al., 2018). Ambos compuestos han sido encontrados en las muestras de orina tras el consumo de ajo negro y cebolla negra.

Los diferentes compuestos excretados en orina durante las 24 horas posteriores a la ingesta de ajo negro y cebolla negra han permitido plantear rutas de degradación tentativas de los compuestos organosulfurados, dando la oportunidad a futuros estudios de profundizar en el metabolismo, distribución y excreción de estos compuestos, así como de ahondar en el potencial saludable de estos productos derivados del ajo y la cebolla.

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CAPÍTULO 8

CAPÍTULO 8: CONCLUSIONES

De los resultados obtenidos durante el desarrollo de la presente Tesis Doctoral se ha llegado a las siguientes conclusiones siguiendo el orden de los objetivos propuestos:

PRIMERA. Se han optimizado y validado dos métodos UHPLC-HRMS selectivos, sensibles y precisos para identificar y cuantificar 10 compuestos fenólicos, 27 compuestos organosulfurados y 21 aminoácidos en cebolla negra y cebolla fresca, estableciéndose las principales diferencias en cuanto a su composición en compuestos fitoquímicos entre la cebolla fresca y la cebolla negra.

SEGUNDA. Se han caracterizado por primera vez los cambios producidos en el perfil de compuestos fenólicos, organosulfurados, aminoácidos, azúcares y ácidos orgánicos durante el proceso de elaboración de la cebolla negra en tres variedades de cebolla (chalota, chata y echalion). El proceso térmico de obtención de cebolla negra produce cambios en el perfil de compuestos fitoquímicos, aumentando el contenido de azúcares y ácidos orgánicos y disminuyendo la concentración de flavonoides y de compuestos organosulfurados, siendo la isoaliína el compuesto organosulfurado mayoritario en cebolla negra.

TERCERA. La digestión gastrointestinal *in vitro* afectó de forma similar el contenido fenólico total en ajo fresco y ajo negro, presentando unos índices de bioaccesibilidad del 59 y el 47%, respectivamente. Mientras que el contenido de compuestos organosulfurados del ajo negro se vio afectado en menor medida que el de ajo fresco por las condiciones de la digestión gastrointestinal *in vitro*, presentando un índice de bioaccesibilidad del contenido total del 15% en ajo fresco y del 55% en ajo negro.

CUARTA. La digestión gastrointestinal *in vitro* afectó en mayor medida al perfil de compuestos fenólicos de la cebolla fresca que al de la cebolla negra. No obstante, tras la digestión gastrointestinal *in vitro,* la cebolla fresca (500 nmol/g peso fresco) seguía presentando una mayor concentración de compuestos fenólicos que la cebolla negra (21 nmol/g peso fresco). En la cebolla fresca, la quercetina y el ácido vanílico fueron los principales compuestos fenólicos

presentes tras la digestión gastrointestinal; en cuanto a la cebolla negra, el compuesto fenólico mayoritario tras el proceso digestivo fue la quercetina.

QUINTA. Respecto al contenido total de organosulfurados, la cebolla negra se vio afectada en menor medida que la cebolla fresca por el proceso de digestión gastrointestinal *in vitro*, presentando una bioaccesibilidad total final de 83 y 31%, respectivamente. En la cebolla fresca, el compuesto mayoritario tras dicha digestión fue el γ-glutamil-*S*-(1-propenil)-L-cisteína sulfóxido (G1PCS), mientras que en la cebolla negra fue la isoaliína.

SEXTA. El proceso de elaboración de ajo negro y cebolla negra podría tener un impacto positivo en la bioaccesibilidad del perfil de compuestos organosulfurados, formal contrario de lo que sucede con el perfil de compuestos fenólicos.

SÉPTIMA. La microbiota intestinal afectó de forma similar al contenido fenólico del ajo fresco y el ajo negro, con una disminución del 44 y el 42%, respectivamente. Sin embargo, en la cebolla fresca se produjo un aumento del contenido total de compuestos fenólicos, hasta un 45%, mientras que en cebolla negra produjo una disminución de hasta el 22%. El ácido 4-hidroxibenzoico fue el compuesto fenólico mayoritario tras la fermentación colónica en el ajo fresco y el ajo negro, siendo también mayoritario el ácido 4-hidroxifenilacético en el ajo negro. Por otro lado, en la cebolla fresca resultaron mayoritarios la isorhamnetina, la quercetina y el ácido 3,4-dihidroxibenzoico y, en cebolla negra, el ácido 3,4-dihidroxibenzoico y el ácido 4-hidroxifenilacético.

OCTAVA. De igual manera, la microbiota intestinal causó una disminución significativa de los compuestos organosulfurados del ajo negro y cebolla negra, aunque no en ajo fresco y cebolla fresca. Los principales compuestos organosulfurados presentes en el ajo fresco, el ajo negro y la cebolla fresca tras la fermentación colónica *in vitro* fueron la *S*-alilcisteína (SAC) y el sulfóxido de metionina; mientras que el sulfóxido de metionina y la *S*-propilmercapto-L-cisteína (SPMC) fueron los principales compuestos encontrados al final del proceso fermentativo de la cebolla negra.

