Efecto del aceite en la biodisponibilidad de los compuestos fenólicos del tomate

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ABREVIATURAS

AUC_{last}	Curva de concentración *vs.* tiempo desde tiempo 0 hasta la última concentración detectable
CBG	β-glucosidasa citosólica
C_{max}	Concentración máxima
COMT	Catecol-*O*-metiltransferasa
HPLC	Cromatografía líquida de alta resolución
HPLC-MS/MS	Cromatografía líquida de alta resolución acoplada a espectrometría de masas
LOD	Límite de detección
LOQ	Límite de cuantificación
LPH	Lactasa-floricin hidrolasa
MS	Espectrometría de masas
Mt	Millones de toneladas
OF	Salsa de tomate sin aceite
ROOE	Salsa de tomate con aceite de oliva refinado
ROS	Especies reactivas de oxígeno
SPE	Extracción en fase sólida
SULT	Sulfotransferasas
SGLT1	Transportador de glucosa asociado a sodio
t_{max}	Tiempo máximo para llegar a la concentración máxima
UGT	Uridina-5'-difosfato glucuronosiltransferasas
UHPLC	Cromatografía líquida de ultra alta resolución
UHPLC-MS/MS	Cromatografía líquida de ultra alta resolución acoplada a espectrometría de masas
UV	Ultravioleta
VOOE	Salsa de tomate con aceite de oliva virgen extra

ABBREVIATIONS

AUC_{last}	Concentration-*versus*-time curve from time 0 until the last detectable concentration
CBG	Cytosolic β-glucosidase
C_{max}	Maximum plasma concentration
COMT	Catechol-*O*-methyl transferase
HPLC	High performance liquid chromatography
HPLC-MS/MS	High performance liquid chromatography coupled to tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
LPH	Lactase-phlorizin hydrolase
MS	Mass spectrometry
Mt	Million tons
OF	Tomato sauce without olive oil
ROOE	Tomato sauce enriched with refined olive oil
ROS	Reactive oxygen species
SPE	Solid-phase extraction
SULT	Sulfotranferase
SGLT1	Sodium-glucose linked transporter
t_{max}	Time needed to reach the maximum plasma concentration
UGT	Uridine 5'-diphospho-glucuronosyltransferase
UHPLC	Ultra-high performance liquid chromatography
UHPLC-MS/MS	Ultra-high performance liquid chromatography coupled to tandem mass spectrometry
UV	Ultraviolet
VOOE	Tomato sauce enriched with virgin olive oil

ÍNDICE

RESUMEN

1. Resumen

El tomate es la hortaliza más consumida en España (14.3 Kg/cápita/año), con una repercusión económica muy importante[1]. En el ámbito de la fruta/hortaliza transformada, el tomate también es el número uno en consumo, donde la ingesta per cápita está cuantificada aproximadamente en 3.8 Kg/año para la salsa de tomate y 1.5 Kg/año para el tomate natural envasado[2].

El tomate es uno de los alimentos de origen vegetal más ampliamente utilizados y con muchos usos. Se consume fresco o como productos procesados (enlatados, salsas, zumos, kétchups, etc.)[1]. Los tomates y los productos a base de tomate son ricos en carotenoides, vitamina C, vitamina E, folato y compuestos fenólicos. La cantidad de compuestos bioactivos está muy influenciada por las distintas variedades que existen, además de los factores agronómicos, geográficos y ambientales[3,4]. El consumo regular de tomate se ha correlacionado con una reducción del riesgo de padecer varios tipos de cáncer y enfermedades cardiovasculares[5–7]. Estos efectos beneficiosos se han atribuido a los compuestos bioactivos, particularmente a los carotenoides, licopeno y β-caroteno, y a los compuestos fenólicos[8–10].

Los tomates son una buena fuente de compuestos fenólicos, como los flavonoides y los ácidos fenólicos, entre los que destacan la naringenina (flavanona), el ácido 5-cafeoilquínico (ácido hidroxicinámico) y la rutina (flavonol)[4]. La biodisponibilidad de los compuestos fenólicos en los alimentos viene determinada por diferentes factores tanto del alimento como del individuo[11]. La matriz alimentaria es uno de los factores externos que más condiciona la biodisponibilidad de los diferentes componentes funcionales, pero los datos publicados de los efectos del tratamiento térmico sobre la matriz no son consistentes[12,13].

A pesar de que hoy en día se conoce que el procesado y la presencia de lípidos en la comida[14–18] aumenta la biodisponiblilidad de los carotenoides, existe todavía poca información sobre cómo afectan los tratamientos del alimento al contenido fenólico del tomate[4], y se han realizado pocos estudios en humanos para conocer cómo afecta la forma de administración (tomate crudo o procesado) a la absorción y la excreción de los compuestos fenólicos del tomate[19–21]. También se ha observado que la presencia de ácidos grasos (matriz lipídica) aumenta la biodisponibilidad de los fenoles[22], pero no se

conoce cuál sería el efecto del enriquecimiento con aceites en la biodisponibilidad de los polifenoles del tomate tras su administración en humanos.

1. Abstract

Tomato is the most consumed vegetable in Spain (14.3 kg/capita/year), with a substantial economic impact[1]. In the field of transformed fruit/vegetable, tomato is also number one in consumption, where the intake is quantified approximately in 3.8 kg/capita/year for tomato sauce and 1.5 kg/capita/year for natural canned tomatoes[2].

Tomatoes are one of the most widely used plant-based foods and with many applications. They are eaten raw or as processed products (canned, sauces, juices, ketchups)[1]. Tomatoes and tomato based products are rich in carotenoids, vitamin C, vitamin E, folic acid and phenolic compounds. The amount of bioactive compounds is strongly influenced by the different varieties, and also agronomic, geographic and environmental factors[3,4]. Regular consumption of tomatoes has been correlated with a reduced risk of developing several types of cancer and cardiovascular diseases[5-7]. These beneficial effects are attributed to bioactive compounds, particularly carotenoids, lycopene and β-carotene, and phenolic compounds[8-10].

Tomatoes are a good source of phenolic compounds such as flavonoids and phenolic acids, as naringenin (flavanone), 5-caffeoylquinic acid (hydroxycinnamic acid) and rutin (flavonol)[4]. The bioavailability of phenolic compounds depends on different factors related to the food as well as each individual consumer[11]. The food matrix is one of the external factors that influences the bioavailability of different functional components, but published data on the effects of thermal treatment are not consistent[12,13].

Although it is known that processing and the presence of lipids in food increases the bioavailability of carotenoids[14-18], there is still little information on how treatments affect tomato polyphenol content[4], and few studies have investigated the absorption and excretion of phenolic compounds according to how raw tomato or processed are administered to humans[19-21]. It has also been found that fatty acids increase the bioavailability of phenolics[22], but little is known about the effect of oil addition in the bioavailability of tomato polyphenols in humans.

HIPÓTESIS Y OBJETIVOS

2. Hipótesis y Objetivos

Hipótesis

El procesado de tomate y la adición de una matriz lipídica durante el tratamiento del fruto favorecen la extractabilidad y por lo tanto, la biodisponibilidad de los compuestos fenólicos contenidos en los productos.

Objetivos

El objetivo general de esta tesis doctoral fue verificar si la biodisponibilidad de los compuestos fenólicos presentes en el tomate y derivados del tomate está influenciada por la tipología de la ingesta y la adición de una matriz lipídica durante el procesado.

Para poder alcanzar dicho objetivo general se plantearon los siguientes objetivos específicos:

- Desarrollar y validar una metodología óptima para la identificación y cuantificación de los polifenoles provenientes de tomate y sus subproductos a través de UHPLC-MS/MS.

- Desarrollar y validar un método analítico por HPLC-MS/MS y UHPLC-MS/MS para identificar y cuantificar los polifenoles y metabolitos derivados del consumo de tomates y sus subproductos en muestras de orina y plasma humano.

- Realizar un ensayo de biodisponibilidad en humanos sanos para estudiar los efectos de la adición de diferentes matrices lipídicas (aceite de oliva virgen extra o aceite de oliva refinado) a salsas de tomate.

- Evaluar los efectos de la tipología de la ingesta (tomate y salsa de tomate) en la biodisponibilidad de los polifenoles en un estudio agudo de biodisponibilidad en humanos sanos.

2. Hypothesis and Aims

Hypothesis

Tomato processing and the addition of a lipid matrix during the treatment of the fruit favor the extractability and therefore, the bioavailability of the phenolic compounds contained in the products.

Aims

The aim of this thesis is to verify whether the bioavailability of phenolic compounds present in tomatoes and tomato products is influenced by the type of intake and the addition of a lipid matrix during processing.

In order to achieve this aim, the following specific aims were proposed:

- Development and validation of an optimal methodology for the identification and quantification of polyphenols from tomato and by-products through UHPLC-MS/MS.

- Development and validation of an analytical method by HPLC-MS/MS and UHPLC-MS/MS to identify and quantify the polyphenol metabolites derived of the consumption of tomato and tomato by-products in human urine and plasma.

- Carry out a bioavailability study in healthy humans to study the effects of adding different lipid matrices (extra virgin olive oil or refined olive oil) to tomato sauces.

- Evaluate the effects of the type of intake (tomato and tomato sauces) in the bioavailability of polyphenols in a accute bioavailability study in healthy human.

INTRODUCCIÓN

3. Introducción

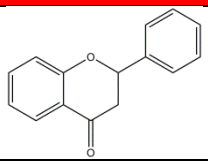
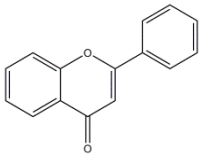
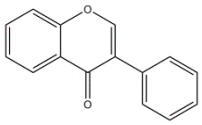
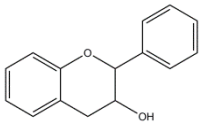
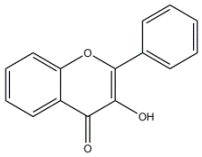
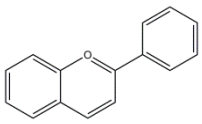
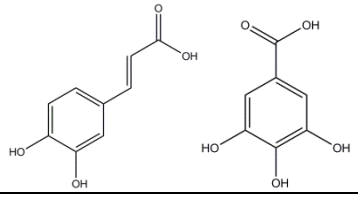
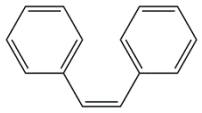
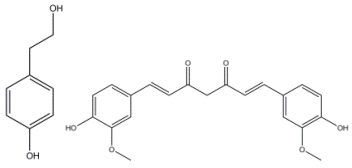
3.1. Compuestos bioactivos

Los compuestos bioactivos están presentes en pequeñas concentraciones en una gran variedad de frutas, hortalizas y verduras y han despertado un gran interés científico debido a que presentan actividades biológicas y efectos beneficiosos en la salud humana[23–27]. Dentro de los compuestos bioactivos se encuentran las vitaminas (vitamina C, folato y provitamina A), los minerales (potasio, calcio y magnesio), los fitoquímicos (compuestos fenólicos, alcaloides, compuestos nitrogenados, compuestos órgano-sulfurados, fitoesteroles y carotenoides) y la fibra dietética[28]. En la presente tesis doctoral nos hemos focalizado en los fitoquímicos.

3.1.1. Compuestos fenólicos

Los compuestos fenólicos son metabolitos secundarios de las plantas y los compuestos bioactivos más abundantes de la dieta. A pesar de su extrema variedad (alrededor de 8000 estructuras) los compuestos fenólicos poseen en común al menos un anillo aromático con uno o más grupos hidroxilo unidos. La diferenciación entre los compuestos viene dada por el número de anillos fenólicos y los grupos (azúcares, ácidos orgánicos o metilos) unidos a ellos. La clasificación generalmente más usada es la que separa los flavonoides de los no flavonoides. Los flavonoides tienen en común una estructura C_6-C_3-C_6, representada por dos anillos bencénicos unidos entre sí por un anillo piránico. Entre los flavonoides, podemos encontrar a las flavanonas, flavonas, isoflavonas, flavanoles, flavonoles y antocianidinas. Los no flavonoides se clasifican según el número de carbonos y dentro de este grupo hallamos a los ácidos fenólicos, estilbenos y fenoles no carboxílicos[30,31]. En la **Tabla 1**, se muestra la clasificación más aceptada de los compuestos fenólicos.

Tabla 1. Clasificación de los compuestos fenólicos.

FLAVONOIDES		
Subgrupo	**Estructura**	**Ejemplos**
Flavanonas		**Naringenina, hesperitina, eriodictiol**
Flavonas		**Apigenina, luteolina, tangerenina**
Isoflavonas		**Genisteína, daidzeína, glicetina**
Flavanoles		**Catequina, epicatequina, galocatequina**
Flavonoles		**Quercetina, caemferol, isorhamnetina**
Antocianidinas		**Delfinidina, cianidina, petunidina**
NO FLAVONOIDES		
Subgrupo	**Estructura**	**Ejemplos**
Ácidos fenólicos		**Ácido cafeico, ácido homovanílico, ácido gálico**
Estilbenos		**Resveratrol, palidol, piceatanol**
Fenoles no carboxílicos		**Tirosol, oleuropeina, curcumina**

Los compuestos fenólicos de los alimentos son comúnmente encontrados conjugados con azúcares y ácidos orgánicos. Los azúcares y los grupos hidroxilo incrementan la solubilidad de los flavonoides, mientras que los grupos metilo provocan una menor solubilidad, haciendo al compuesto más lipofílico[30]. Los compuestos fenólicos intervienen en la pigmentación de las plantas, como agentes protectores contra la luz ultravioleta o presentan actividades anti-mutagénicas, antivirales, antibacterianas (bactericidas o bacteriostáticas), alguicidas, antifúngicas, insecticidas, estrogénicas y quelantes que sirven para proteger al organismo de los que compiten en su entorno biológico[30,32,33]. Los grupos hidroxilo unidos al compuesto fenólico también son buenos donadores de hidrógeno y, por lo tanto, ayudan a eliminar las especies reactivas de oxígeno (ROS) del organismo rompiendo, de este modo, el ciclo de generación de nuevos ROS que provocan la oxidación de las células del organismo. Los compuestos fenólicos también inhiben la oxidación de lípidos, proteínas y ADN provocada por los radicales libres y modulan la actividad enzimática[30]. De esta forma, los polifenoles pueden ayudar a la prevención de diversas enfermedades cardiovasculares y algunos tipos de cáncer[24,28–31,34].

3.1.1.1. Biodisponibilidad de los compuestos fenólicos

La biodisponibilidad se define como la fracción de nutrientes o no nutrientes que es disponible para realizar las funciones fisiológicas y/o de almacenaje del cuerpo humano[11]. Existen diversos factores que pueden afectar a la biodisponibilidad directamente o variando el contenido fenólico en los alimentos previo a su consumo. En la **Figura 1,** se muestra un resumen de estos factores.

- *Factores intrínsecos y extrínsecos del alimento*

  Numerosos factores pueden influir en el contenido fenólico de las plantas, como la genética, además de los factores agronómicos, geográficos y ambientales[3,4]. También, el grado de madurez afecta a las concentraciones y proporciones de los diversos fenoles de diferentes maneras: en general, las concentraciones de ácidos fenólicos disminuyen durante la maduración, mientras que las concentraciones de antocianinas aumentan[35].

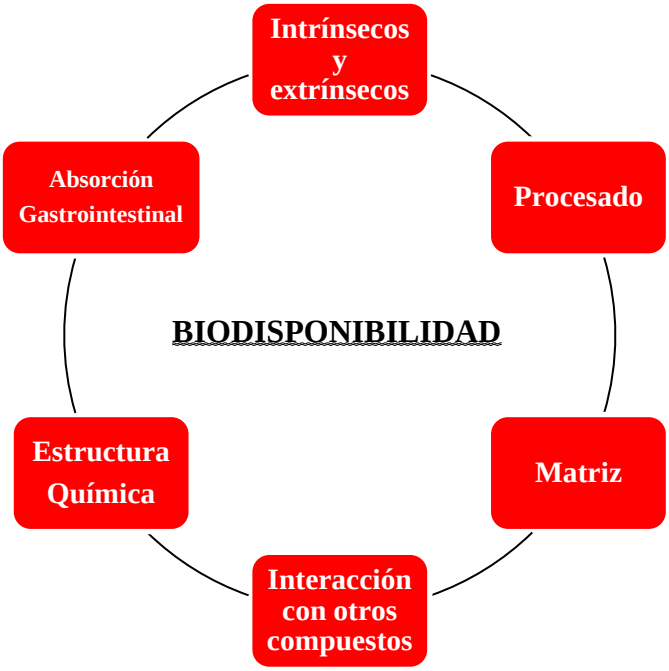


Figura 1. Factores que afectan la biodisponibilidad de los compuestos fenólicos.

- *Factores relacionados con el procesado del alimento*

  En general, los tratamientos térmicos, los métodos culinarios, la deshidratación, la molienda, la homogeneización, la encapsulación y el almacenaje afectan al contenido fenólico de los alimentos, variando su biodisponibilidad y bioactividad[36,37]. Aunque el estudio de estos compuestos bioactivos durante los tratamientos térmicos es bastante amplio, no existe un consenso respecto si el nivel de fenoles aumenta o disminuye después de dicho tratamiento, sino que varía según el alimento o la familia de polifenoles que se estudie[35]. El aumento de temperatura aumenta el riesgo de degradación y oxidación de los polifenoles, pero puede, por otro lado, romper la interacción de éstos con la pared celular de la matriz del alimento facilitando su liberación durante la digestión[11]. Del mismo modo, los métodos culinarios también presentan un efecto controvertido sobre la biodisponibilidad de los compuestos fenólicos. Según el método utilizado, ya sea, hervido, al vapor, frito, ahumado, y dependiendo del alimento estudiado, se llegan a resultados distintos. En un estudio llevado a cabo por Miglio *et al.*[38], se vio disminuida la concentración de polifenoles tras el hervido de zanahorias, mientras que un incremento en el contenido de estos compuestos y su capacidad antioxidante se observó al cocinar al vapor brócoli. Los métodos de deshidratación, utilizados para aumentar la vida media del alimento y evitar contaminación microbiológica, producen una pérdida en la concentración de

polifenoles posiblemente a través de la rotura de los compartimentos celulares o vía degradación enzimática[11]. También se ha demostrado que tanto la molienda como la homogeneización ayudan a aumentar la biodisponibilidad por la alteración de la matriz del alimento. Estos procesos permiten obtener partículas más pequeñas aumentando el área de superficie ayudando al acceso de las enzimas digestivas[11,35]. Otro método de procesado es la encapsulación que se utiliza para la estabilización del producto, para aumentar su biodisponibilidad o para tener controlados los polifenoles durante la digestión. En concreto, se utiliza para aumentar la solubilidad de polifenoles poco solubles como el resveratrol o la curcumina[11]. Por último, el método de almacenaje también afecta al contenido fenólico y de la misma manera que ocurría con los tratamientos térmicos, según el alimento y compuestos fenólico estudiados, la concentración de los compuestos aumenta o disminuye[35].

- *Factores relacionados con la matriz del alimento*

  La matriz del alimento es uno de los principales factores que pueden influir en la biodisponibilidad de los compuestos fenólicos. La absorción de los compuestos fenólicos se ve alterada dependiendo del estado de la matriz del compuesto, ya sea, líquido o sólido. La mayoría de los alimentos en estado líquido poseen una menor viscosidad, pasando a través del estómago más rápidamente que los sólidos, y tienen un menor porcentaje de proteínas o hidratos de carbono que pueden unirse a los polifenoles[11]. Aunque muchos estudios han concluido que la biodisponibilidad de los compuestos fenólicos es mayor cuando se consumen en estado líquido, existen otros que proponen el estado sólido como el ideal para una mayor biodisponibilidad[11]. También existe variabilidad entre las diferentes matrices líquidas en las que se pueda presentar un alimento, variando de este modo la absorción y biodisponibilidad del fenol según en qué tipo de líquido se encuentre el compuesto. Por ejemplo, en un estudio en humanos se investigó la absorción de quercetina, catequina y resveratrol tras el consumo de vino blanco, zumo de uva y zumo de vegetales, presentando una absorción diferente para cada tipo de alimento. Lo mismo ocurrió cuando se estudió la adición de leche a café y té donde en el primero se observaron diferencias en las concentraciones de epicatequinas mientras que con el segundo no se produjo ningún cambio[11,35].

- *Interacciones con otros compuestos*

  A parte del estado líquido o sólido, también pueden afectar otros componentes de la matriz como las proteínas, los hidratos de carbono, la fibra, las grasas, o los minerales, además de la interacción con otros compuestos bioactivos[11,35,39]. En el caso de la fibra dietética, estudios en los que se investiga la adición de fibra al alimento, se observa que disminuye la disponibilidad de los fenoles debido al atrapamiento físico y/o al incremento de la viscosidad y la masa[11]. Por otro lado, la grasa, contenida o administrada en los alimentos, ha demostrado aumentar la absorción de los fenoles más apolares, aunque según el polifenol estudiado aumenta el tiempo de absorción. Esto sería debido posiblemente a la creación de micelas que con la ayuda de las grasas del alimento provocarían una estabilización o solubilización de la mezcla de éstas[11,35]. En el caso de las proteínas, se han demostrado efectos negativos al disminuir la biodisponibilidad de los polifenoles en la mayoría de los estudios realizados. Las proteínas presentan mayor afinidad por los compuestos fenólicos con más grupos hidroxilo como por ejemplo los taninos, formando complejos que reducen la absorción de los fenoles[11]. Pocos estudios han investigado el efecto de los hidratos de carbono y los minerales sobre la absorción de los polifenoles. En ambos casos se observaron efectos adversos, provocando una disminución en la absorción de los hidratos de carbono, posiblemente por la inhibición de la amilasa, y de los minerales, por la formación de quelatos[11]. Por último, la mayoría de estudios revelan que la biodisponibilidad de los polifenoles es más elevada cuando son ingeridos juntamente con otros polifenoles que cuando se consumen solos[11]. También la presencia de algunas vitaminas, como por ejemplo, el ácido ascórbico, ayudan a prevenir la oxidación de algunos fenoles, sugiriendo una mejora en la biodisponibilidad de estos compuestos[11].

- *Estructura química*

  En los alimentos, la mayoría de los polifenoles se encuentran como polímeros o en forma glicosilada. En estas formas, la mayoría de los compuestos no pueden ser absorbidos y deben ser hidrolizados por enzimas intestinales o por la microbiota del colon antes de su absorción, dando lugar a las correspondientes agliconas. La estructura química específica de los polifenoles, así como el tipo de azúcar al que estén unidos, determinan su velocidad y el grado de absorción intestinal[35,40].

- *Absorción gastrointestinal*

La digestión comienza en la cavidad oral, donde encontramos el enzima amilasa. Dado que el tiempo que el alimento se encuentra en la boca es corto, la función de dicho enzima es considerada escasa. La reducción del tamaño de partícula del alimento, ayuda a incrementar el acceso de los enzimas en los siguientes pasos de la digestión[11]. La mayoría de los polifenoles parecen liberarse de la matriz durante la fase gástrica gracias a la digestión con la pepsina conjuntamente con los movimientos peristálticos y el bajo pH, los cuales ayudan a disminuir el tamaño de partícula[11]. Al pasar del estómago al intestino delgado, el pH se incrementa aproximadamente de 2 a 7, permitiendo activar a los enzimas segregados por el páncreas o la bilis y formar micelas solubles en agua[11]. Como se ha explicado anteriormente, la mayoría de polifenoles necesitan ser hidrolizados para que puedan ser absorbidos. Existen dos posibles mecanismos: por acción de la lactasa-floricin hidrolasa (LPH) presente en el borde del enterocito o por la β-glucosidasa citosólica (CBG) presente dentro del mismo[11,31,35,40,41]. Tras la desglicosilación por parte de la LPH, las agliconas liberadas pueden entrar en el enterocito por difusión pasiva, como resultado de su mayor lipofilia[11,31,35]. La CBG, en cambio, actúa dentro del enterocito transportando, a través del transportador de glucosa asociado a sodio (SGLT1), los glucósidos más polares[11,31,35]. No todos los polifenoles son hidrolizados en el intestino delgado, dado que los que están unidos a ramnosas, no se hidrolizan. Estos compuestos deben alcanzar el colon para que se produzca la hidrólisis a través del α-ramnosidasa secretado por la microbiota del colon a fin de ser absorbidos[41].

Los polifenoles una vez transformados en agliconas simples, en el enterocito, son sometidos a otras modificaciones estructurales como la metilación, sulfatación y/o glucuronidación, obteniéndose metabolitos de fase II[11,31,35,40,41]. Los enzimas encargados de metabolizar las agliconas son: catecol-*O*-metiltransferasa (COMT), el cual cataliza la transferencia de un grupo metilo de adenosilmetionina a las agliconas que contienen un resto difenólico, sulfotransferasas (SULT) que producen la transferencia de un sulfato de la fosfoadenosina-fosfosulfato a un grupo hidroxilo de las agliconas, y uridina-5'-difosfato glucuronosiltransferasas (UGT) que cataliza la transferencia del ácido glucurónico del ácido UDP-glucurónico a los compuestos fenólicos [31,35,40,41]. Los polifenoles que atraviesan la membrana basolateral del enterocito, se introducen en el flujo sanguíneo, y a través de la vena porta llegan al hígado donde son sometidos a nuevas conjugaciones de metabolismo de fase II[11,31,41].

Siguiendo el transporte por el flujo sanguíneo, los polifenoles pueden ser distribuidos a la mayoría de tejidos e incluso atraviesan la barrera hematoencefálica, la cual solo son capaces de traspasar los compuestos lipídicos[11]. Algunos de los fenoles conjugados, que no atraviesan la membrana basolateral, son transportados mediante la acción de transportadores otra vez hacia el lumen intestinal del intestino delgado. Al mismo tiempo, la conjugación de los fenoles en el hígado facilita su excreción a través de la bilis mediante circulación enterohepática pudiendo volver a ser absorbidos por el intestino delgado. Los polifenoles que no se absorben en el intestino delgado pasan intactos al colon, conjuntamente con los compuestos que fueron absorbidos, metabolizados en el hígado y excretados en la bilis o directamente excretados del enterocito de vuelta al intestino delgado[40]. La microbiota intestinal del colon hidroliza los glucósidos a agliconas y las degrada a ácidos fenólicos simples. Estos compuestos pueden ser absorbidos y metabolizados por el hígado antes de ser excretados por la orina[31,35]. Los compuestos no absorbidos son eliminados a través de las heces[41].

Los metabolitos de fase II varían según la naturaleza del sustrato, la dosis ingerida, la especie y el sexo[35]. La dosis administrada juega un papel crucial para determinar el primer sitio de metabolización. Las dosis altas permiten la metabolización en el hígado, mientras que en una ingesta más pequeña, la metabolización se produce en el enterocito, siendo el hígado el segundo mecanismo para la modificación de los compuestos fenólicos conjugados por el intestino delgado[40]. Cabe destacar que los mecanismos de conjugación son altamente eficaces y que en plasma las agliconas pueden encontrarse en muy bajas concentraciones o ausentes[35,39–41]. En la **Figura 2**, se presenta un esquema de la absorción y biotransformación de los compuestos fenólicos de la dieta.

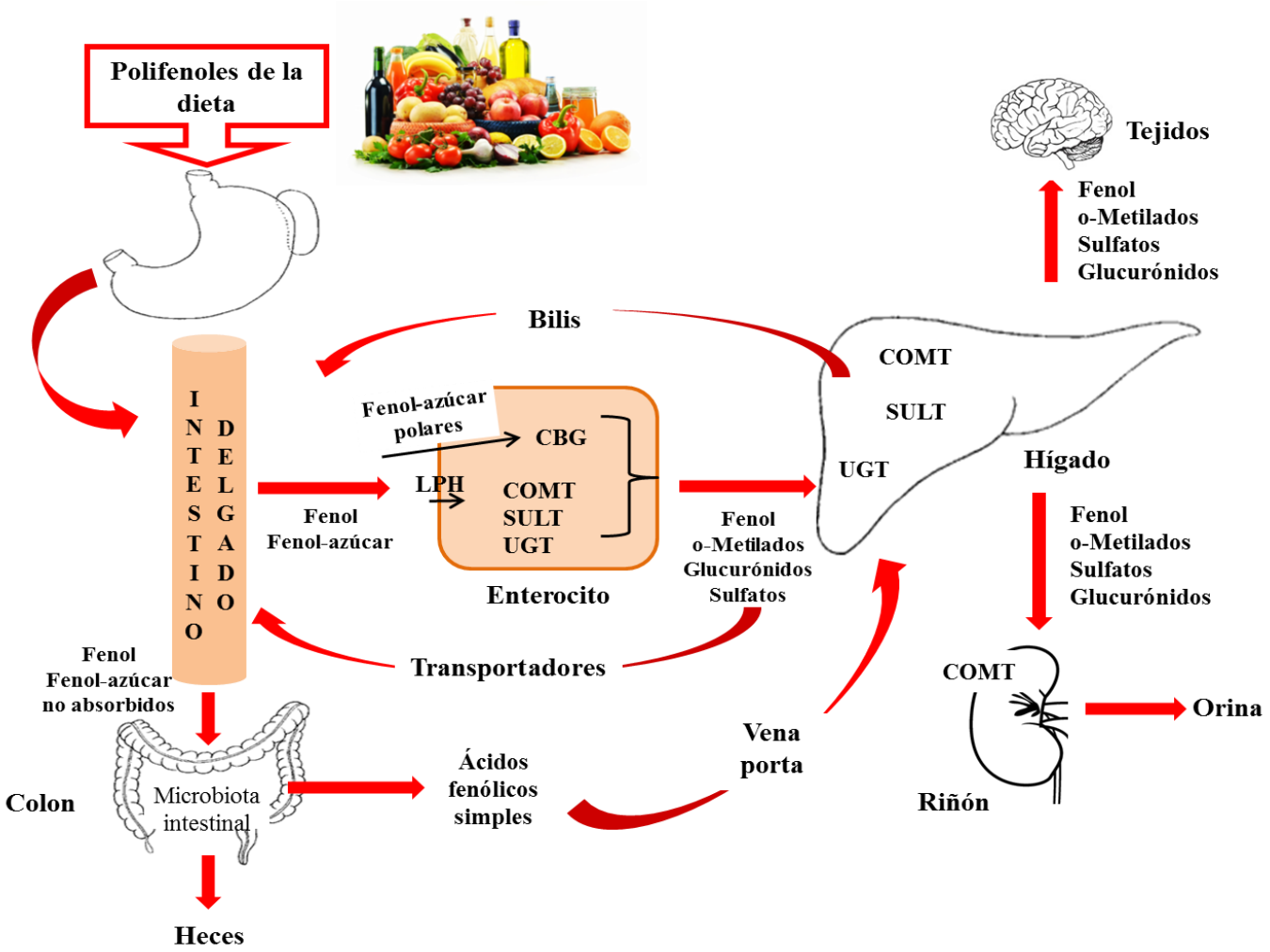


Figura 2. Esquema de la absorción y biotransformación de los compuestos fenólicos de la dieta.

3.1.1.2. Extracción y análisis de compuestos fenólicos en alimentos y muestras biológicas

La identificación y cuantificación de los compuestos fenólicos en alimentos y muestras biológicas es una tarea difícil debido a la amplia variedad de estructuras que se encuentran en la naturaleza y la falta de estándares comerciales disponibles. Se han utilizado varios métodos, incluidos los métodos espectrofotométricos, la electroforesis capilar, la espectroscopia de resonancia magnética nuclear, la espectroscopia de infrarrojos, y las técnicas cromatográficas tales como la cromatografía de fluidos supercríticos, cromatografía de gases, cromatografía líquida de alta resolución (HPLC) y, más recientemente la cromatografía líquida de ultra alta resolución (UHPLC).

Sin embargo, se requieren extractos limpios antes de que las muestras se puedan analizar. Existen varios métodos de extracción entre los que se incluyen la extracción líquido-líquido, la extracción sólido-líquido, la extracción con fluido supercrítico, y la extracción en fase sólida (SPE).

Una de las técnicas más aplicadas para su determinación es el HPLC que permite el acoplamiento con diferentes detectores, como el ultravioleta (UV), la fluorescencia, índice de refracción, dispersión de la luz, electroquímica, y espectrometría de masas (MS). Actualmente, la mejor herramienta de análisis para cuantificar y caracterizar los compuestos fenólicos es el HPLC acoplado a MS, debido a la detección de los compuestos a muy bajas concentraciones.