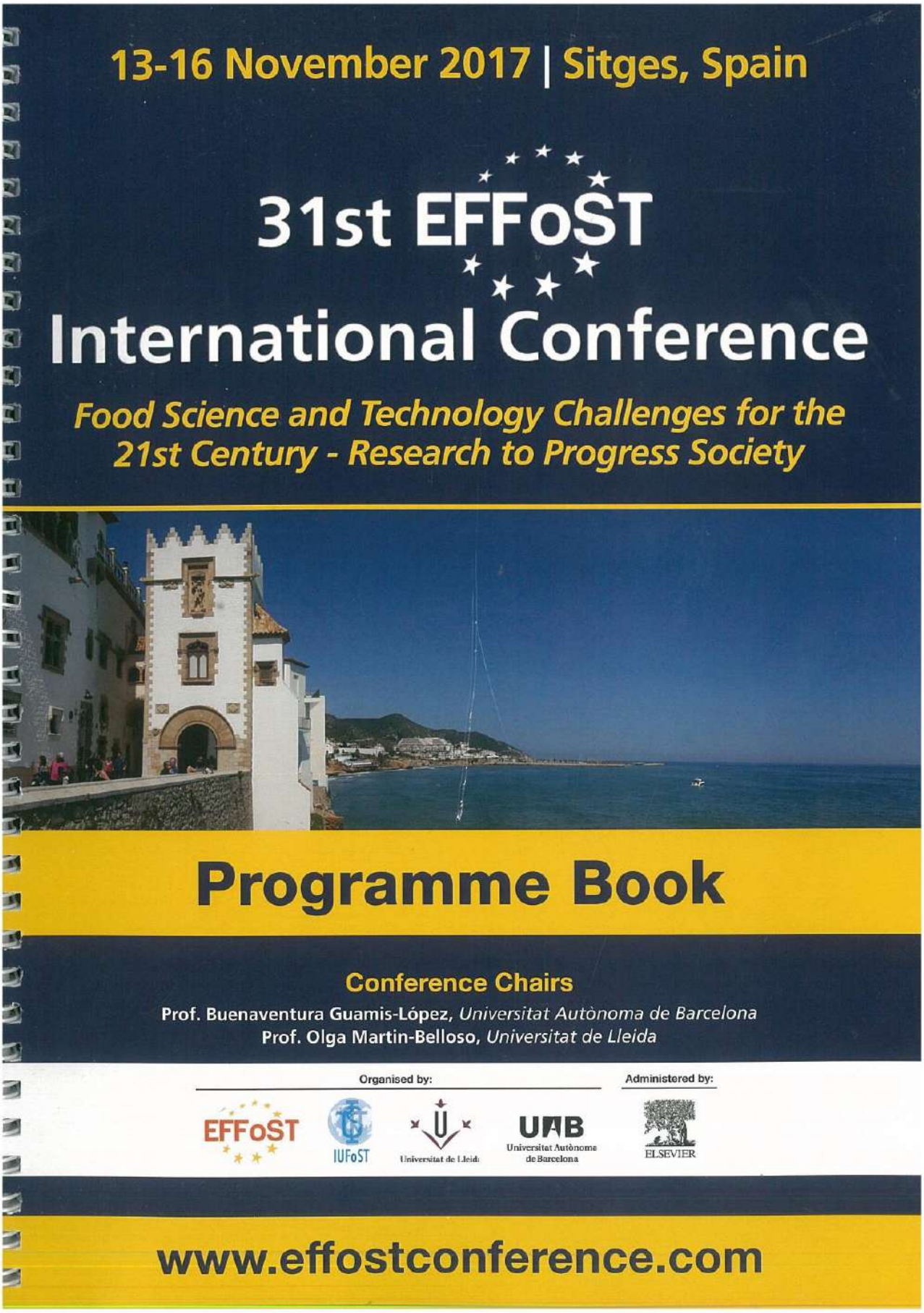
NOVENA. Se han identificado los principales compuestos organosulfurados en orina tras la ingesta de ajo negro y cebolla negra en humanos sanos. Entre los

compuestos mayoritarios, destacan la isoaliína, la metiína y el S-(2-carboxipropil)-L-cisteína tras el consumo de ajo negro y la isoaliína, la metiína y el trans-S-(1-propenil)-L-cisteína tras el consumo de cebolla negra. Además, se han propuesto, por primera vez, rutas de degradación de los compuestos organosulfurados en el organismo tras el consumo de estos productos.

DÉCIMA. Los cambios producidos en el perfil de compuestos fenólicos y organosulfurados durante el proceso de elaboración de ajo negro y cebolla negra tienen un importante efecto en la bioaccesibilidad, metabolismo y excreción de los mismos, lo que implicaría una modificación en su potencial biológico.

ANEXOS

ANEXO I: COMUNICACIONES A CONGRESOS



ANEXO I

[P2.096]	**Improving the nutritional and functional potential of fermented cereal-based beverages by new technological approaches** M.L. Pacala*[1], L. Favier[2], Y. Kadmi[2,3], A.S. Sipos[1], *[1]Lucian Blaga University of Sibiu, Romania, [2]Ecole Nationale Superieure de Chimie de Rennes, France, [3]Institut Charles Viollette, France, [4]Universite d'Artois, France, [5]Institut Charles Viollette de Lille, France, [6]Université de Lille, France*
[P2.097]	**Plant sterol enriched milk-based fruit beverages with or without galactooligosaccharides: Plant sterol stability and bioaccessibility** V. Blanco-Morales*, G. Lopez-Garcia, A. Cilla, G. Garcia-Llatas, R. Barbera, M.J. Lagarda, A. Alegria, *University of Valencia, Spain*
[P2.098]	**European and US food sanitary measure relating Listeria monocytogenes and Salmonella spp. in certain RTE meat products: An equivalence study** D. Neri*[1], S. Antoci[1], M. Di Leonardo[1], A.B. Ciorba[2], R. D'Aurelio[1], G.A. Santarelli[1], A. Giovannini[1], I. Del Matto[1], V.A. Prencipe[1], F. Pomilio[1], L. Lannetti[1], G. Migliorati[1], *[1]Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Italy, [2]Ministero della Salute Direzione Generale per l'Igiene e la Sicurezza degli Alimenti e la Nutrizione (DGISAN) - Ufficio 2 - Igiene degli alimenti ed esportazione Roma, Italy*
[P2.099]	**A multiscale approach to understand dough microstructure formation during process; from protein network to dough rheology** J. Fontaine*[1,2], E. Lancelot[1], D. Dellavalle[1], J. Grua-Priol[1], J. Cheio[2], A. Le-Bail[1], *[1]UBL (Universite Bretagne Loire), France, [2]VMI, France*
[P2.100]	**Characterization of primary and secondary metabolites in black onion, a novel derived product from fresh shallot onions (*Allium ascalonicum* L.)** J.M. Moreno-Rojas*, A. Moreno-Ortega, J.L. Ordoñez, G. Pereira-Caro, *IFAPA, Spain*
[P2.101]	**Targeted emulsion design for controlling lipolysis and carotenoid bioaccessibility kinetics** S.H.E. Verkempinck*[1], L. Salvia-Trujillo[1], C. Carrillo[2], L.G. Moens[1], M.E. Hendrickx[1], T. Grauwet[1], *[1]KU Leuven, Belgium, [2]University of Burgos, Spain*
[P2.102]	**Rheological and surface properties of edible coating suspensions with liposomal and non-liposomal antioxidant rutin** A. Silva-Weiss, A. Celedón, W. Silva, F. Osorio*, B. Giménez, *Universidad de Santiago de Chile, Chile*
[P2.103]	**Analysis of α-dicarbonyl compounds and volatile flavor compounds using Maillard reaction model systems** K-G. Lee*[1], J-Y. Cha[1], M-K. Kim[2], *[1]Dongguk University, Republic of Korea, [2]Chonbuk National University, Republic of Korea*
[P2.104]	**Combining cryogenic freezing and modified atmosphere packaging: an innovative process for improving food preservation quality** S. Guri*[1], A. Callens[2], M.J. Pons[1], *[1]Carburos Metálicos-Air Products Group, Spain, [2]Air Products N.V./S.A.,, Belgium*
[P2.105]	**Development of an in vitro model to form mature biofilms of *Listeria* spp. on food-contact surfaces** C. Ripolles-Avila, A.S. Hascoët, A.E. Guerrero-Navarro, M. Martínez-Garcia*, J.J. Rodríguez-Jerez, *Universitat Autònoma de Barcelona, Spain*
[P2.106]	**Babassu oil extraction with pressurized ethanol - batch process** N.A. Oliveira*, Y.D. Jacon, H. Fukumasu, A.L. Oliveira, *University of São Paulo, Brazil*
[P2.107]	**Effect of High intensity Light Pulses (HILP) on the surface microflora of salmon (*Salmo salar*) and cod (*Gadus morhua*)** S. Pedros-Garrido*[1,2], J.A. Beltran[2], J.G. Lyng[3], D. Bolton[4], N. Brunton[3], P. Whyte[1], *[1]University College Dublin, Ireland, [2]University of Zaragoza, Spain, [3]University College Dublin, Ireland, [4]Teagasc Food Research Centre, Ireland*
[P2.108]	**Multiple emulsions with olive leaves extract as fat replacers in meat systems** B. Gimenez[1], E. González[2], A. Silva-Weiss[1], F. Osorio*[1], S. Cofrades[3], P. Robert[2], *[1]Universidad de Santiago de Chile, Chile, [2]Universidad de Chile, Chile, [3]ICTAN-CSIC, Spain*
[P2.109]	**Screening of probiotic bacteria for the fermentation of goat's milk** R. Muelas, P. Monllor, A. Martí, G. Romero, J.R. Diaz, E. Sendra*, *Universidad Miguel Hernández, Spain*
[P2.110]	**Application of pulses into the gluten free biscuit formulation: Challenges and Opportunities** C.B. Barnard*, V.S. Stojceska, *Brunel Univeristy London, UK*
[P2.111]	**Project HELIX: development, implementation and management of a food safety and technical knowledge transfer approach for food-sector small and medium sized enterprises (SMEs) in Wales, UK** E.C. Redmond*, D. Mumford, S. Mayho, D.C. Lloyd, *Zero2Five Food Industry Centre, Cardiff Metropolitan Univeristy, UK*