Toda esta información referente a la extracción y análisis de los compuestos fenólicos en alimentos y muestras biológicas se presenta a continuación bajo el título "*Improved Characterization of Polyphenols Using Liquid Chromatography*" capítulo 14 del libro "*Polyphenols in Plants: Isolation, Purification and Extract Preparation*" (Elsevier, 2014 – ISBN: 978-0-12-397934-6)[42].

CHAPTER

14 Improved Characterization of Polyphenols Using Liquid Chromatography

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CHAPTER OUTLINE HEAD

14.1 Introduction

Polyphenols are plant secondary metabolites and the most abundant dietary bioactive compounds. Nowadays, it is estimated that 100,000 to 200,000 secondary metabolites exist (Metcalf, 1987). Despite their extreme variety, polyphenols possess a common carbon skeleton building block: the C6–C3 phenylpropanoid unit. Biosynthesis by this pathway leads to a wide range of plant phenols: cinnamic acids (C6–C3),

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benzoic acids (C6–C1), flavonoids (C6–C3–C6), proanthocyanidins [(C6–C3–C6)*n*], coumarins (C6–C3), stilbenes (C6–C2–C6), lignans (C6–C3–C3–C6) and lignins [(C6–C3)*n*] (Seabra *et al.*, 2006).

An exhaustive identification of polyphenols in food and biological samples is of great interest due to their health-promoting effects. Notably, they have an important protective role against a number of pathological disturbances, such as atherosclerosis, brain dysfunction, and cancer (Ignat *et al.*, 2011). It is well known that the protective effects of polyphenols *in vivo* depend on their accessibility and extractability from food, intestinal absorption, metabolism, final biological action in the human body, and potential interaction with target tissues (Tulipani *et al.*, 2012). Phenolics may also act as antifeedants, contributors to plant pigmentation and protective agents against UV light, amongst other activities (Ignat *et al.*, 2011). Nevertheless, the lack of commercially available standards and the wide range of phenolic structures found in nature make identification of phenolic compounds a complex task.

Polyphenol extraction is a crucial step in the development of an analytical method sensitive enough to determine these substances at low concentrations. Several extraction methods are described in the literature (Ignat *et al.*, 2011), but the most common are liquid–liquid extraction (Baydar *et al.*, 2004; Vallverdú-Queralt *et al.*, 2010), solid–liquid extraction (Martinez-Huelamo *et al.*, 2012; Medina-Remon *et al.*, 2009), and extraction with supercritical fluid (Palenzuela *et al.*, 2004; Palma and Taylor, 1999).

Diverse methods have been reported for the identification and quantification of phenolic compounds (Ignat *et al.*, 2011), including spectrophotometry (Huang *et al.*, 2009; Medina-Remon *et al.*, 2009), capillary electrophoresis (CE) (Herrero-Martinez *et al.*, 2005), nuclear magnetic resonance spectroscopy (NMR) (Slimestad *et al.*, 2008), near-infrared spectroscopy (NIR) (Chen *et al.*, 2009), and chromatographic techniques like high-performance liquid chromatography (HPLC) (Martinez-Huelamo *et al.*, 2012; Vallverdú-Queralt *et al.*, 2010), ultra-high-performance liquid chromatography (UHPLC) (Epriliati *et al.*, 2010; Gruz *et al.*, 2008), high-speed counter-current chromatography (HSCCC) (Cao *et al.*, 2009; Yanagida *et al.*, 2006), supercritical fluid chromatography (SFC) (Kamangerpour *et al.*, 2002), and gas chromatography (GC) (Friedman, 2004; Lu and Foo, 1998), although in this chapter we will focus only on liquid chromatography.

Available HPLC detectors have various limitations. Although low detection limits and good sensitivity are obtained by UV, fluorescence, refractive index, light scattering or electrochemical detectors, the structural information they provide lacks detail. The introduction of methods that combine two or more analytical techniques, such as HPLC-UV coupled with photodiode array detection (HPLC-UV-DAD) and HPLC coupled with mass spectrometry (HPLC-MS), has improved structural elucidation of metabolites (Marston and Hostettmann, 2009).

Nowadays, the best analytical tool to quantify and characterize phenolic compounds is considered to be liquid chromatography coupled with ultraviolet-photodiode array detection (UV-DAD) (Chen *et al.*, 2009; Crozier *et al.*, 1997; Epriliati *et al.*, 2010; Fang *et al.*, 2009; Kerem *et al.*, 2004; Liu *et al.*, 2008; Sakakibara *et al.*,

2003; Sun *et al.*, 2007; Wang *et al.*, 2009) or mass spectrometry (MS) (Cao *et al.*, 2009; Chiva-Blanch *et al.*, 2011; Cimpan and Gocan, 2002; Gruz *et al.*, 2008; Han *et al.*, 2008; Martinez-Huelamo *et al.*, 2012; Sanchez-Rabaneda *et al.*, 2003a; Tsao and Deng, 2004; Tulipani *et al.*, 2012; Urpi-Sarda *et al.*, 2009; Vallverdú-Queralt *et al.*, 2010; Volpi and Bergonzini, 2006).

14.2 Sample preparation

14.2.1 Analyte isolation

Accurate identification and quantification of analytes greatly depends on the extraction step. The lack of a standard extraction procedure, which is due to the variability and complexity of phenolic chemical structures and the matrices in which they are found, has led to the proliferation of multiple extraction techniques and methods (Table 14.1).

Extraction can also ensure a more sensitive determination of phenolic compounds and metabolites found at very low concentrations by eliminating interfering components, especially in biological matrices.

Liquid–liquid (LLE) and solid–liquid extraction (which may be followed by solid-phase extraction (SPE) to purify the extract) are the most widely used techniques. Common extraction solvents are methanol, ethanol, acetone, ethyl acetate, and diethyl ether, containing only a small amount of acid. However, polar phenolic acids such as cinnamic acids cannot be extracted with pure organic solvents, and require alcohol–water or acetone–water mixtures.

Liquid samples are usually centrifuged and/or filtered and then the sample is either directly injected into the separation system or analytes are isolated using LLE or SPE.

Conventional methods such as boiling, heating or refluxing can be used to extract natural phenolic compounds from samples, but polyphenols can be lost due to hydrolysis, ionization and oxidation during the process (Li *et al.*, 2005). In recent years, other techniques have been developed for polyphenol extraction, including ultrasound-assisted, microwave-assisted, supercritical fluid, and high hydrostatic pressure extraction (HHP) (Wang and Weller, 2006).

Supercritical fluid extraction is being increasingly used in food and pharmaceutical industries as it is more environmentally friendly, avoiding the use of large amounts of toxic solvents, as well as being rapid, automatable, and selective (Bleve *et al.*, 2008; Maróstica-Junior *et al.*, 2010). The intrinsic low viscosity and high diffusivity of supercritical CO_2 has permitted faster and more efficient separation, and relatively clean extracts. In addition, the absence of light and air during extraction reduces the degradation of analytes that occur in traditional extraction techniques. Supercritical fluids have solvating powers similar to organic solvents but with higher diffusivity, lower viscosity, and lower surface tension. However, the solvating power of a supercritical fluid needs to be controlled by temperature and pressure, or by adding organic modifiers such as methanol. For example, owing to the polarity of

Table 14.1 Examples of Extraction and Analysis of Polyphenols in Food and Biological Samples

Matrix	Analytes	Extraction	Analysis	LOD (mg/l)	References
Food and Beverage Samples					
Fruits and fruit juices	Phenolic acids, anthocyanins, hydroxybenzoic acids, flavan-3-oles, hydroxycinnamic acids, coumarins, flavanones, flavones, dihydrochalcones, flavonols	SE LLE	HPLC-UV HPLC-ESI-MS	0.03–0.005	Abad-García *et al.*, 2007; Liu *et al.*, 2012; Sakakibara *et al.*, 2003; Xu *et al.*, 2012; Fang *et al.*, 2009
Grapes and grape juices	Anthocyanins, flavanols, flavonols, hydroxycinnamates	SE LLE	HPLC/Q-TOF HPLC-MS/MS HPLC-DAD HPLC-MS	3–0.5	Liang *et al.*, 2011; Muñoz *et al.*, 2008; Xu *et al.*, 2012
Vegetables	Quercetin glycosides, hydroxycinnamic acids, phenolic acids, flavanols, flavonols, flavones	SE SPE	HPLC-UV CE UHPLC-DAD	0.62–0.005	Caridi *et al.*, 2007; Silva *et al.*, 2012
Tea leaves and derived products	Flavanols, hydroxycinnamic acids, phenolic acids, flavones, phenolic terpenes, hydroxybenzoic acids	SE	HPLC-DAD-ESI-MS/MS UHPLC-UV UHPLC-MS/MS	0.048–0.0301	Aura *et al.*, 2002 ; Spáčil *et al.*, 2010; Wang *et al.*, 2008a
Apples	Flavanols, flavonols, hydroxycinnamates, anthocyanins, dihydrochalcones	SE	HPLC-DAD HPLC-MS	3×10^{-7}– 3×10^{-8}	Alonso-Salces *et al.*, 2005; Vrhovsek *et al.*, 2004
Wine	Flavanols, flavonols, phenolic acids, stilbenes, hydroxycinnamates, hydroxybenzoic acids, procyanidyns, cinnamic acids	LLE MEPS Filtration	HPLC-UV-FLD UHPLC-DAD HPLC-UV-DAD	0.54–0.02 0.2–0.01 0.05–0.003	Bétes-Saura *et al.*, 1996; Gonçalves *et al.*, 2013; Rodrí;guez-Delgado *et al.*, 2001
Alcohol-free beer	Flavanols, hydroxycinnamates	SPE	HPLC-UV	0.2–0.01	Garcia *et al.*, 2004

Beans, soy beans and derived products	Flavanols, phenolic acids, hydroxycinnamates, isoflavones	SE	HPLC-UV-DAD HPLC-ESI-MS UHPLC-UV	<0.5	Griffith and Collison, 2001; Ross *et al.*, 2009; Toro-Funes *et al.*, 2012
Cocoa and chocolate	Catechin and epicatechin, procyanidins	LLE SE	HPLC-FLD UHPLC-MS/MS	$0.002–2\times10^{-6}$ 20–9	Machonis *et al.*, 2012; Ortega *et al.*, 2010
Olive oil	Tyrosols and flavonols	LLE	HPLC-ECD	<4	Capannesi *et al.*, 2000
Water	Phenolic acids, flavonols, hydroxycoummarics	SE	HPLC-DAD	0.3–0.1	Liu *et al.*, 2008
Propolis	Flavonoids	LLE	HPLC-MS	<0.0025	Volpi and Bergonzini, 2006
Tomato and derived products	Phenolic acids, flavonols, flavanones, hydroxy-cinnamic acids	LLE	HPLC-MS/MS, HPLC-QTOF, HPLC-Orbitrap	1.7×10^{-5} 3×10^{-7}	Vallverdú-Queralt *et al.*, 2010, 2011a, 2011b; 2011d, e 2012b, c
Biological Samples					
Rat urine and plasma	Isoflavones and its metabolites Puerarin (daidzein-8-C-glucoside) and its metabolites Epicatechin, epigallocatechin and its metabolites	SPE Protein precipitation LLE	HPLC-MS/MS HPLC-ESI-MS/MS HPLC-UV	0.125–0.025	Fang *et al.*, 2002; Fu *et al.*, 2008; Prasain *et al.*, 2004a
Serum	Isoflavones and lignans Procyandin B1	SPE LLE	HPLC-ESI-MS/MS HPLC-MS	$<1\times10^{-10}$	Grace *et al.*, 2003; Sano *et al.*, 2003
Urine	Phenolic acids, Flavanols, flavonols Flavanols, flavonols, phenolic acids and related metabolites	LLE Protein precipitation SPE	HPLC-ESI-MS/MS UHPLC-ESI-MS/MS	1.3×10^{-7}– 1×10^{-10}	Magiera *et al.*, 2012; Martinez-Huelamo *et al.*, 2012; Rios *et al.*, 2003
Plasma	Epicatechin and related metabolites Quercetin Flavanols, flavonols, phenolic acids and related metabolites	SPE	HPLC-UV/Vis-FLD-ECD HPLC-ECD HPLC-ESI-MS/MS	<0.2 2.9×10^{-7}– 5×10^{-9}	Erlund *et al.*, 1999; Martinez-Huelamo *et al.*, 2012; Ottaviani *et al.*, 2012

LLE, liquid–liquid extraction; SE, solid extraction; SPE, solid-phase extraction.

anthocyanins, their extraction by the SC–CO_2 method requires high pressures and the presence of methanol or ethanol (Bleve *et al.*, 2008).

Ultrasound-assisted extraction is an inexpensive, simple, and efficient alternative to conventional extraction techniques (Wang *et al.*, 2008b). This method extracts non-volatile and semi-volatile compounds from the matrix. The ultrasonic process facilitates contact between the sample matrix and extraction solvent. Ultrasonication is often carried out to improve phenolic compound extraction from plants; for example, a study with *Folium eucommiae* (Huang *et al.*, 2009) found it to be more efficient than conventional extraction techniques.

Another promising approach for extracting phenolic compounds is microwave-assisted extraction, which was satisfactorily used to analyze gallic acid, protocatechuic acid, chlorogenic acid and caffeic acid in Eucommia ulmodies (Li *et al.*, 2004). Zhang *et al.* extracted polyphenols from Camellia oleifera fruit hull using microwave-assisted extraction (Zhang *et al.*, 2011), finding the optimal conditions to be a liquid : solid ratio of 15.33 : 1 (ml/g), extraction time of 35 min and extraction temperature of 76°C. The same method has also been used to extract polyphenols from green tea (Nkhili *et al.*, 2009) and found to be more efficient than conventional heating.

Enzymatic release of phenolic compounds is also employed to extract phenolic compounds, for example, from grape pomace (Maier *et al.*, 2008). Another study investigated the ability of three enzymes (Ultraflo L, Viscozyme L, and a-Amylase) to release phenolic compounds from *Ipomoea batatas* L. (sweet potato) stems (Min *et al.*, 2006). Ferulic acid release rate was optimal when Ultraflo L (1.0%) was used, whereas Viscozyme L was the most effective for the release of vanillic acid and vanillin.

Another technique that enhances the extraction of polyphenols is HPP. Studies carried out by Shouqin *et al.* (2005) have demonstrated the benefits of hydrostatic pressure for the extraction of flavanols.

14.2.2 Analyte purification. Solid phase Extraction

SPE is an extraction technique used as a clean-up procedure and a pre-concentration step with crude plant, biological, environmental, food, and pharmaceutical samples (Ho *et al.*, 2012; Kerio *et al.*, 2012; Martinez-Huelamo *et al.*, 2009; Navas, 2012; Olmos-Espejel *et al.*, 2012).

Alkyl-bonded silica or copolymer sorbents are commonly used to extract analytes, reverse-phase sorbents being most chosen for polyphenols. Samples and solvents are usually slightly acidified to prevent ionization of phenolic compounds, which would lead to a weaker analyte retention in the sorbent (Navas, 2012; Vinas *et al.*, 2011).

In a recent study, reverse-phase HLB cartridges were used to extract phenolic compounds and metabolites from the urine and plasma of volunteers who had consumed different types of tomato sauce: without oil, or containing 5% of virgin olive oil or 5% of refined olive oil (Martinez-Huelamo *et al.*, 2012). Urpi-Sardà *et al.* (2009) also used HLB cartridges to extract conjugated phenolics from urine and

plasma after regular consumption of cocoa. SPE is essential when working with LC-MS in order to reduce the matrix effect, especially when analytes are found in low concentrations, as occurs in biological samples. However, HLB cartridges have also been used to determine resveratrol and piceid in beer matrices (Chiva-Blanch *et al.*, 2011).

Mix-mode cation/anion exchange reverse-phase sorbents have also been applied to extract phenolic compounds from biological matrices due to their higher capacity to clean up samples. In a study by Medina-Remon *et al.* (2009), HLB, MCX (mix-mode cation-exchange reverse-phase sorbent) and MAX (mix-mode anion-exchange reverse-phase sorbent) were compared in the extraction of 10 representative polyphenols from urine samples, and the best recoveries were obtained with MAX cartridges. Vallverdú-Queralt *et al.* used MAX cartridges to analyze phenolic compounds of different tomato varieties (Vallverdú-Queralt *et al.*, 2011e) and to distinguish between organic and conventional tomatoes (Vallverdú-Queralt *et al.*, 2012a). In a study carried out to analyze colonic microbial metabolites, MCX was used to extract polyphenols from urine and plasma after regular consumption of cocoa (Urpi-Sarda *et al.*, 2009).