ANEXO I

Characterization of Primary and Secondary Metabolites in Black Onion, a novel derived product from Fresh Shallot Onions (*Allium ascalonicum* L.)

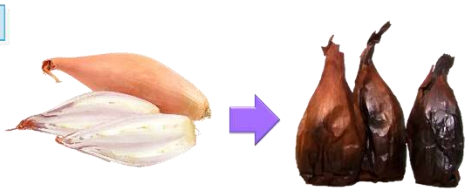
IFAPA Instituto de Investigación y Formación Agraria y Pesquera

José Manuel Moreno-Rojas, Alicia Moreno-Ortega, José Luis Ordoñez, Gema Pereira-Caro

Department of Food and Health, IFAPA-Alameda del Obispo, 14071, Córdoba, Spain

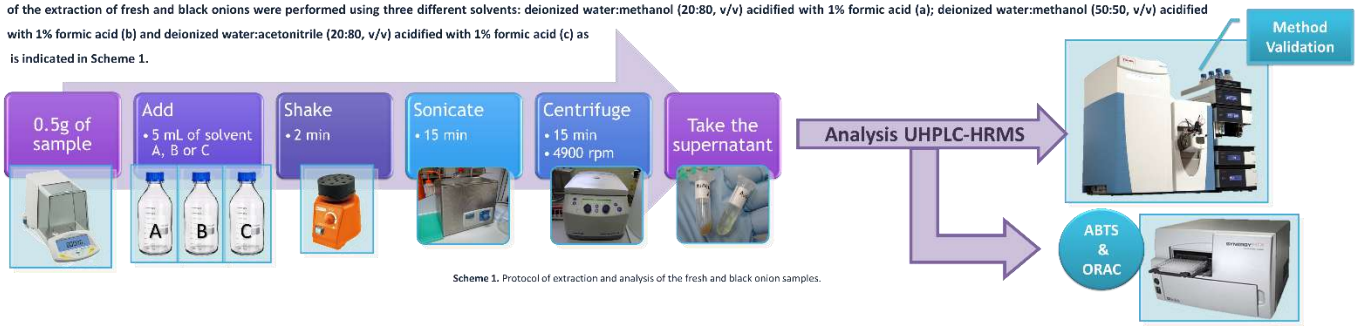
INTRODUCTION

Onions are one of the most important horticultural crops and is used as food, spice and medicinal plant almost worldwide. Onion bulbs are a rich source of fructooligosaccharides and amino acids as well as health-promoting constituents such as flavonoids and a huge variety of organosulfur compounds accounting to its well-known nutritional properties[1]. Black onion, made from fresh onion with an industrial process involving controlled temperature and humidity[2], is a novel derived product potentially used as functional food with improved organoleptic and nutritional properties. The aim of this work was to identify and quantify the phenolic compounds, amino acids and organosulfur compounds of fresh shallot onion and black onion by UHPLC-PDA-HRMS. Also, the antioxidant activity measured by ABTS and ORAC assays were carried out.



MATERIALS AND METHODS

Fresh shallot onions and black onions were obtained from local markets. Both fresh and black onions were first frozen in liquid N_2 to avoid enzymatic activity, freeze dried and grinded afterwards. The optimization of the extraction of fresh and black onions were performed using three different solvents: deionized water:methanol (20:80, v/v) acidified with 1% formic acid (a); deionized water:methanol (50:50, v/v) acidified with 1% formic acid (b) and deionized water:acetonitrile (20:80, v/v) acidified with 1% formic acid (c) as is indicated in Scheme 1.



Scheme 1. Protocol of extraction and analysis of the fresh and black onion samples.