14.3 High performance liquid chromatography (HPLC)

The type of column used to separate phenolics and their glycosides is almost exclusively a reverse-phase C18-bonded silica column ranging from 100 to 300 mm in length and with an internal diameter of 2–4.6 mm (Merken and Beecher, 2000; Stalikas, 2010; Tsao and Deng, 2004; Tulipani *et al.*, 2012), although occasionally C8 columns are used to separate phenolic acids. Columns are maintained from room temperature to 40°C during the analysis but thermostated columns give more repeatable elution times and greater resolution, and allow the backpressure of the LC column to be reduced at high flow rates.

The use of a binary system is essential for the separation of structurally varied phenolic compounds. Gradient elution is usually performed with a solvent A, including an aqueous acidified polar solvent or water-containing buffer, and a solvent B, which can be an organic solvent such as methanol or acetonitrile, pure or acidified (Merken and Beecher, 2000; Tsao and Deng, 2004). The volume of injection ranges from 1 to 100 µl, depending on the internal diameter of the column used (Merken and Beecher, 2000).

14.3.1 Ultraviolet detection

Ultraviolet (UV) is the simplest and most commonly used HPLC detector due to its greater sensitivity, linearity, versatility, and reliability (Wolfender, 2009). The existence of conjugated double and aromatic bonds in phenolic compounds allows them to absorb UV or UV-VIS light (Stalikas, 2010). There are different types of UV detectors: fixed wavelength, multiple wavelength, or photodiode array (DAD)

(Wolfender, 2009), the last one being the most frequently used to detect phenolic compounds.

Polyphenols absorb light at different wavelengths. Flavonoids have two characteristic absorption bands: the first has a maximum in the 240 to 285nm range, corresponding to the A-ring, while the second band has a maximum in the 300 to 550nm range, which is attributed to the substitution pattern and conjugation of the C-ring. Anthocyanins also present two absorption bands, in the regions of 265–275 and 465–560 nm. Flavones, flavonols, and flavonols are detected at 280 and 350 nm. UV spectra of catechins give peaks at 210, 278, and 280 nm. Flavones and flavonols have bands in the ranges of 240–280 nm and 300–380 nm. Flavanones and isoflavones are detected at 280–290 nm and 236–262 nm, respectively (Crozier *et al.*, 1997; Sakakibara *et al.*, 2003). Figure 14.1 shows the UV spectra of representative polyphenols.

UV detection became the preferred detector in LC analysis since it is cheap and robust, especially for food matrices containing high phenol concentrations (Table 14.1). In a study by Bétes-Saura *et al.* (1996) an HPLC coupled with a UV-DAD detector was used to identify and quantify 30 polyphenols in white wines. The column used was a C18 (250×4 mm), with 5 µm particle size. Flow rate was set at 1.5 ml/min and gradient elution was performed with glacial acetic acid in water at pH 2.65 (phase A) and 20% solvent A mixed with 80% acetonitrile (phase B). The chromatogram was monitored simultaneously at three wavelengths: 280, 320, and 365 nm. Benzoic acids, tyrosol, flavan-3-ols, and the oligomeric procyanidins were quantified at 280 nm, cinnamic acids and their tartaric esters at 320 nm and flavonols at 365 nm (Caporaso *et al.*, 2011). The method was validated, providing good precision and linearity and low limits of detection, which varied from 0.003 mg/l for cis-caftaric acid to 0.051 mg/l for tyrosol.

Liu *et al.* (2008) developed a method to determine polyphenols in water by HPLC-DAD. The separation of phenolic compounds was carried out in a C18 column (150×4.6 mm, 5 µm). Gradient elution was performed using acetic acid/water solution (1:99, v/v) as the aqueous mobile phase and methanol as the organic phase. The photodiode array detector operated between 210 and 400 nm. The method was validated, with recoveries between 83 and 95% and limits of detection ranging from 0.1 to 0.3 mg/l. The developed method allowed the identification and quantification of seven polyphenols (chlorogenic acid, esculetin, caffeic acid, scopoletin, rutin, quercetin hydrate, kaempferol) in tobacco-polluted water.

Another study by Lachman *et al.* (2009) used HPLC-DAD to analyze anthocyanidins in red- and purple-fleshed potatoes from 15 cultivars. Anthocyanidins were determined using a reverse-phase column C18 (4×250 mm, 7 µm). Solvent A was aqueous 1% (v/v) phosphoric acid, 10% (v/v) acetic acid, 5% acetonitrile (v/v), and solvent B was 100% HPLC grade acetonitrile at a flow rate of 1 ml/min. The quantification of anthocyanidins was set at $\lambda = 530$ nm. Prior to HPLC analysis, samples were hydrolyzed by acidic hydrolysis. The results showed that individual cultivars differed significantly in the relative proportion of anthocyanidins. However, the most abundant anthocyanidin in red- and purple-fleshed potatoes was petunidin (46.9%),

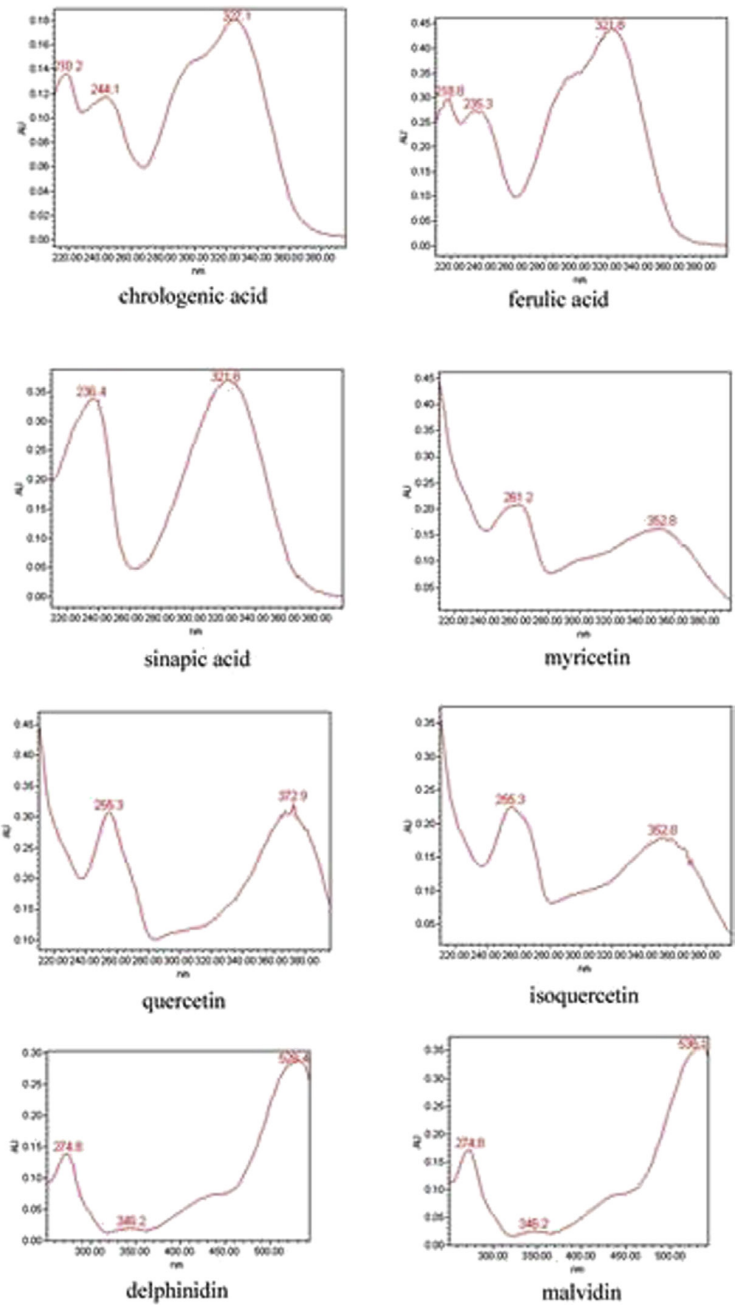

FIGURE 14.1

UV spectra of some polyphenols.

followed by malvidin (22.8%) and pelargonidin (22.1%), cyanidin (5.38%), peonidin (2.74%), and delphinidin (0.15%).

14.3.2 Fluorescence detection

Fluorescence detection is rarely used to analyze polyphenols since only a few exhibit natural fluorescence, including isoflavones without an OH group at position 5 (de Rijke *et al.*, 2002) and flavonoids with an OH group at position 3 (Sengupta and Kasha, 1979), such as catechin and methoxylated flavones (Huck and Bonn, 2001). The analysis of these compounds by HPLC-fluorimetric detection is a more selective and sensitive technique for complex mixtures and provides lower limits of detection (LODs) than UV detection. Moreover, the combination of UV detection and fluorescence makes it possible to distinguish between fluorescent and non-fluorescent co-eluting compounds, and allows a more sensitive detection of the former (Rodriguez-Delgado *et al.*, 2001).

De Quirós *et al.* (2009) proposed a new method for the analysis of flavanol, procyanidin, hydroxycinnamate, flavonol, and stilbene derivatives in white wines based on HPLC-UV-fluorimetric detection. A reverse-phase (250 × 4.0 mm, 5 µm) column was used, and the mobile phases consisted of (A) water–acetonitrile–acetic acid, 67 : 32 : 1 v/v/v, and (B) water–acetic acid, 99 : 1 v/v at 0.8 ml/min. The identification and quantification of phenolic compounds was achieved by setting the UV-Vis and fluorimetric detectors at selected wavelengths. The fluorescence detector was set at λ_{em} 360 nm and λ_{ex} 278 nm for (+)-catechin, (−)-epicatechin, procyanidin B1 and procyanidin B2, and at λ_{em} 392 nm and λ_{ex} 300 nm for trans-resveratrol. The UV–Vis detector system was set at 280 nm for flavanols, procyanidins, and trans-resveratrol, 320 nm for caftaric acid and 360 nm for flavonols. The method was fully validated, providing great repeatability with an RSD lower than 5%, limits of detection from 0.02 to 0.2 µg/ml, and recoveries of 97.3%.

Another study (Ottaviani *et al.*, 2012) used an HPLC-fluorimetric detection technique to determine epicatechin metabolites in human plasma after a dietary intervention of a dairy-based cocoa drink rich in epicatechin and procyanidins. The separation was achieved with a C18 column (150 × 4.6 mm, 3 µm) using 4% (v/v) methanol and 42 mM sodium acetate and an acetonitrile gradient with a flow rate of 0.8 ml/min. The detection of (−)-epicatechin and its related metabolites was achieved following the traces of fluorescence at 276 nm excitation and 316 nm emission and UV absorption at 280 nm. This method demonstrated that (−)-epicatechin-3′-β-D-glucuronide, (−)-epicatechin-3′-sulfate, and 3′-O-methyl-(−)-epicatechin-5/7-sulfate are the predominant (−)-epicatechin-related metabolites in humans, and confirmed the relevance of the stereochemical configuration in the context of flavanol metabolism.

Nevertheless, when working with fluorescence detection, it is necessary to take into account that emission spectra of polyphenols may show pH-dependence or might undergo solvent-dependent dual emission.

To implant fluorescence detection of phenolic compounds that do not exhibit natural fluorescence, derivatization with metal cations has been used. For example,

quercetin and kaempferol can form highly fluorescent complexes with metal cations such as Al (III) (Hollman *et al.*, 1996).

14.3.3 Electrochemical detection

Electrochemical detection is another technique that can be used to analyze polyphenols in food and biological matrices, since most flavonoids are electroactive due to the presence of phenolic groups. Electrochemical detection is a selective technique and can be very sensitive for compounds that are oxidized or reduced at low-voltage (Milbury, 2001). Most flavonoids show two values of maximum detector response: the first corresponds to the oxidation of phenolic substituents on the B-ring, while the second might be due to the other, less oxidizable, phenolic groups.

In a study by Aaby *et al.* (2004), the electrochemical behavior of 20 flavonoids and cinnamic acid derivatives was correlated with antioxidant activity measured by FRAP, DPPH, and ORAC assays. The aim of the work was to determine whether the analysis of phenolic compounds by HPLC coupled with a coulometric array detector could be used to predict antioxidant activity assessed by the three tests. It was concluded that the electrochemical response at a relatively low oxidation potential (300 mV) and the cumulative response at medium oxidation potential (400 and 500 mV) showed considerable correlation with antioxidant activities, with the highest correlations being found with FRAP and DPPH assays after short reaction periods.

Another example of polyphenol analysis by electrochemical detection is given by Peng *et al.* (2005). The aim of the study was to determine phloridzin, (−)-epicatechin, chlorogenic acid and myricetin in apple juice and cider by capillary electrophoresis with electrochemical detection. The analytes were separated in 20 min in a 75cm length capillary at a separation voltage of 18 kV in a 50 mmol/l borate buffer (pH 8.7). The limits of detection ranged from 1×10^{-7} to 5×10^{-7} g/ml for all analytes. The proposed method gave high recoveries of 95–98% and good reproducibility, with an RSD lower than 3.6%.

Capannesi *et al.* (2000) compared different techniques to evaluate the phenolic content of an extra-virgin olive oil with varying storage time and conditions. The techniques used were a disposable screen-printed sensor coupled with differential pulse voltammetry, and a tyrosinase-based biosensor operating in an organic solvent, using an amperometric oxygen probe as the transducer. Electrochemical detection revealed the degradation reaction of large molecules (such as oleuropein derivatives) into smaller ones.

Electrochemical detection has also been applied to biological samples, as described by Jin *et al.* The aim of the study was to validate and apply a method for the quantification of quercetin in human plasma after the ingestion of a commercial canned green tea (Jin *et al.*, 2004). The analysis was performed by an HPLC system coupled with electrochemical detection. A microbore octadecylsilica column (150 × 1.0 mm, 3 µm) was used and the mobile phase was methanol–water (4 : 6, v/v) containing 0.5% phosphoric acid, with a flow rate of 25 µl/min. Quercetin was oxidized at a detection potential of +0.5 V *versus* Ag/AgCl. The method proved highly selective and sensitive with a detection limit of 0.33 pg.

14.3.4 Mass spectrometry

Liquid chromatography coupled with mass spectrometry is an efficient method to detect and quantify phenolic compounds in plant extracts and biological fluids. The mass spectrometer ionizes the compounds to generate charged molecules and molecule fragments, measuring their mass-to-charge ratios (Ignat *et al.*, 2011; Marston and Hostettmann, 2009; Stalikas, 2010; Wolfender, 2009). Different sources can be used for compound ionization: fast atom bombardment (FAB), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photo-ionization (APPI), and matrix-assisted laser desorption ionization (MALDI). The detection of the compounds can be performed in positive or negative ion mode, the latter being more common in polyphenol analysis (Ignat *et al.*, 2011; Magiera *et al.*, 2012; Prasain *et al.*, 2004b; Schieber *et al.*, 2000; Sporns and Wang, 1998; Stobiecki, 2000). Table 14.2 shows a list of fragment ions of representative polyphenols and their metabolites in negative mode.

Different types of mass analyzers can be used in polyphenol analysis: single quadrupole (MS), triple-quadrupole (MS/MS), ion-trap mass spectrometers (MS^n), time-of-flight (TOF), quadrupole-time-of-flight (QTOF), Fourier transform mass spectrometry (FTMS), and *Orbitrap-based* hybrid mass spectrometers (LTQ-Orbitrap) (Liang *et al.*, 2012; Meda *et al.*, 2011; Mikulic-Petkovsek *et al.*, 2012; Van Der Hooft *et al.*, 2012; Xie *et al.*, 2011).

Quadrupoles consist of four parallel rods connected together, with voltages applied between one pair of rods and the other. Ions with a specific mass-to-charge ratio (*m/z*) will pass through the quadrupole when a particular voltage is applied. This enables quadrupoles to filter the ions en route to the detector. As well as single, triple quadrupole systems are also available, in which the first (Q1) and third quadrupole (Q3) work as filters while Q2 acts as the collision cell. The generic mode for screening in MS systems is the full scan, where a mass spectrum is acquired every few seconds, thus allowing the identification of the protonated or deprotonated molecule and consequently the calculation of the molecular weight of the substance. More sensitive modes of working in quadrupole systems include selected ion monitoring (SIM) in single quadrupole instruments and multiple reaction monitoring (MRM) mode in triple quadrupole instruments. In SIM experiments, the use of a fixed voltage allows the detection of a single *m/z,* whereas in MRM experiments, Q1 filters a precursor ion, and the Q2 is the collision cell, which produces a product ion by collision of the precursor ion with a neutral collision gas. The product ion is transferred into Q3 where only a specific *m/z* is allowed to pass.

Tandem mass spectrometry enables polyphenols to be detected and quantified in complex matrices through MS/MS techniques such as product ion scan, precursor ion scan, and neutral loss scan. A product ion scan mass spectrum contains the fragment ions generated by the collision of the molecular ion. A precursor ion mass spectrum is obtained by limiting the fragment ion to a single ion of interest. Parent ions (molecular ions) are scanned to determine which of them give the target fragment ion. Neutral loss mass spectra show fragment ions with a particular loss of mass, for example, glucoside polyphenols would have a mass loss of 162 μ, which corresponds to a glucoside. Working in MRM mode in combination with precursor

Table 14.2 List of Fragment Ions of some Polyphenols and Related Metabolites Obtained Working in Negative-Ion ESI Mode

Compound	MW	$[M-H]^-$	*m/z* ions
3,3/4-Hydroxyphenyl propionic acid glucuronide	342	341	165
3,3/4-Hydroxyphenyl propionic acid sulfate	246	245	165
3/4-Hydroxyphenyl acetic acid glucuronide	328	327	151
3/4-Hydroxyphenyl acetic acid sulfate	232	231	151
3-Hydroxybenzoic	138	137	93
4-Hydroxyhippuric acid	195	194	100
4-Hydroxybenzoic	138	137	93
8-Prenylnaringenin	340	339	219, 175, 119
Apigenin-*C*-hexoside-hexoside	594	593	503, 473, 383, 353
Apigenin-*C*-hexoside-pentoside	564	563	563, 503, 473, 443, 353
Caffeic acid	180	179	135, 107
Caffeic acid-*O*-hexoside 1	342	341	179, 135
Caffeic acid glucuronide	356	355	179
Caffeic acid sulfate	230	259	179
Carboxyacteyl tryptophan	290	289	203, 159, 142
Chlorogenic acid	354	353	191
Coumaric acid glucuronide	340	339	163
Coumaric acid sulfate	244	243	163
Cryptochlorogenic acid	354	353	191, 173, 135
Dihydrocaffeic acid sulfate	262	261	181
Dihydrocaffeic acid glucuronide	358	357	181
Dihydrocaffeic acid, 3,4-dihidroxyphenylpropionic acid	182	181	137
Dihydroxyphenyl acetic acid, homoprotocatechuic acid	168	167	123
Ethyl galate	198	197	169
Ferulic acid	194	193	134
Ferulic acid-*O*-hexoside	356	355	193, 178, 149
Ferulic acid glucuronide	370	369	193
Ferulic acid sulfate	274	273	193
Glutamylphenylalanine	294	293	164, 147, 103
Hydroferulic acid	196	195	136
Homovanillic acid	182	181	137
Hydroferulic acid 3,4-*O*-glucuronide	372	371	195
Hydroferulic acid 3,4-*O*-sulfate	276	275	195
Hydroxyphenyl acetic acid	152	151	107

Continued

Table 14.2 List of Fragment Ions of some Polyphenols and Related Metabolites Obtained Working in Negative-Ion ESI Mode—cont'd

Compound	MW	$[M-H]^-$	*m/z* ions
Hydroxyphenyl propionic acid	166	165	121
Isoferulic acid	194	193	134
Isorhamnetin	316	315	301
Isorhamnetin glucuronide	492	491	315
Isorhamnetin sulfate	396	395	315
Isoxanthohumol	354	353	233, 165, 119
Kaempferol	286	285	251
Kaempferol-3-*O*-rutinoside	594	593	593, 285
Kaempferol-*O*-rutinoside-hexoside	756	755	593, 285
m-Coumaric acid	164	163	119
Naringenin	272	271	151, 119
Naringenin 4′-glucuronide	448	447	271
Naringenin 7-glucuronide	448	447	271
Naringenin-7-*O*-glucoside (prunin)	434	433	433, 271
Neochlorogenic acid	354	353	191, 179, 135
p-Coumaric acid	164	163	119
Phenyl acetic acid	136	135	91
Phenyl acetic glucuronide	312	311	135
Phenyl acetic sulfate	216	215	135
Phloretin-*C*-diglycoside	598	597	477, 387, 357, 417
Piceid	390	389	227, 185
Quercetin	302	301	301, 151
Quercetin sulfate	382	381	301
Quercetin-3-*O*-glucuronide	478	477	301
Resveratrol	228	227	185, 143
Rutin	610	609	609, 300
Rutin-*O*-hexoside-pentoside	904	903	741, 609, 300
Taxifolin	304	303	285
Xanthohumol	354	353	233, 119

ion scan and product ion scan can be helpful in characterizing a particular compound found in complex mixtures. This kind of mass analyzer is the most commonly used.

An ion-trap mass spectrometer (MS^n) consists of a chamber with two electrodes and two end pieces that trap ions with a series of electromagnetic fields. Once the ions are inside, another magnetic field is applied, and only selected ions remain in the chamber. This mass analyzer is useful for structural elucidation purposes, performing multiple stage MS^n (Anari *et al.*, 2004; Wolfender, 2009).

The introduction of high-resolution spectrometers like TOF, QTOF, FTMS, and LTQ-Orbitrap has provided increased resolution and mass accuracy. A TOF mass

analyzer consists of an ion source and a detector. The ions are accelerated towards the detector with the same amount of energy through an accelerating potential. Ions with different *m/z* reach the detector at different times, with lighter ions arriving first due to their greater velocity. This spectrometer permits the analysis of a wide mass range, supplying molecular formula information and precise ion trace extraction.

The QTOF is a hybrid configuration of the TOF spectrometer. The ions are filtered in Q1, the collision takes place in Q2, and finally the product ion is determined by TOF. Compared to a triple quadrupole spectrometer, the QTOF offers greater sensitivity and accuracy when working in full scan mode, and unlike TOF equipment, measures MS/MS. The QTOF spectrometer is useful for the characterization of molecules with a wide range of mass.

FTMS is based on the effect of a magnetic field on an ion rotating in a radiofrequency field. Using the magnetic field, the ions are directed to a chamber where they rotate, describing small orbits with minimum frequency. The application of a radio frequency signal excites the ions to describe spiral orbits with increasing amplitude. When the diameter of the orbit is equal to the distance between the two electrodes, the ions are detected, generating an image of power, which is a direct function of their *m/z* relationship. This image is integrated by a Fourier transformation and converted to a signal proportional to its intensity. The full spectrum is obtained by scanning a radio frequency field that varies between 8 kHz and 100 MHz. The main advantages of this type of analyzer are its high precision mass measurements (0.001% and above) and almost unlimited resolving power.

The LTQ-Orbitrap, which combines an ion-trap analyzer with FTMS, allows MS and MS^n analysis with an error of less than 2 ppm. LTQ-Orbitrap-MS is a good tool for qualitative analysis, facilitating the structural elucidation of unknown compounds (Peterman *et al.*, 2006).

14.3.4.1 HPLC-MS

A liquid chromatograph coupled to a single quadrupole mass spectrometer allowed the identification of trans-resveratrol and cis-resveratrol (up to 10 µg/l) and trans-piceid and cis-piceid (up to 3 µg/l) in red and white wines. Separation was performed in a C18 (50 × 2.0 mm i.d., 5 µm) column, using a mobile phase of (A) water (0.5 ml/l acetic acid) and (B) acetone : acetonitrile : acetic acid (70 : 30 : 0.4 ml). Flow-rate was set at 500 µl/min.

A large number of phenolic compounds were successfully identified in propolis samples from different countries using HPLC-MS. The flavonoids were separated in a C18 column (150 × 4.6 mm, 4 µm) with (A) 0.25% acetic acid and (B) methanol as the mobile phase at a flow rate of 0.5 ml/min (Volpi and Bergonzini, 2006).

Anthocyanidins of 15 grape juice samples, four grape berries, and four grape skins were quantified using LC-MS. Separation was performed in an amide-C18 column (250 × 4.6 mm, 5 µm) with a mobile phase of (A) 0.4% TFA (v/v) in water and (B) 0.4% TFA (v/v) in acetonitrile at 1 ml/min. The results indicated that anthocyanidin concentration was higher in grape skins than the corresponding berries, and varied among the different grape juice samples (Xu *et al.*, 2012).

14.3.4.2 HPLC-MS/MS

The triple quadrupole mass spectrometer has been widely used because it provides higher selectivity, accuracy, and reproducibility, and better limits of detection and quantification, compared with a single quadrupole mass spectrometer.

A liquid chromatograph coupled to a triple quadrupole mass spectrometer equipped with a Turbo Ionspray source in negative-ion mode was used to study the levels of phenolics in different varieties of tomato (Vallverdú-Queralt *et al.*, 2011e), diced tomatoes (Vallverdú-Queralt *et al.*, 2011c), and tomato sauces (Vallverdú-Queralt A. *et al.*, 2012b). It was also used to evaluate the effects of storage on phenolic compounds (Vallverdú-Queralt *et al.*, 2011a) and the effects of pulsed electric fields on tomato polyphenols (Vallverdú-Queralt *et al.*, 2012c). Separation was performed in a C18 column (50×2.0 mm i.d., 5 µm) with a flow rate of 0.4 ml/min. Mobile phases consisted of (A) 0.1% formic acid in Milli-Q water and (B) 0.1% formic acid in acetonitrile. These conditions varied slightly depending on the product being analyzed. First, the presence of polyphenolic compounds was tested by MS/MS experiments of precursor ion scan, neutral loss scan, and product ion scan. The main objective of precursor ion scan experiments is to identify compounds belonging to a group of substances. In neutral loss experiments, the loss of 162 µ or 176 µ is used to confirm the loss of glucose, or galactose and glucuronides, respectively. Finally, product ion scan allows the identification of aglycones by comparison of their MS/MS spectra with those corresponding to the standards after typical fragmentations. MS/MS experiments were carried out by collision-activated dissociation (CAD) of selected precursor ions in the collision cell of the triple quadrupole mass spectrometer and the mass was determined by the second analyzer of the instrument. MS/MS has been used to analyze phenolic compounds in cocoa (Sanchez-Rabaneda *et al.*, 2003b), fennel (Parejo *et al.*, 2004), and artichoke (Sanchez-Rabaneda *et al.*, 2003a). Data was collected in MRM mode for quantification purposes, tracking the transition of the specific parent and product ions for each compound.

HPLC-MS/MS techniques can also be applied to determine phenolic compounds as potential taxonomical markers in food and plant samples (Andres-Lacueva C. *et al.*, 2002; De la Presa-Owens *et al.*, 1995; Romero-Perez *et al.*, 1996; Russo *et al.*, 1998; Singleton and Trousdale, 1983; Vallverdú-Queralt *et al.*, 2011e). For instance, phenolic and hydroxycinnamic acids, flavonoids, total polyphenols, and hydrophilic antioxidant capacity can be used as chemotaxonomic tomato markers to distinguish between tomato varieties (Vallverdú-Queralt *et al.*, 2011e). The polyphenolic profile determined by HPLC revealed a similarity within grape varieties and differences between varieties. Similarly, varieties of white musts (De la Presa-Owens *et al.*, 1995), wines (Romero-Perez *et al.*, 1996), and sparkling wines (Parejo *et al.*, 2004) have been shown to have different phenolic profiles.

Apart from food analysis, HPLC-MS/MS is widely used for biological fluid analysis due to its high sensitivity. In this way, an HPLC-MS/MS was used to identify and quantify phenolic compounds and metabolites from different tomato sauces in human plasma and urine in an intervention study (Tulipani *et al.*, 2012). There were three interventions in this prospective randomized, cross-over study: tomato sauce elaborated without oil, and with the addition of 5% virgin olive oil or refined olive

oil. Chromatographic separation was achieved in a C18 (50×2.0 mm, 5 µm) column, with a gradient elution of 0.1% aqueous formic acid and 0.1% formic acid in acetonitrile, and a flow-rate of 0.6 ml/min. Naringenin, ferulic acid, caffeic acid, and their corresponding glucuronide metabolites were detected in urine after the ingestion of the tomato sauces, while only two of the six urinary phenolic metabolites were identified in plasma as can be seen in Figure 14.2. Polyphenol levels of between 300 and 727 nmol/l have been detected using HPLC-MS/MS with a triple quadrupole instrument, thus showing the high sensitivity and selectivity of this system in the analysis of polyphenols in biological samples with a simple SPE extraction and clean-up process.

Using a triple quadrupole mass spectrometer, Aura *et al.* (2002) demonstrated that fecal microflora can deconjugate rutin, isoquercitrin, and quercetine glucuronides *in vitro* due to the presence of β,D-glucosidase, α,L-rhamnosidase, and β,D-glucuronidase. Fecal samples were freeze-dried before analysis. Then, polyphenol metabolites underwent liquid–liquid extraction using methanol/water (90 : 10, v/v) and were concentrated with a rotary evaporator. Samples were filtered and injected into the HPLC system. Chromatographic separation was performed using a reverse-phase column (100×1 mm). Mobile phases consisted of (A) 10 mmol/l ammonium acetate in water with 0.2% (v/v) acetic acid and (B) 10 mmol/l

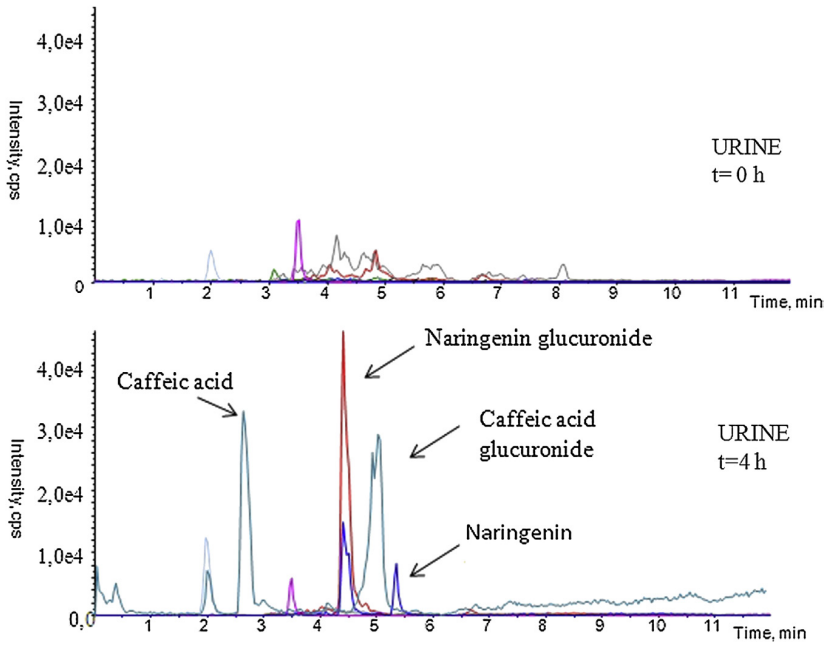

FIGURE 14.2

MRM chromatograms of urine sample at baseline (t = 0 h) and at 4 h after tomato sauce consumption.

ammonium acetate in methanol with 0.2% (v/v) acetic acid. Methanol with 0.1% (v/v) ammonium hydroxide was added as a post-column solvent (30 µl/min) to promote the desprotonation process of phenols previous to the ESI source. The measurements were performed in negative-ionization mode, and analyses were conducted in MRM. One or two fragment ions from the product ion spectra of the metabolites were used to identify the metabolites. This study showed that deconjugation and conversion of isoquercitrin and quercetin glucuronides to hydroxyphenylacetic acid occurs very rapidly in *in vitro* colonic fermentation. In contrast, rutin is deglycosylated at a slower rate, suggesting that rutin would be hydrolyzed at a slower rate than the other substrates. Therefore, the resulting quercetin aglycone appeared only transiently before further metabolism.