RESULTS

OPTIMIZATION OF THE EXTRACTION METHOD

A total of 7 flavonoids, 21 amino acids and 2 organosulfur compounds were selected to perform the optimization of the extraction method from fresh onion and black onion and the validation study. Figure 1 shows the extraction yields or recovery (%) of the selected compounds as representative components of flavonoids, amino acids and organosulfur compounds in black and fresh onion.

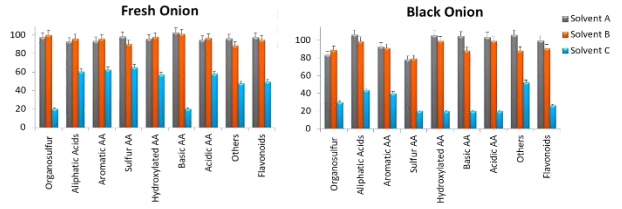


Figure 1. Recovery of flavonoids, amino acids and organosulfur compounds using three different solvents.

Based on these results, the subsequence steps for the method validation and quantification were done using as extraction solvent A which is a mixture of deionized water and methanol (20:80, v/v) acidified with 1% formic acid

ANTIOXIDANT CAPACITY

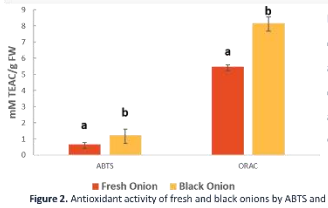
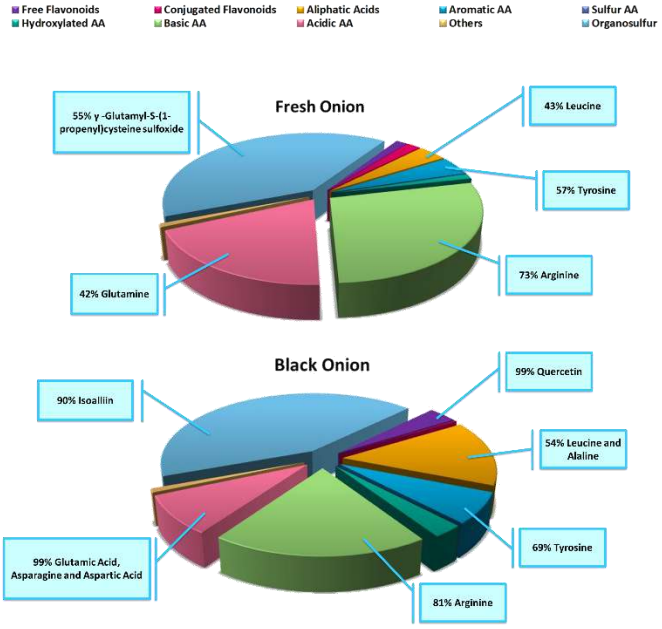


Figure 2. Antioxidant activity of fresh and black onions by ABTS and ORAC assays.

In order to determine the antioxidant potential of black onion compared with fresh onion, two antioxidant assays were performed, ABTS and ORAC assays. Black onion extract showed significant higher antioxidant activity measured by ABTS and ORAC assays compared with fresh onion samples (Figure 2).

IDENTIFICATION AND QUANTIFICATION

A total of 10 flavonoids, 21 amino acids and 22 organosulfur compounds were identified and quantified in black and/or fresh onions. Figure 3 shows the difference on the composition of flavonoids, amino acids and organosulfur compounds between both onions. The main flavonoids in fresh onion are quercetin-4-*O*-glucoside and two isomers of quercetin-*O*-diglucoside. Free quercetin is predominant in black onion. Aliphatic and hydroxilated amino acids are presented in higher quantities in black onion while basic and acidic amino acids are higher in fresh onions. Further, the main organosulfur compound in black onion is isoalliin.



CONCLUSIONS

Two selective, sensitive, and precise UHPLC-HRMS methods were successfully adapted and validated to identify and quantify phenolic compounds, amino acids and organosulfur compounds in black and fresh onions, allowing the determination of 53 primary and secondary metabolites in both types. These results gives a detailed profile of potential bioactive metabolites in black onion, providing a basis for the potential health benefits of a novel product from fresh shallot onion, highlighting its use as functional food.

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2.Toledano-Medina, M.A., et al.Food Chemistry, 199, 135-139 (2016).

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ACKNOWLEDGEMENTS

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This work has been funded by IFAPA through the Project PP.AVA.AVA201601.20.

ANEXO I

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ANEXO I

Changes in bio-functional compounds and antioxidant capacity during the production of three varieties of black onion

IFAPA Instituto de Investigación y Formación Agraria y Pesquera

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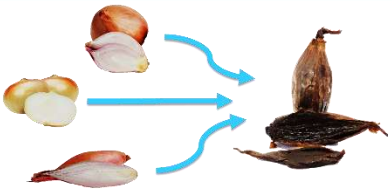
INTRODUCTION

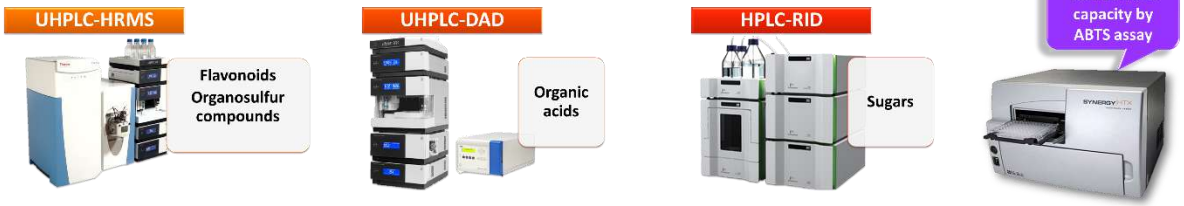
A new derived product from onion, black onion, has been developed by processing (aging) raw onion in a temperature- and humidity-controlled room without using any artificial additives[2]. During the manufacturing process, a series of modifications on the compositional and sensory characteristics of the fresh product will be produced. The aim of this work was to study the impact of the heat treatment on the antioxidant activity and on flavonoids, organosulfur compounds, organic acids and sugars profiles during the production of black onion from raw onion of three different onion varieties.