14.3.4.3 HPLC-HRMS

High-resolution mass spectrometry (HRMS) is used for qualitative analysis. A widely used technique is liquid chromatography/electrospray ionization–time-of-flight–mass spectrometry (HPLC-ESI-QTOF). QTOF technologies allow exact mass measurements of both MS and MS/MS ions. QTOF-MS has been used to determine phenolic compounds in gazpachos, ketchups, and tomato juices (Vallverdú-Queralt A. *et al.*, 2011b), resulting in the identification for the first time of apigenin-*C*-hexoside-hexoside and apigenin-*C*-hexoside-pentoside in tomato-based products. These compounds were distinguished by the presence of the ion $[M-H-60]^-$ (Figure 14.3A and B), following the method of Han *et al.* (2008) which involves liquid chromatography coupled with electrospray ionization mass spectrometry. Vallverdú-Queralt *et al.* (2011b) reported for the first time the presence of protocatechuic acid-*O*-hexoside,

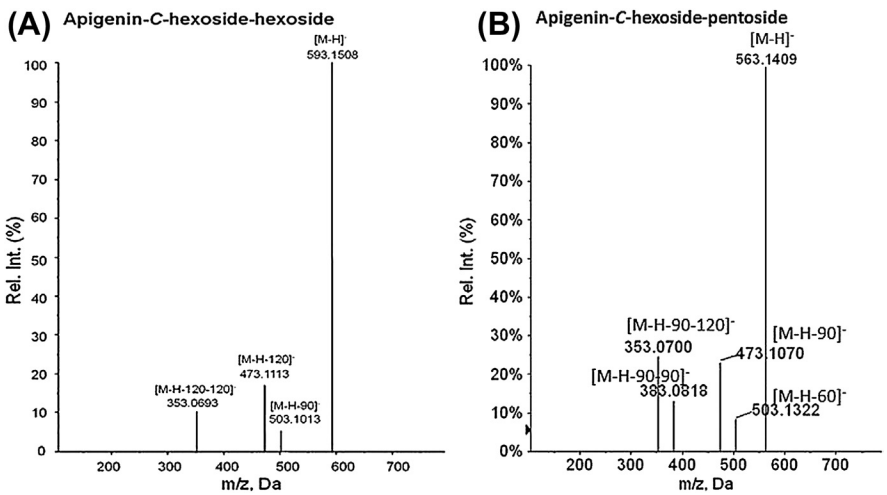

FIGURE 14.3

(A) Mass spectrum of apigenin-*C*-hexoside-hexoside. (B) Mass spectrum of apigenin-*C*-hexoside-pentoside.

caffeic acid-*O*-dihexoside, apigenin-*C*-hexoside-hexoside and apigenin-*C*-hexoside-pentoside in tomato-based products.

Metabolomics, a combination of analytical and statistical techniques, facilitates sample differentiation by quantitatively and qualitatively measuring the dynamic range of metabolites. With the recent developments in plant metabolomic techniques, it is possible to detect several metabolites simultaneously and reliably compare samples for differences and similarities in a semi-automated and untargeted manner. Metabolomics is predicted to play a crucial role in "bridging the phenotype–genotype gap" and in achieving complete genome sequence annotation and the understanding of gene function (Hall, 2006).

Metabolomics has also been used in the quality control of medicinal plants (Kim *et al.*, 2011). An ultraperformance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF MS)-based metabolomic technique was applied for the metabolite profiling of 60 *Panax ginseng* samples of 1 to 6year-old plants. After submitting the data for classification by various metabolite selection methods, the results showed variations according to the age of the samples, especially for those of 4 to 6year-old plants. Thus, a UPLC-QTOF MS-based metabolomics approach was able to quickly and accurately distinguish between *P. ginseng* samples according to their cultivation period.

HPLC-ESI-QTOF has also been used as a non-targeted strategy to differentiate between organic and conventional ketchups (Vallverdú-Queralt *et al.*, 2011d). Interpretation of the observed MS/MS spectra in comparison with results found in the literature was the main tool for putative identification of metabolites. The compounds found in significantly higher ($p<0.05$) amounts in organic than in conventional ketchups were: caffeoylquinic and dicaffeoylquinic acids, caffeic and caffeic acid hexosides, kaempferol-3-*O*-rutinoside, ferulic-*O*-hexoside, naringenin-7-*O*-glucoside, naringenin, rutin, and quercetin. Examination of the chromatograms in TOF-MS mode also suggested the presence of glutamyl phenylalanine (*m/z* 293) and *N*-malonyltryptophan (*m/z* 289).

Recently, LTQ-Orbitrap-MS has been proposed as one of the most suitable strategies for qualitative analysis, since it routinely delivers the highest resolution and mass accuracy, which are necessary to reduce analysis times and increase confidence in results. Due to its ability to eliminate interference in the initial mass selection stage and to the specificity of MS/MS measurements, this spectrometer facilitates qualitative analysis of nontarget compounds. Elemental composition assignment and exact mass measurements are essential for molecule characterization. The structural elucidation of unknown compounds is easily accomplished by using accurate mass measurement of the product ions formed in MS^n experiments.

Thus, accurate mass experiments have yielded the elemental composition of polyphenol compounds, with MS^n fragment ions providing additional structural confirmation. The LTQ Orbitrap provides accurate mass MS and MS^n spectral data on a chromatographic time-scale with scan cycles of 1 s (at R = 60,000) or less. Up to five to eight sequential fragmentation spectra can be obtained,

depending on the concentration and ionization efficiency of the compound. Using multiple-stage mass spectra it is possible to generate spectral trees of the compounds (Sheldon *et al.*, 2009). Van der Hooft *et al.* (2011) validated and applied an accurate mass MS^n spectral tree approach to 121 polyphenolic compounds of different chemical flavonoid subclasses, including isomeric forms. The study focused on the possibility of discriminating between positional and stereoisomeric forms. Accurate mass spectra of polyphenols were obtained using an LTQ-Orbitrap hybrid mass spectrometer in negative and positive ionization mode. The accurate MS^n fragmentation spectra enabled isomeric compounds to be differentiated. Spectral trees of 119 polyphenols (except catechin and epicatechin) showed unique fragments and differences in relative intensities of fragment ions. Thus, spectral trees constitute a potent tool for the identification of phenolic compounds or their metabolites. This tool could be applied to generate an MS^n metabolite database based on MS^n fragmentation and exact mass measurement.

Another study used liquid chromatography coupled with an LTQ Orbitrap to analyze polyphenols in tomato samples (Vallverdú-Queralt *et al.*, 2010). A C18 column (50 × 2.0 mm i.d, 5 µm) was used to separate the compounds. Gradient elution was performed with water/0.1% formic acid and acetonitrile/0.1% formic acid at a constant flow rate of 0.4 ml/min. A total of 38 compounds were identified in the tomato samples with very good mass accuracy (< 2 mDa). The spectra generated for cinnamic and benzoic acids showed the deprotonated molecule $[M\text{-}H]^-$ and some fragments. The typical loss of CO_2 was observed for gallic, protocatechuic, caffeic, and ferulic acids, giving $[M\text{-}H\text{-}44]^-$ as a characteristic ion, and loss of a methyl group $[M\text{-}H\text{-}15]^-$ was observed for ferulic acid. Flavonol aglycones such as quercetin gave the deprotonated molecule $[M\text{-}H]^-$ as a characteristic ion and ions corresponding to retro-Diels Alder fragmentation in the C-ring involving 1,3 scission, as described by other authors (Gruz *et al.*, 2008). The LTQ-Orbitrap was crucial for the structural determination of kaempferol-*O*-rutinoside-hexoside and rutin-*O*-hexoside-pentoside, which were not discernible under lower-resolution conditions.

Phloridzin-*C*-diglycoside (*m/z* 759) was only identified in the LTQ-Orbitrap due to the lack of sensitivity of the triple quadrupole. Figure 14.4 shows the MS^2 of *m/z* 759 of phloridzin-*C*-diglycoside, displaying losses of 90 u and 120 u from *m/z* 759 and 639, respectively, which confirmed the presence of two hexose units. Moreover, loss of H_2O was observed in the product ion spectra of *m/z* 759, showing an ion at *m/z* 741, which displayed a loss of 120 u (*m/z* 621). Losses of 90 and 120 u are characteristic fragment ions in the MS/MS mode of *C*-glycosides (Parejo *et al.*, 2004; Sanchez-Rabaneda *et al.*, 2003a).

In another study, 53 *O*-glycosyl-*C*-glycosyl flavones with *O*-glycosylation on phenolic hydroxyl or on the *C*-glycosyl residue, or a combination of both forms, were studied by liquid chromatography-UV diode array detection-electrospray ionization mass spectrometry ion trap in the negative mode. The study of the relative abundance of the main ions from the MS preferential fragmentation on $-MS^2$ and/or $-MS^3$ events allowed the differentiation of the *O*-glycosylation position,

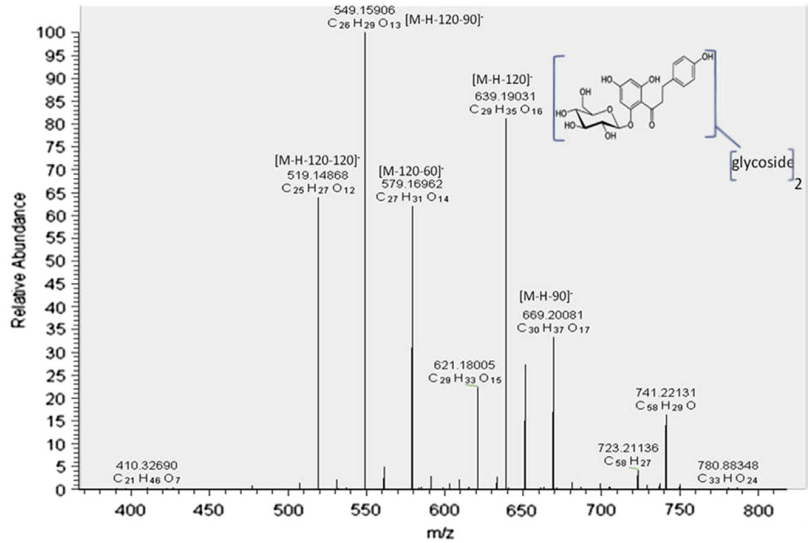

FIGURE 14.4

Identification of phloridzin-*C*-diglycoside in tomato samples. The MS^2 in product ion scan of *m/z* 759 shows the characteristic fragment ions of a *C*-diglycoside polyphenol.

either on a phenolic hydroxyl or on the sugar moiety of *C*-glycosylation (Ferreres *et al.*, 2007).

De Paepe *et al.* (2013) developed and validated a method to identify phenolic compounds in apple extracts using UHPLC coupled with an Orbitrap. An accurate mass spectrometry technique allowed the identification of 39 phenolic compounds in apples, including flavonoids, proanthocyanidins, and phenolic acids.

14.4 Ultra-high-performance liquid chromatography (UHPLC)

Liquid chromatographic performance has been improved by the introduction of UHPLC. To improve chromatographic separation, new columns with a very small particle packing size (1.7 μm) have been developed. Column efficiency is inversely proportional to particle size as the Van Deemter equations prove, so columns with 1.7μm particles provide higher resolution and better efficiency than the conventional ones (Novakova *et al.*, 2006). Improved mobile phase systems can operate at high backpressures (15000 psi), thus enhancing mobile phase viscosity and the capacity to dissolve analytes (Epriliati *et al.*, 2010). Due to these high pressures, new hardware in LC technology has been developed. As a result of the combination of columns and high pressure, UHPLC has enhanced sensitivity and peak resolution, and reduced

both analysis time and costs (Gruz *et al.*, 2008; Leandro *et al.*, 2006; Ortega *et al.*, 2010; Wu *et al.*, 2008).

14.4.1 UHPLC-UV

An ultra high pressure liquid chromatography technique was used to develop a new method for analyzing conjugated isoflavones in commercial soy milks (Toro-Funes *et al.*, 2012) using UV detection. This approach allowed the determination of 12 isoflavones in less than 8 min in a single run. The method was fully validated, with limits of detection lower than 0.05 mg/l and a limit of quantification below 0.2 mg/l. Chromatographic separation of analytes was achieved using a C18 column (50 × 2.1 mm, 1.7 µm). Solvent A was ultrapure water with 0.1% formic acid, and solvent B was acetonitrile with 0.1% formic acid, and the flow rate was set at 0.6 ml/min. The quantification of isoflavones was performed at 262 nm.

Gonçalves *et al.* (2013) also developed a new method to analyze hydroxybenzoics and hydroxycinnamics acids in wine samples using UHPLC coupled to a photodiode array detector in 11 min. The method was validated and showed limits of detection of 0.01–0.2 mg/l and a limit of quantification of 0.03–0.7 mg/l. A C18 (100 × 2.1 mm, 1.8 µm) column was used, and the gradient elution was performed with 0.1% formic acid and methanol as mobile phases at 0.25 ml/min. Before UHPLC analysis, phenolic compounds were extracted using microextraction by packed sorbent optimized for hydroxybenzoic and hydroxycinnamic acids.

Another study used UHPLC coupled with a UV detector to identify and quantify 58 polypyhenols in sage tea (Zimmermann *et al.*, 2011) within 28 min. Separation of the phenolic compounds was carried out using a reverse-phase column (150 × 2.1 mm, 1.7 µm). 0.1% formic acid was used as the aqueous mobile phase, and acetonitrile containing 0.1% formic acid as the organic mobile phase, at a flow rate of 0.4 ml/min. Wavelengths of the UV detector were set for quantification at 273, 320, and 360 nm. The method was applied to characterize 16 commercial brands of sage tea.

14.4.2 UHPLC-MS/MS

Coupling UHPLC with electrospray ionization tandem mass spectrometry offers a strong alternative to conventional HPLC-MS/MS in terms of analysis time, costs, and improved resolution and sensitivity.

Using UHPLC-MS/MS, 17 phenolic acids were quantified in white wine, grapefruit juice and green tea infusion within 10 min. Separation was performed in a reverse-phase column C8 (2.1 × 150 mm, 1.7 µm) with a mobile phase of aqueous 7.5 mM HCOOH (A) and acetonitrile (B) at a flow rate of 250 µl/min (Gruz *et al.*, 2008). The validated method offered good precision and accuracy, and limits of

detection of 0.15–15 pmol/injection. A UHPLC-MS/MS system is also suitable for routine analysis in laboratories.

A UHPLC-MS/MS was used to analyze procyandins and alkaloids (caffeine and theobromine) in samples of cocoa nibs (Ortega *et al.*, 2010). A high-strength silica separation column (100 × 2.1 mm i.d., 1.8-mm particle size) was used with (A) water/acetic acid (99.8/0.2, v/v) and (B) acetonitrile at a flow rate of 0.4 ml/min. This method allowed catechin and epicatechin to be quantified separately, which was not possible with HPLC-UV due to the coelution of the compounds. Also, the total analysis time for a cocoa phenolic extract was reduced from 80 min by HPLC to 12.50 min by UHPLC, and limits of detection of the procyanidin were enhanced from 0.009–0.02 mg/ml with HPLC-MS/MS to 0.007–0.01 mg/ml with UHPLC-MS/MS.

Twenty-six phenolic compounds, including 15 isoflavonoids, five flavones, four flavanones, a coumestan, and a coumarine of plant material, were analyzed in 17 min by UHPLC–ESI–MS/MS. The 26 compounds were separated with a C8 column (2.1 × 150 mm, 1.7 mm), using a mobile phase of methanol (A) and 10 mM aqueous formic acid (B) 100% A at a flow rate of 0.2 ml/min. The validated method achieved good accuracy and precision, with limits of detection ranging between 0.0001 and 10 pmol/injection. Solvent consumption and time were reduced compared to conventional HPLC systems (approximately 30–70 min) (Prokudina *et al.*, 2012).

14.5 Conclusions

Polyphenol characterization is a difficult task due to the large number of phenolic compounds present in nature and the few available commercial standards.

Efficient identification and quantification involves pretreating the samples to avoid interference. Food and beverage samples are normally analyzed after liquid–liquid or solid–liquid extraction, but biological samples such as plasma, urine, or serum usually require solid-phase extraction, since they contain far lower levels of compounds than food samples. Solid-phase extraction procedures are also used to clean up the samples by eliminating interfering compounds, thus avoiding the matrix effect.