MATERIALS AND METHODS

Three varieties of onion (Shallot, Chata and Echalion) were processed to obtain black onion. The manufacture process consisted in a heating process where bulbs were subjected to relative humidity conditions close to saturation (90-95% RH) and at a temperature between 65 and 70ºC in the presence of oxygen for 28 days. A final drying step were applied to obtain the final product (24 h at 50ºC). Samples were taken during the manufacturing process at different time points. Samples were grinded by using a cryogenic grinder with liquid nitrogen mill equipment and kept at -80˚C until analysis of flavonoids, organosulfur compounds, organic acids and individual sugars by using UHPLC-HRMS, UHPLC-DAD and HPLC-RID analysis, respectively. In addition, the antioxidant capacity was measured by ABTS assay.

T0 → Raw Onion
T1 → 7 days
T2 → 14 days
T3 → 21 days
T4 → 28 days
T4S → Black Onion



RESULTS: EFFECT OF THE HEATING PROCESS

ON FLAVONOIDS

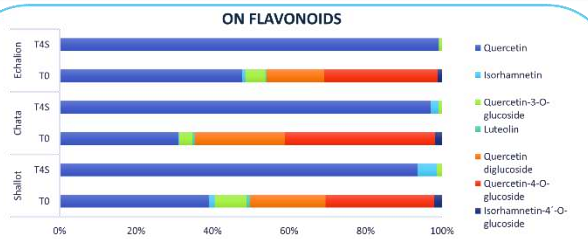


Figure 1. Percentage of individual flavonoids quantified in raw (T0) and black onion (T4S).

There were important changes on flavonoid composition during the production of black onion. As shown in Figure 1, principal flavonoids in fresh onion samples were free quercetin, quercetin-3-*O*-glucoside and quercetin-4-*O*- glucoside for the three varieties. At stage T4S, quercetin was the major flavonoid present in black onion samples in all varieties.

ON ORGANOSUFUR COMPOUNDS

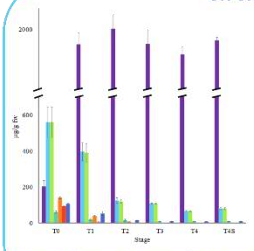


Figure 2. Changes on organosulfur compound profiles during the elaboration of black onion from shallot variety

During the production of black onion there was a considerably decrease of two isomers γ-glutamil-*S*-(propenyl)-L-cysteine sulfoxides and a concomitant significant increase of isoalliin in all varieties. This compound, predominant in black onion, accounted for more than 80% of the total content of organosulfur compounds in all onion varieties (Figure 2).

ON SUGAR PROFILES

Figure 3 showed that fructose content was significantly increased from T0 to T1 with a concomitant decrease of sucrose levels, which was then constant until the end of the production process. A similar behavior was observed for glucose content in all onion varieties.

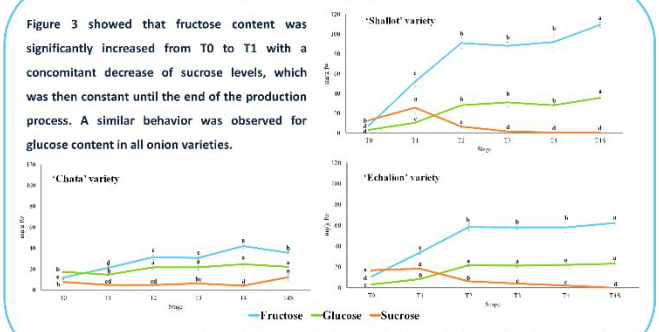


Figure 3. Evolution of sugars during production process of black onion for 'Shallot', 'Chata' and 'Echalion' onions.

ORGANIC ACIDS & ANTIOXIDANT ACTIVITY

The most abundant organic acids in raw onion samples were malic, tartaric and oxalic acids which their concentration increased significantly from T0 to T4S in all samples varieties. Moreover, onion samples showed significant increase on the antioxidant activity during the heating process (from T0 to T4S) by ABTS assay.

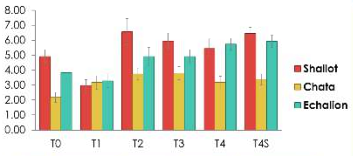


Figure 4. Antioxidant activity during production process of black onion measured by ABTS

OVERALL CHANGES DURING BLACK ONION PRODUCTION

In order to explore the influence of the heating process during the production of black onion a principal component analysis (PCA) was applied. Figure 4 shows a clear discrimination among samples based on the different stages of the process and revealed that the main differences between onion samples was between T0 (initial stage) and the rest of time points while less differences were found among T1 up to T4S.

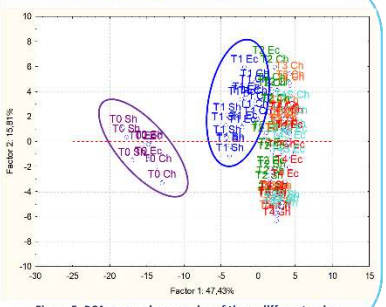


Figure 5. PCA comparing samples of three different onion varieties during black onion production.

CONCLUSIONS

The heating process applied to black onion production induces several phytochemical changes in the three onion varieties which turn into total flavonoids losses and gains in organosulfur compounds including isoalliin as well as into fructose and glucose content.

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ACKNOWLEDGEMENTS

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ANEXO I

Sociedad Española de Dietética y Ciencias de la Alimentación

Ilustre Colegio Oficial de Médicos de Madrid

El presente certificado acredita que

Dª Alicia Moreno Ortega

ha participado como **PONENTE**, en el curso

XXIII Jornadas de Nutrición Práctica
XIII Congreso Internacional de Nutrición, Alimentación y Dietética

incluido en el **PROGRAMA DE FORMACIÓN CONTINUADA** de este ilustre Colegio, celebrado en Madrid, el 3 y 4 de abril de 2019, con una duración total de **15 horas lectivas.**

Y para que conste, firmo el presente certificado en Madrid, a 4 de abril de 2019.

ILUSTRE COLEGIO OFICIAL DE MÉDICOS DE MADRID · FORMACIÓN CONTINUADA ·

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II Congreso de Jóvenes Investigadores en Ciencias Agroalimentarias

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La Universidad de Almería certifica que

Alicia Moreno Ortega

presentó la comunicación oral titulada:

"Evolución del perfil de compuestos y de la actividad antioxidante durante el proceso de elaboración de la cebolla negra"

en el Panel Científico de Seguridad y Calidad Alimentaria, Química Agroambiental, Nutrición y Salud del II Congreso de Jóvenes Investigadores en Ciencias Agroalimentarias, celebrado el 17 de octubre de 2019 en la Universidad de Almería.

En Almería, a 17 de octubre de 2019,

Diego Luis Valera Martínez
Vicerrector de Investigación, Desarrollo e Innovación
Universidad de Almería

Juan Reca Cardeña
Director de CIAIMBITAL Centro de Investigación en Agrosistemas Intensivos Mediterráneos y Biotecnología Agroalimentaria
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ANEXO I

UNIVERSIDAD D CÓRDOBA

La Vicerrectora de Posgrado e Innovación Docente de la Universidad de Córdoba

ACREDITA que

Alicia Moreno Ortega, con DNI **nº 32730168H ha asistido al IX Congreso Científico de Investigadores en Formación**, con el título NUEVOS DESAFÍOS, NUEVAS OPORTUNIDADES, organizado por las Escuelas de Doctorado Educo y eidA3 (sede Córdoba) de la Universidad de Córdoba, celebrado en Córdoba los días 3 a 6 de mayo de 2021 y **ha presentado la comunicación en formato poster** titulada "Caracterización Funcional De Un Nuevo Producto, La Cebolla Negra. Estudios De Bioaccesibilidad Y Potencial Saludable "

Fdo: Julieta Mérida García

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ANEXO I

IX Congreso Científico de Investigadores en Formación de la Universidad de Córdoba

Córdoba, 3 a 6 de mayo 2021

CARACTERIZACIÓN FUNCIONAL DE UN NUEVO PRODUCTO, LA CEBOLLA NEGRA. ESTUDIOS DE BIOACCESIBILIDAD Y POTENCIAL SALUDABLE.

***Moreno-Ortega, A.*[1,2], *Moreno-Rojas, R.*[1], *Pereira-Caro, G.*[2], *Moreno-Rojas, J.M.*[2]**

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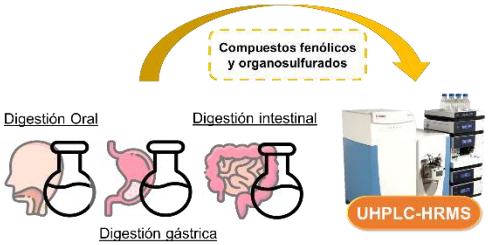
INTRODUCCIÓN

La cebolla negra se obtiene sometiendo la cebolla fresca a condiciones de temperatura y humedad controladas durante un periodo de tiempo determinado, obteniendo finalmente un nuevo producto de color oscuro, sabor más dulce y menos pungente y con una textura más suave que permite, además, aprovechar los excedentes de producción de este cultivo y aumentar su vida útil [1]. Dada la novedad de este producto, el objetivo principal era la caracterización de su composición, haciéndose necesario saber cómo afecta el proceso de elaboración de la cebolla negra a estos compuestos, entre ellos, compuestos bioactivos como la quercetina o la isoaliína, ya identificados en la cebolla fresca y cuyo consumo está relacionado con diversos efectos beneficiosos para la salud [2]. Sin embargo, para que estos compuestos de interés tengan efectos beneficiosos *in vivo*, es necesario que sean bioaccesibles, es decir que, tras la ingesta de cebolla fresca o negra, sean liberados de la matriz alimentaria para estar disponibles para su absorción en el tracto gastrointestinal, otro de los objetivos de este estudio.

MATERIAL Y MÉTODOS

Se tomaron muestras a lo largo del proceso de elaboración de la cebolla negra (0, 7, 14, 21 y 28 días). Estas muestras fueron molturadas con un molino criogénico y se mantuvieron a -80ºC hasta su extracción y análisis mediante diferentes técnicas.



La evaluación de la bioaccesibilidad y de la evolución del perfil de compuestos bioactivos presentes en **cebolla fresca** y **cebolla negra** se realizó sometiendo estas matrices alimentarias a un proceso de digestión gastrointestinal *in vitro*.

RESULTADOS Y DISCUSIÓN

CARACTERIZACIÓN DEL PERFIL DE COMPUESTOS DURANTE EL PROCESO DE ELABORACIÓN DE LA CEBOLLA NEGRA

La composición de flavonoides cambia drásticamente durante el proceso de elaboración, disminuyendo la concentración total hasta 12 veces, y siendo la quercetina libre el compuesto fenólico mayoritario en la cebolla negra.

En cuanto a los compuestos organosulfurados, se produce un incremento significativo de la concentración de isoaliína, con la consecuente disminución de sus precursores γ-glutamil -S- (propenil)-L-cisteína sulfóxidos (Fig. 1). Asimismo, la concentración de fructosa aumentó hasta 14 veces durante este proceso, contribuyendo al dulzor característico de este producto (Fig. 2)

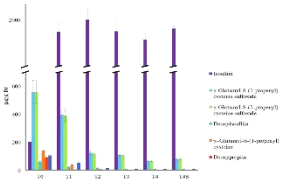


Figura 1. Cambios en el perfil de compuestos organosulfurados durante el proceso de elaboración de la cebolla negra

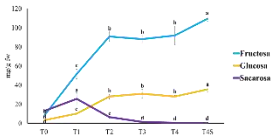


Figura 2. Evolución de los azúcares durante el proceso de elaboración de la cebolla negra

Por otro lado, el ácido orgánico mayoritario que encontramos en el producto final fue el ácido tartárico, mientras que se observa un descenso muy marcado en el contenido de glutamina (>99%), ya que es uno de los precursores principales de la reacción de Maillard, responsable del color oscuro tan particular de la cebolla negra. [3].

EVALUACIÓN DE LA BIOACCESIBILIDAD Y EL PERFIL DE COMPUESTOS BIOACTIVOS MEDIANTE UNA DIGESTIÓN GASTROINTESTINAL *IN VITRO*

La cebolla fresca presenta una disminución significativa de flavonoides glucosilados durante el proceso digestivo, mientras que la quercetina y la isorhamnetina libres aumentan su concentración, presentando índices de bioaccesibilidad de 165 y 210.7%, respectivamente. Mientras que, en la cebolla negra, el contenido de polifenoles de partida era considerablemente inferior al de la cebolla negra, disminuyendo progresivamente durante el proceso digestivo y mostrando un índice de bioaccesibilidad total del 41.1%.