Polyphenols can be identified or quantified with various analytical techniques, but the most commonly used are liquid chromatography coupled with UV detection, and liquid chromatography coupled with mass spectrometry.

The most widespread technique for analyzing polyphenols in food and plant samples is HPLC-UV, which also constitutes an excellent option for routine analysis in analytical laboratories and the food industry. In contrast, when working with biological samples, a more sensitive technique like mass spectrometry is required due to the complexity of the matrix and because mass spectrometry offers lower limits of detection than HPLC-UV (Table 14.1).

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3.2. El tomate

3.2.1. Origen y distribución

El tomate es un fruto originario de América, concretamente de México y Centroamérica. La procedencia de las primeras zonas de cultivo todavía es controversial, ya que existen dos hipótesis: la peruana y la mexicana. Si bien no existe una evidencia científica sobre ello, México presume de ser el primer país cultivador de tomate, siendo Perú el centro de la mayor diversidad de especies silvestres de este fruto[43–45].

Hernán Cortés introdujo el tomate en España tras la conquista en 1521 de Tenochtitlán, ciudad Azteca conocida actualmente como Ciudad de México. Desde España, el tomate llegó a Italia, Francia, e Inglaterra. Tras la conquista del sur de América, y Filipinas, el tomate se distribuyó por las colonias americanas y se introdujo en Asia. A través de Inglaterra, el tomate llegó a América del norte y los países colonizados en Asia por este país[44]. Los tomates se cultivaron en gran parte de Europa como un alimento a partir del siglo XVI, aunque en algunos países como Inglaterra y sus colonias norteamericanas se utilizaban como plantas ornamentales, ya que se consideraron venenosas al contener glicoalcaloides tóxicos[46].

3.2.2. Descripción botánica

El tomate (*Solanum lycopersicum*) pertenece a la familia de las Solanáceas, la cual incluye más de 98 géneros y 3000 especies originarias de la China, India, Centroamérica y Suramérica[43,44]. Dentro de la familia de las Solanáceas destaca el género *Solanum*, con unas 1250 a 1700 especies, donde encontramos las patatas, berenjenas y los tomates[43,44]. Las especies del género *Solanum* se cultivan en zonas cálidas o tropicales y son notables por su diversidad morfológica y ecológica. Existen una gran variedad de tomates que se clasifican en dos grupos según sea su destino de producción: tomate para consumo en fresco y tomate para la industria del procesado[1]. Los tomates de consumo en fresco se pueden clasificar en: redondos lisos, asurcados, oblongos o alargados y tomates cereza o cherry[1]. En el caso del tomate destinado para usos industriales pueden distinguirse dos tipos según el mercado de destino: tomate para concentrado y salsas y tomate pelado[1]. Dentro del tomate concentrado encontramos las variedades H-9036, Incas, Odín, Perfect peel, Riel, Tenorio y UC-82, mientras que para

el tomate pelado las variedades son: Ercole, Gladis, Peralta, Red Spring y Supermarzano[1].

3.2.3. Importancia económica

El tomate es uno de los alimentos de origen vegetal más distribuido en todo el mundo y de mayor valor económico. Su demanda aumenta continuamente y con ella su cultivo, producción y comercio. Según datos de la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAOSTAT) en 2013, su producción llegó a ser de 164 millones de toneladas (Mt)[44,47]. En los últimos 20 años, Asia ha pasado a ser el principal continente productor de tomate con un 51.2%, superando en gran medida a América (19%) y Europa (17.3%) (**Figura 3**), los cuales habían sido hasta el momento los mayores productores de este fruto.

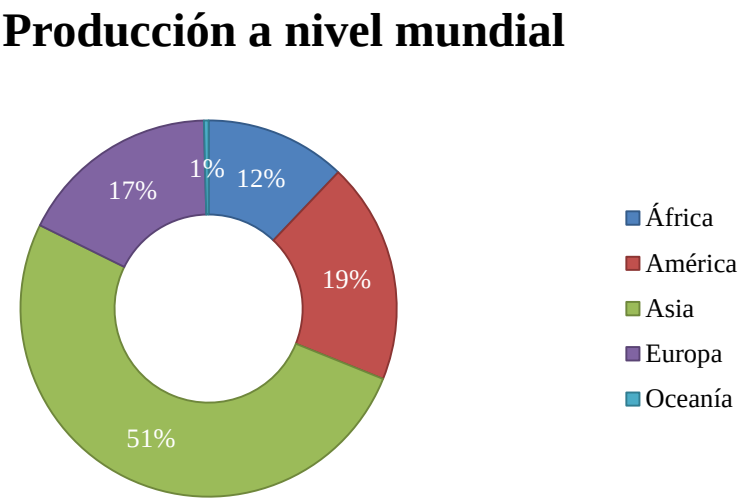

Figura 3. Producción de tomate fresco a nivel mundial.

Los principales países de producción de tomate son: China, con 50 Mt, seguido de la India, EEUU, Turquía y Egipto. España se encuentra entre los 10 primeros países con mayor producción de tomate a nivel mundial, hallándose en noveno lugar (**Figura 4**)[47].

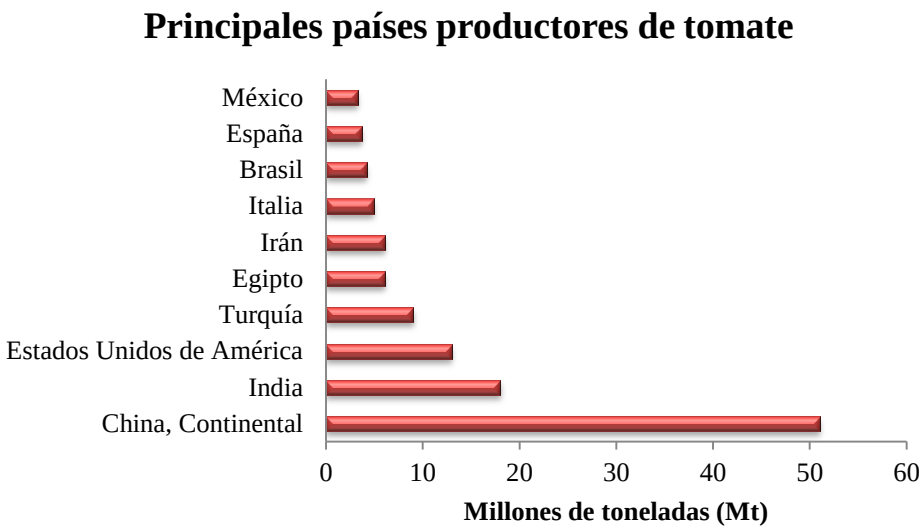

Figura 4. Los 10 principales países productores de tomate a nivel mundial.

Aunque el continente asiático es el principal productor, México con 1.5 Mt, los Países Bajos con 0.98 Mt, y España con 0.91 Mt son los tres principales exportadores, según datos de la FAOSTAT del 2012[47].

En el caso de España, en 2013, el tomate llegó a ser el quinto alimento a nivel de producción con 3.7 Mt, detrás de la oliva, el trigo, la uva y la leche de vaca[47]. Las principales comunidades autónomas productoras se hallan en el sur de España, siendo Andalucía y Extremadura las que presentan una mayor producción (**Figura 5**).

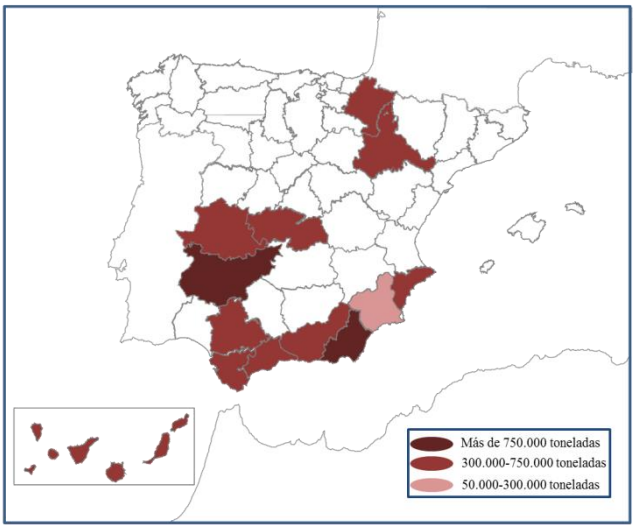

Figura 5. Principales regiones productoras de tomate en España.

La mayor parte del tomate producido en Extremadura se destina a la industria, igual que en Navarra y Zaragoza. En cambio, en Andalucía, donde destaca Almería, su cultivo se destina principalmente al consumo en fresco y sobre todo a la exportación. Otras provincias destacadas en producción de tomate fresco son Granada, Málaga, Murcia y Canarias. Cabe matizar que el mayor destino de las exportaciones son los Países Bajos, Alemania, Francia, Reino Unido y Polonia. Por lo que respecta al consumo, en España se ingiere una media de 14.3 kg de tomate por habitante al año. El consumo por regiones es heterogéneo, siendo superiores a la media nacional en Cataluña, Murcia y Aragón e inferiores en La Rioja, Navarra, Galicia, Asturias, Canarias y País Vasco[2].

3.2.4. Tomate procesado

Los tomates principalmente se consumen en crudo, a pesar de que se ha observado un aumento significativo en su ingesta a través de productos procesados[46]. El tomate para procesado industrial incluye una gran variedad de usos, entre los que destacamos: tomate concentrado, tomate triturado, tomate en polvo, tomate pelado, enteros o dados, kétchup, zumos o salsas[2,36].

En España, el producto de tomate procesado más consumido es la salsa de tomate, seguida del tomate natural, tomate natural triturado, kétchup, tomate natural entero pelado y zumo de tomate con un 3.76, 1.53, 1.33, 0.52, 0.21 y 0.1 kg/cápita/año, respectivamente[1].

3.2.5. Composición y valor nutricional

Los tomates representan una importante fuente de nutrientes, minerales y vitaminas. En la **Tabla 2**, se muestra la composición nutricional del tomate y su mayor subproducto, la salsa de tomate.

Tabla 2. Composición nutricional del tomate y salsa de tomate[1,48].

	Por 100 g		Por ración (245 g)	
	Tomate	**Salsa de tomate**	**Tomate**	**Salsa de tomate**
Energía (Kcal)	16	24	38	59
Proteínas (g)	0.79	1.20	1.90	2.94
Lípidos (g)	0.25	0.30	0.60	0.74
Saturados (g)	0.034	0.041	0.082	0.100
Monoinsaturados (g)	0.040	0.045	0.096	0.110
Poliinsaturados (g)	0.101	0.121	0.242	0.296
Hidratos de carbono (g)	3.47	5.31	8.33	13.01
Fibra (g)	1.9	1.5	4.6	3.7
Agua (g)	94.75	91.28	227.40	223.64
MINERALES				
Calcio (mg)	33	14	79	34
Hierro (mg)	0.57	0.96	1.37	2.35
Magnesio (mg)	10	15	24	37
Zinc (mg)	0.12	0.22	0.29	0.54
Sodio (mg)	115	474	276	1161
Potasio (mg)	191	297	458	728
Fósforo (mg)	17	27	41	66
VITAMINAS				
Tiamina (mg)	0.575	0.024	1.380	0.059
Riboflavina (mg)	0.055	0.065	0.132	0.159
Equivalentes niacina (mg)	0.712	0.991	1.709	2.428
Vitamina B_6 (mg)	0.111	0.098	0.266	0.240
Folatos (µg)	8	9	19	22
Vitamina C (mg)	12.6	7	30.2	17.2
Vitamina A: Equivalentes Retinol (µg)	20	22	48	54

El agua es el principal componente de los dos productos representando un 95% del total en el tomate y un 91% en la salsa. Dentro de los macronutrientes, destacan los hidratos de carbono, con un 3.5% para el tomate, y los lípidos, con un 5.3% en la salsa. Entre los minerales, el potasio es el elemento encontrado con mayor proporción en el tomate, alcanzando 191 mg/100 g mientras que en la salsa destaca el sodio con 474 mg/100 g. En lo que respecta a las vitaminas, cabe destacar la vitamina A con 20 y 22 µg/100 g para tomate y salsa respectivamente, y la vitamina C con 12.6 mg/100 g para el tomate y 7 mg/100 g para la salsa[1,44].

3.2.6. Efectos beneficiosos del tomate sobre la salud

El consumo regular de tomates y sus productos se asocia a un menor riesgo de padecer ciertos tipos de cáncer[8,49,50], y enfermedades cardiovasculares[5,51–55]. Existen diversos estudios *in vitro*, en animales y en humanos en los que se estudian los efectos

beneficiosos del tomate o sus nutrientes sobre este tipo de enfermedades. En la **Tabla 3**, se muestra una representación de los estudios realizados con tomate y sus derivados sobre la salud.

Tabla 3. Estudios de los efectos beneficiosos del tomate y sus derivados sobre la salud.

Sujetos	Intervención	Efecto en la salud	Referencia
Estudio *in vitro* (células humanas de cáncer de próstata)	Células tratadas con concentraciones crecientes de licopeno (0.3-3.0 µM) durante la noche.	El licopeno a las concentraciones estudiadas no afectó a la proliferación celular o promovió la necrosis, pero alteró la función mitocondrial y la apoptosis inducida en células de cáncer de próstata humano LNCaP.	8
Estudio *in vitro* (monocitos humanos y células epiteliales vasculares)	Extractos de Kétchup *vs* compuestos bioactivos del kétchup (7.5 µM licopeno, 1.4 µM α-tocoferol o 55 µM ácido ascórbico individuales).	Protección en el efecto antiinflamatorio contra la quimiotaxis en los extractos de kétchup.	52
Ratas macho Wistar	Dieta hipercalórica *vs* dieta hipercalórica suplementada con oleorresina de tomate rica en licopeno (10 mg/Kg/día).	Incremento de adiponectina en el tejido adiposo, en plasma y en la expresión de mRNA.	51
Cerdos	Sofrito (100 g/día).	Preservación de la función endotelial coronaria y mejora del perfil de la apolipoproteína AI y apolipoproteína J en las lipoproteínas de alta densidad.	53
Ratas macho	Dieta basal *vs* dieta basal y 2% de colesterol *vs* dieta basal y 2% de colesterol con 20% tomate *vs* dieta basal y 2% de colesterol con 20% salsa de tomate durante 1 mes.	Disminución de triglicéridos, LDL y colesterol total en las intervenciones con tomate y salsa, con mejores resultados en salsa.	55
Mujeres de mediana edad sanas	Consumo regular de tomate y subproductos (1.5, 1.5 a 4, 4 a 7, 7 a 10 y >10 porciones/semana).	A mayor consumo de tomate hay una mayor reducción de riesgo cardiovascular.	5

Hombres y mujeres sanos	Zumo de tomate fortificado con germen de soja (300 mL (66 mg isoflavonas y 22 mg licopeno)/día/8 semanas).	Gran biodisponibilidad de las isoflavonas y licopeno tras su administración.	6
Individuos con síndrome metabólico	1 zumo de tomate comercial al día, 4 veces por semana durante 2 meses (2.5 mg licopeno/100 g zumo y 312 µg β-caroteno/100 g zumo).	Reducción de las concentraciones séricas de factores de riesgo cardiovascular y mejora de la disfunción endotelial y el control glucémico al disminuir la resistencia a la insulina.	54
Individuos con alto riesgo cardiovascular	Consumo regular de gazpacho (250 g/semana).	Disminución de la presión arterial sistólica y diastólica tras la ingesta de gazpacho.	56
Mujeres con sobrepeso u obesas	330 mL/día de zumo de tomate *vs* control.	Disminución de concentraciones séricas de IL-8 y TNF-α en el grupo intervención al comparar el tiempo basal y después de la intervención. Disminución de la concentración sérica de IL-6 en el grupo de intervención respecto al grupo control.	57
Pacientes con diabetes tipo 2	200 g de tomate fresco/día durante 8 semanas.	Disminución de la presión arterial sistólica y diastólica. Incremento en apoA-I al final del estudio en comparación con los valores iniciales.	58
Hombres sanos	200 g/día de salsa de tomate amarillo (0 mg de licopeno) *vs* 200 g/día de salsa de tomate rojo (16 mg de licopeno) durante 1 semana. Después el primer grupo siguió una dieta suplementada con licopeno (16 mg/día) y el segundo grupo recibió placebo. Se recogió el suero antes y después de la intervención y se incubó con células de cáncer de próstata y se midió la expresión génica de 45 genes.	El licopeno afecta a la expresión génica tanto al ser administrado en la salsa como en forma de suplemento.	50

La mayoría de los estudios atribuyen los efectos beneficiosos a los carotenoides, sobre todo al licopeno, y pocos de ellos tienen en cuenta la posible sinergia que puede existir entre estos compuestos y los polifenoles a la hora de actuar sobre los posibles factores de ciertas enfermedades crónicas.