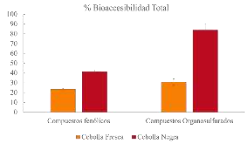


Figura 3. Índices de bioaccesibilidad del total de compuestos fenólicos y organosulfurados en cebolla fresca y cebolla negra

Por otro lado, el contenido de compuestos organosulfurados de la cebolla fresca se vio afectado en mayor medida durante las etapas de digestión oral e intestinal, que en la gástrica. Sin embargo, durante la digestión de la cebolla negra se observa una tendencia más estable de los compuestos durante el proceso digestivo, con una bioaccesibilidad total final del 83.3%.

CONCLUSIONES

El proceso térmico aplicado a la cebolla fresca durante la elaboración de cebolla negra induce una serie de cambios fitoquímicos importantes los cuales han sido caracterizados durante este estudio, mostrando, además, que el proceso de obtención de cebolla negra ejerce un efecto positivo sobre la bioaccesibilidad de los compuestos bioactivos de este nuevo producto. Asimismo, se ha evaluado la influencia del proceso digestivo en el perfil de compuestos bioactivos presentes en cebolla fresca y cebolla negra.

REFERENCIAS

[1] K.L. Ríos-Ríos et al. *Trends Food Sci Technol*, 88 (2019) 459-467. [2] P. S Bisen, M. Emerald. *Curr Nutr Food Sci*. 12(3) (2016) 190-199. [3] A. Moreno-Ortega et al. *Food Chem*. 311 (2020) 125958. [4] A. Moreno-Ortega et al. *Foods*. 10 (2021) 337.

AGRADECIMIENTOS

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ANEXO II: CONVENIO DE COTUTELA

UNIVERSIDAD D CÓRDOBA

UNIVERSITÀ DI PARMA

CONVENIO DE COTUTELA DE TESIS

El Rector de la Universidad de Córdoba, España (UCO), Dr. José Carlos Gómez Villamandos

Y el Rector de la Universidad de Parma, Prof. Paolo Andrei.

Las dos partes se reconocen la capacidad legal necesaria para formalizar y firmar este Convenio,

De acuerdo con las regulaciones siguientes:

- En Italia, la ley 210 del 03/07/1998, por el que se indica que los procedimientos para la activación de Estudios de Doctorados están regulados por cada universidad en total autonomía organizativa, didáctica e investigadora; y el Reglamento Ministerial para Estudios de Doctorados aprobado por el Decreto Ministerial número 45.del 02.08.2013

- En España, el Real Decreto 99/2011, de 28 de enero, por el que se regulan los Estudios de Doctorado; la Normativa Reguladora de los Estudios de Doctorado de la Universidad de Córdoba; los Estatutos de la Universidad de Córdoba; así como la Ley Orgánica 6/2001, de 21 de diciembre, de Universidades.

DECLARAN:

Que las dos instituciones desean instaurar y desarrollar una cooperación científica que favorezca la movilidad de los candidatos al doctorado, así como iniciativas de colaboración en materia de investigación.

En este contexto, las dos instituciones

ACUERDAN

Establecer un convenio para la cotutela de la Tesis Doctoral de Dª **Alicia Moreno Ortega**, con el Título: **Caracterización nutricional de un nuevo producto, cebolla negra, derivada de la cebolla (*Allium cepa*)**

CONDICIONES ADMINISTRATIVAS

Artículo 1. Inscripción

La doctoranda Dª **Alicia Moreno Ortega** deberá estar inscrito para la realización de su tesis en régimen de cotutela, a partir del curso académico **2019-2020**, durante un periodo no inferior a dos cursos académicos ni superior a cinco. El periodo de trabajo estará repartido entre las dos instituciones. El tiempo de estancia en cada una de las dos universidades no será inferior a doce meses; podrá realizarse de una sola vez o por periodos de al menos 2 meses.

AGREEMENT FOR THE JOINT SUPERVISION OF A THESIS

The Chancellor of the University of Córdoba, Spain (UCO), Dr. José Carlos Gómez Villamandos

And the Chancellor of the University of Parma, Prof. Paolo Andrei.

Each acknowledging that the other is legally empowered to enter into this agreement,

In due observance to the following regulations:

- The Law no. 210 of 03/07/1998, which provides that the procedures for the activation of Ph.Ds are regulated by each university in full organizational autonomy, teaching and research;

- the Ministerial Regulations for PhD, approved by Ministerial Decree n. 45 of 02.08.2013

- The Spanish Royal Decree 99/2011, of January 28, by which the PhD is regulated; the regulations of Doctoral Studies at the University of Córdoba; the Statutes of the University of Cordoba; as well as the Organic Law 6/2001 of 21 December, of Universities.

HEREBY DECLARE:

That the two institutions seek to develop and enhance their scientific cooperation with a view to favouring the mobility of PhD candidates, and to embark upon joint research initiatives.

Accordingly, the two institutions

AGREE

To undertake the joint supervision of the Doctoral Thesis of Ms **Alicia Moreno Ortega**, entitled: **Evaluation of nutritional properties of black onion, a new product derived from fresh onion (*Allium cepa*).**

ADMINISTRATIVE TERMS

Article 1. Registration

The PhD Candidate, Ms. **Alicia Moreno Ortega** shall be registered for the preparation of a jointly-supervised thesis, from the academic year **2019-2020** onwards, for a period of no less than two academic years and no more than five. The study period will be divided between the two institutions. A minimum of twelve months shall be spent at each University all at once, or in periods of at least two months.

La doctoranda Dª **Alicia Moreno Ortega** deberá cumplir todos los requisitos establecidos por ambas instituciones en relación con la admisión al programa de doctorado, su progreso y evaluación.

Artículo 2. Derechos de inscripción

La doctoranda Dª **Alicia Moreno Ortega** estará inscrito en las dos universidades. Abonará los derechos de inscripción en la **Universidad de Córdoba** y será exonerado de los mismos en la **Universidad de Parma.**

Artículo 3. Cobertura social

La doctoranda Dª **Alicia Moreno Ortega** se beneficiará del régimen de seguridad social vigente en la institución de origen **(Universidad de Córdoba)**

El doctorando será responsable de su cobertura sanitaria en caso de enfermedad y accidente durante su estancia en cada una de las universidades. Así mismo, será responsable de adquirir un seguro de repatriación en caso de enfermedad o accidente durante los periodos de movilidad, de acuerdo con la normativa de cada universidad. Finalmente, estará obligado a ser titular de una garantía de responsabilidad civil válida para la duración de su estancia en el seno de la universidad en la que realice las estancias.

Artículo 4. Financiación de los gastos del tribunal

La universidad en que se celebre la Tesis financiará los gastos del Tribunal, de acuerdo con lo estipulado en la misma para la modalidad de mención Doctorado Internacional.

CONDICIONES ACADÉMICAS

Artículo 5. Elaboración de la tesis

El doctorando efectuará su trabajo de investigación bajo el control y responsabilidad de los siguientes directores de tesis, en cada una de las dos universidades:

Por la **Universidad de Córdoba**
Director: Dr. **José Manuel Moreno Rojas**
NIF:80.146.931-G
Programa de Doctorado: **Biociencias y Ciencias Agroalimentarias**
Co-directora: **Mª Gema Pereira Caro**
NIF: **77.337.783-F**
Programa de Doctorado: **Biociencias y Ciencias Agroalimentarias**
Por la **Universidad de Parma**
Director: Prof. **Daniele Del Rio**
Passport number: YA4098699
Departmento: **Scienze Medico-Veterinarie**
Co-director: Dr. **Pedro Miguel Mena Parreño**
Passport number: PAA589895
Departmento: **Scienze Medico-Veterinarie**

Estos profesores se comprometen a ejercer plena, coordinada y conjuntamente la dirección de la citada

The PhD Candidate, Ms. **Alicia Moreno** Ortega must meet all the requirements of both institutions regarding admission to the doctoral program, their progress and evaluation.

Article 2. Registration Fees

The PhD Candidate, Ms. **Alicia Moreno Ortega** shall be registered at both Universities. She will pay registration fees at the **University of Córdoba** and will be exempted from the payment of fees at the **University of Parma**.

Article 3. Social Security Cover

The PhD Candidate, Ms. **Alicia Moreno Ortega** will be covered by the social security regime in force at the home institution **(University of Córdoba)**

The doctorate student will be responsible for their health coverage in case of illness or accident during their stay at each of the universities. Likewise, they are responsible for purchasing repatriation insurance in case of illness or accident during periods of mobility, according to the rules of each university. Finally, they will be obliged to hold valid civil liability insurance for the duration of their stay within the university where they are studying.

Article 4. Payment of the expenses for the PhD Examining Committee

The university where the thesis is defended shall finance the committee's expenses, according to the provisions on theses determined under the International Doctorate framework.

ACADEMIC TERMS

Article 5. Thesis preparation

The PhD candidate will undertake research work under the guidance and responsibility of the following thesis supervisors at each University:

At the **University of Cordoba**
Supervisor: Prof. **José Manuel Moreno Rojas**
NIF 80.146.931-G
Doctoral Program; **Biociencias y Ciencias Agroalimentarias**
Co-supervisor: **Mª Gema Pereira Caro**
NIF: **77.337.783-F**
Programa de Doctorado: **Biociencias y Ciencias Agroalimentarias**
At the **University of Parma**
Supervisor: Prof. **Daniele Del Rio**
Passport number: YA4098699
Department: **Scienze Medico-Veterinarie**
Co-supervisor: Dr. **Pedro Miguel Mena Parreño**
Passport number: PAA589895
Department: **Scienze Medico-Veterinarie**

The above supervisors undertake to provide the fully coordinated joint supervision of the mentioned Doctoral Thesis. The positive assessment of both thesis

Tesis Doctoral. El Informe positivo de ambos supervisores será un prerrequisito necesario para la admisión de la Tesis a su examen final.

Artículo 6. Condiciones de presentación y defensa

El tribunal ante el que se defenderá la Tesis se decidirá de mutuo acuerdo entre las dos universidades y su composición se regulará por la normativa vigente en la universidad en que se presente. Debe incluir a profesores de ambas instituciones.

La tesis: Será defendida en la universidad de **Córdoba** Será redactada y defendida en español y completada por un resumen en inglés

El título: Aprobada la tesis, ambas universidades se comprometen a expedir los correspondientes títulos de Doctor. En el título de Doctor se incluirá la mención de "Tesis en régimen de cotutela con la Universidad de **Parma**"

Registro, derechos de autoría y reproducción: El doctorando se compromete a respetar la normativa vigente en cada uno de los países para el registro, derechos de autoría y reproducción de la Tesis Doctoral.

Artículo 7. Vigencia del Convenio

Este acuerdo entrará en vigor el día en que esté firmado por ambas partes. Será válido por 5 años, renovado o terminado en cualquier momento, por mutuo acuerdo entre las partes o a instancias de cualquiera de las dos universidades.

Firmas

En prueba de lo cual, este Acuerdo se firma por duplicado con el mismo contenido, en español y en inglés, quedando un original en poder de cada institución.

supervisors shall be a necessary prerequisite for admission to the final examination

Article 6. Submission and defence of the thesis

The panel before which the thesis must be defended will be appointed by agreement between the two universities and its composition shall be governed by the laws in force in the country of the university where the thesis defense will take place. It should include professors from both universities

Thesis: The thesis shall be read at the **University of Córdoba** It will be written and read in **Spanish** and will include an extended summary in English

Degree: Once the thesis has been successfully examined, both universities shall award the degree of Doctor. The PhD degree certificate will include the mention: "Jointly supervised with the University of **Parma**"

Registration and copyright: The PhD candidate undertakes to comply with the regulations in force in each country with regard to the registration and copyright of the Doctoral Thesis.

Article 7. Term of the Agreement

This agreement shall enter into force on the date of signature by both parties. It will be valid for five years, renewable by mutual agreement of the parties or terminated at any time by mutual agreement between the parties or at the request of either of the two universities.

Signature

In witness whereof, this Agreement is hereby signed in duplicate with the same content, in Spanish and English, one being held by each of the parties.

Córdoba, 4/11/2019 Dr. D. José Carlos Gómez Villamandos Rector de la Universidad de Córdoba.	Parma, ... Prof. Paolo Andrei. The Chancellor of the University of Parma
Dr. Arturo F. Chica Pérez Director de la Escuela de Doctorado Universidad de Córdoba.	Prof. Chiara Dall'Asta Director of the Graduate School University of Parma

Dr. José Manuel Moreno Rojas Director de Tesis Universidad de Córdoba	Prof. Daniele Del Rio Thesis supervisor University of Parma
Dra. Mª Gema Pereira Caro Co-Directora de Tesis Universidad de Córdoba	Dr. Pedro Miguel Mena Parreño Thesis co-supervisor University of Parma

Dª **Alicia Moreno Ortega**

The doctorate candidate, NIF 32.730168H