3.2.7. Compuestos fenólicos en tomates y subproductos

Recientemente, se han publicado diversos estudios sobre el contenido polifenólico de diferentes variedades de tomate, salsas, kétchups, zumos o gazpachos[4]. Los principales grupos de flavonoides y no flavonoides encontrados en el tomate y sus derivados son: flavonoles, flavanonas, flavonas y ácidos fenólicos[4,59].

Entre los flavonoles, podemos encontrar la quercetina, caemferol y en gran medida la rutina, en concentraciones que oscilan entre 1 y 20 mg/100 g. Dentro de las flavanonas, encontramos la naringenina y su forma glicosilada y entre las flavonas destaca la apigenina con sus varias formas glicosiladas[4]. Entre los ácidos fenólicos destacan los ácidos hidroxibenzoicos como el ácido gálico o el ácido protocateico y los ácidos hidroxicinámicos como el ácido 5-cafeoilquínico, con concentraciones que oscilan entre 0.14 y 8 mg/100 g[4]. Las concentraciones de los compuestos fenólicos varían según la variedad de tomate, el tipo de cultivo utilizado, el procesado y el almacenaje[60–63].

3.2.8. Biodisponibilidad de los compuestos fenólicos del tomate

La biodisponibilidad, como se ha comentado anteriormente, puede estar alterada por diversos factores: intrínsecos, extrínsecos, relacionados con el procesado, la matriz del alimento, la estructura química y la absorción gastrointestinal. En la bibliografía se han descrito diferentes estudios en los que se evalúa el cambio experimentado en los compuestos fenólicos del tomate teniendo en cuenta algunos de dichos factores[12,19,21,37,63–76].

- *Factores intrínsecos y extrínsecos:*

  Un factor intrínseco es el tipo de variedad de tomate. Distintas publicaciones resaltan la variabilidad del contenido fenólico según la variedad del tomate estudiado con concentraciones que pueden variar de 7 a 44 mg/Kg de quercetina o 14 a 32 mg/Kg de ácido 5-cafeoilquínico[68,69].

Dentro de los factores extrínsecos puede influir el método de cultivo, ya sea orgánico o convencional. En un estudio en el que se valoró durante 3 años el contenido fenólico en tomates sembrados en cultivos orgánicos y convencionales, se observó que el contenido de algunos polifenoles era mayor en cultivos orgánicos que en convencionales, pero estos resultados estaban más condicionados por el año que no por las condiciones de cultivo[64]. Otros estudios similares evaluaron el contenido polifenólico en tomates, kétchups y zumos, demostrando que, en los tres productos hay más polifenoles en los productos procedentes del cultivo orgánico *versus* del convencional[65,67,66].

- *Procesado del alimento*

  Hay descritos en la bibliografía diversos estudios sobre el efecto del tratamiento térmico o mecánico que experimentan los compuestos fenólicos de los tomates al ser tratados, así como su almacenaje. Se estudió el perfil fenólico del efecto de diferentes tratamientos industriales durante el procesado de salsas de tomate y dados de tomate y se determinó que el tiempo de evaporación era un punto crítico que debía ser controlado, ya que hacía disminuir el nivel de compuestos bioactivos tanto como la temperatura. También se concluyó que la adición de un extracto elaborado con pieles y semillas, un paso utilizado normalmente durante el procesado industrial para incrementar el espesor, ayudaba a enriquecer el producto final con estos compuestos[70,77]. En otro estudio similar en el que se estudiaron tres métodos distintos de procesado de tomate fresco a salsa de tomate, se encontró que la naringenina era el compuesto fenólico más afectado durante el tratamiento y que el ácido 5-cafeoilquínico incrementaba su concentración durante el procesado, sugiriendo una mejora en su biodisponibilidad[71]. En el caso del almacenaje, se evaluaron los cambios producidos en el perfil fenólico de kétchups y zumos durante 3, 6 y 9 meses y de gazpachos durante 3 meses y se observó que el nivel de polifenoles disminuía pasado este tiempo pero sin comportar un perjuicio al consumidor[37,63].

- *Absorción gastrointestinal*

  Existen varios ensayos en humanos en los que se ha estudiado la biodisponibilidad de los compuestos bioactivos del tomate, aunque la mayoría de ellos evalúan solamente el efecto en los carotenoides, sobretodo en el licopeno[12,72]. Los resultados encontrados en estos estudios demuestran como los carotenoides son más biodisponibles en tomates

procesados, ya sean zumos o salsas[12,72], que no en el fruto fresco proporcionando así más efectos beneficiosos para la salud[73–76].

Por otro lado, se encuentra un menor número de estudios en los que evalúan el efecto de la administración de tomate en la biodisponibilidad de los polifenoles en seres humanos sanos[19,21]. En un estudio se valoró el nivel de naringenina tras la ingesta de 150 mg de salsa de tomate cocinada y un control el cual no contenía salsa. Tras la ingesta de la salsa se detectó el compuesto en el plasma de los voluntarios mientras que en los controles no se detectó ningún resto del compuesto[21]. Un estudio similar, del mismo grupo de investigación, demostró que tras la ingesta de tomates cherry sin cocinar y cocinados en forma de salsa, las concentraciones de naringenina y ácido 5-cafeoilquínico incrementaban significativamente tras la administración de los tomates cocinados, pero no sin cocinar[19].

3.2.8.1. Efecto de una matriz lipídica en la biodisponibilidad de los compuestos fenólicos del tomate.

Uno de los factores que afectan a la biodisponibilidad de los polifenoles es la matriz en la que se encuentra el alimento. Las interacciones entre los diferentes compuestos o la matriz en la que el compuesto esté adherido afectan mucho en la biodisponibilidad de los compuestos. Recientemente, se ha publicado un estudio del efecto de la adición de aceite de oliva virgen extra (5 y 10%) en el perfil fenólico de salsas de tomate[62]. Los resultados demostraron que al aumentar la concentración de aceite de oliva había un incremento en el nivel de polifenoles, por lo que se demuestra que la matriz del alimento juega un papel crucial en la accesibilidad de los compuestos fenólicos. Por lo que respecta a estudios llevados a cabo con tomate y el efecto de la adición de aceite de oliva en humanos, la bibliografía hasta el momento reporta resultados con carotenoides, pero no con compuestos fenólicos. Los estudios encontrados demuestran que hay un incremento en la concentración de los carotenoides en plasma en las intervenciones en que se ha adicionado aceite[78,79].

RESULTADOS

4. Resultados

En esta sección, se expondrán los trabajos realizados a lo largo de esta tesis doctoral. Cuatro de los seis artículos están publicados en revistas del primer cuartil, el quinto artículo está publicado en la revista *Molecules* del segundo cuartil y el sexto y último artículo, en revisión por la revista *Molecular Nutrition and Food Research*. Previo a cada publicación hay un resumen donde se detallan brevemente los objetivos del estudio, sus resultados y las principales conclusiones.

4.1. Publicación 1. Validación de un nuevo método por LC-MS/MS para la detección y cuantificación de metabolitos fenólicos de salsas de tomate en muestras biológicas.

Validation of a new LC-MS/MS method for the detection and quantification of phenolic metabolites from tomato sauce in biological samples.

Miriam Martínez-Huélamo, Sara Tulipani, Xavier Torrado, Ramón Estruch and Rosa M. Lamuela-Raventós. *Journal of Agricultural and Food Chemistry*. 2012, 60(18):4542-4549.

Para una buena identificación y cuantificación de los compuestos fenólicos se necesita tener un método sensible y selectivo. Por ello, en esta publicación se muestra la optimización de un método para la determinación de 11 compuestos fenólicos derivados del tomate en orina y plasma humano por HPLC-MS/MS. Previo al análisis, se necesitó realizar una SPE para obtener unas muestras limpias para poder ser detectadas con mayor precisión en su posterior inyección en el sistema cromatográfico. El método fue completamente validado obteniéndose resultados de recuperación entre 73 y 104% en plasma y entre 65 y 106% en orina. La precisión varió entre 90.3 y 115% en orina y entre 85.7 y 115% en plasma, mientras que el coeficiente de variación de la precisión fue inferior al 15%. El método permitió límites de detección (LODs) y cuantificación (LOQs) de 0.5 a 29 y 2.0 a 90 ng mL^{-1} en orina, para la naringenina y el ácido isoferúlico, respectivamente, y 0.5 a 30 y 2.0 a 105 ng mL^{-1} en plasma, respectivamente, para los mismos compuestos fenólicos. Una vez validado el método, se realizó un estudio con 8 voluntarios sanos a los que se les suministró 250 mL de salsa de tomate y se les recogió orina y plasma después de la intervención. Además de los compuestos validados, también se buscaron los posibles metabolitos glucurónidados y sulfatados. Se pudieron detectar 4 compuestos en las muestras biológicas tras la ingesta de la salsa de tomate: naringenina, naringenina glucurónido, ácido ferúlico glucurónido y ácido cafeico glucurónido.

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Validation of a New LC-MS/MS Method for the Detection and Quantification of Phenolic Metabolites from Tomato Sauce in Biological Samples

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ABSTRACT: Tomato is a good source of bioactive molecules such as vitamin C, carotenoids, and phenolic compounds. Up to now, only a few studies have evaluated the bioavailability of phenolic compounds from tomato. This paper presents the optimization of a method for the determination of phenolics in tomato and their metabolites in human urine and plasma after ingestion of tomato sauce. The sample preparation includes a SPE step to obtain cleaner extracts for injection in the LC-MS/MS system. The mean recovery of analytes ranged from 73 to 104% in plasma and from 65 to 106% in urine, the accuracy was between 90.3 and 115.0% in urine and between 85.7 and 115.0% in plasma, and the precision coefficient of variation was <15%. The method allowed detection and quantification limits of 0.5−29 and 2.0−90 ng mL^{-1} in urine, respectively, and 0.5−30 and 2.0−105 ng mL^{-1} in plasma, respectively, for the same phenolic compounds.

KEYWORDS: *tomato, phenolic metabolites, LC-MS/MS, plasma, urine*

INTRODUCTION

Tomato (*Solanum lycopersicum*, formerly *Lycopersicon esculentum*) is one of the most popular and extensively consumed vegetable crops worldwide[1−3] and the most exported vegetable from Spain (880630 tons annually, according to FAO data). Tomato fruit is widely consumed either fresh or after processing into various products.[4,5]

Phenolic compounds found in tomato sauce belong to the class of phenolic acids, including hydroxycinnamic acids and their ester conjugates (mono- and dicaffeoylquinic acids, glucosides of caffeic, ferulic, dihydroferulic, and coumaric acids), with chlorogenic acid being the most abundant phenolic acid.[6,7] Tomato products also have a high content of the flavanone naringenin, which has been reported as the most abundant compound in tomato sauce.[6] In addition, recent results from our group show that rutin is a major flavonoid present in tomato products.[8,9]

There is today a growing awareness that the health effects derived from phenolic compound consumption depend on their bioavailability (absorption, distribution, metabolism, and elimination), a factor that is also influenced by their chemical structure.[10−14] Once ingested through food, phenolics are absorbed and then subjected to phase I (hydrolysis) and phase II (conjugation) metabolism in the small intestine and liver, which produces a series of water-soluble conjugate metabolites (methyl, glucuronide, and sulfate derivatives) that may pass into the bloodstream, accumulate in tissues, and then be excreted in urine.[15]

For the determination of phenolics and their metabolites in human urine and plasma, it is necessary to ensure an efficient and reproducible extraction in the sample treatment process. Optimization of this step is key to obtaining a method of analysis sensitive enough to determine these substances at low concentration. One of the most widely used techniques for the preconcentration and cleanup of analytical samples is solid-phase extraction (SPE). Tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS) has been demonstrated to be highly suitable for the analysis of phenolic metabolites[11,16−18] due to its high sensitivity, allowing lower detection limits and adequate selectivity versus interfering substances of the matrix, compared to LC with UV or electrochemical detection.

Previous studies by our group have established that the tomato sauce-making process (for instance, the addition or not of an oil matrix during sauce preparation) may affect the phase II metabolism of tomato phenolics and consequently the urinary excretion and plasma bioavailability. We here present the optimization of a new simple and rapid method for the determination of phenolic compounds and their metabolites in urine and human plasma and additionally report a screening of the occurring conjugated metabolites, which were identified by MS/MS experiments.

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EXPERIMENTAL PROCEDURES

Reagents and Materials. Phenolic standards caffeic acid, chlorogenic acid, dihydrocaffeic acid, ferulic acid, hippuric acid, isoferulic acid, and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA); 3-hydroxyphenylpropionic acid, 4-hydroxyphenylpropionic acid, naringenin, and ethyl gallate (internal standard (IS)) were from Extrasynthèse (Genay, France); and 4-hydroxyhippuric acid was from Phytolab (Vestenbergsgreuth, Germany).

All reagents were of HPLC grade: acetonitrile (MeCN), methanol (MeOH), and formic acid (HCOOH) were purchased from Scharlau Chemie S.A. (Barcelona, Spain); hydrochloric acid 37% (HCl) and orthophosphoric acid 85% (H_3PO_4) were supplied by Panreac Quimica SA (Barcelona, Spain). Human plasma was purchased from Sigma-Aldrich. Ultrapure water was generated by a Millipore System (Bedford, MA, USA).

The SPE cartridges used in this study were Oasis HLB 96-Well Plate 30 μm (30 mg) supplied by Waters (Milford, MA, USA).

Subjects and Study Design. Eight volunteers aged between 27 and 46 years (33 ± 5.9 years) and with a mean body mass index of 23 ± 0.93 kg/m^2 were selected. On the day of intervention, baseline blood and urine samples were collected from the subjects, who then consumed 250 mL of tomato sauce. Blood and urine samples were collected after the ingestion of the sauce. Blood was immediately centrifuged at 1500*g* for 20 min at 4 °C and stored at −80 °C until analysis. The urine bottles were stored at 4 °C and, immediately after the participants had started to collect the next fraction, the amount of urine in each fraction was measured, acidified with 0.2 M HCl, and stored at −80 °C until analysis. The study protocol was approved by the Ethics Committee of Clinical Investigation of the University of Barcelona (Spain), and the clinical trial was registered at the International Standard Randomized Controlled Trial Number (ISRCTN20409295). Informed consent was obtained from all participants.

Preparation of Standard and Stock Solutions. Individual stock solutions of caffeic acid, chlorogenic acid, dihydrocaffeic acid, dihydrophenylpropionic acid, ethyl gallate, ferulic acid, hippuric acid, isoferulic acid, naringenin, quercetin, and 4-hydroxyhippuric acid were prepared at a concentration of 1 mg mL^{-1} in 80% methanol.

The working solution, used to spike plasma and urine samples, was prepared by mixing the individual standard solutions and diluting it with H_2O. A solution of ethyl gallate at a concentration of 400 ng mL^{-1} was used as the internal standard. All standard solutions were stored at −80 °C.

Synthetic human urine was prepared by dissolving 0.65 g/L of $CaCl_2{\cdot}2H_2O$, 0.65 g/L $MgCl_2{\cdot}H_2O$, 4.6 g/L NaCl, 2.3 g/L Na_2SO_4, 0.65 g/L $Na_3C_6H_5O_7{\cdot}2H_2O$, 2.8 g/L KH_2PO_4, 1.6 g/L KCl, 1.0 g/L NH_4Cl, 25 g/L urea, and 1.1 g/L creatinine in Milli-Q water.

Instrumentation. *LC-ESI-MS/MS.* An HP Agilent Technologies 1100 LC system equipped with an autosampler and a column oven set to 30 °C and coupled to an API 3000 triple-quadrupole mass spectrometer (PE Sciex) with a turbo ion spray source was used to identify and quantify the corresponding phenolic metabolites. Chromatographic separation was achieved on a Luna C18 (50 × 2.0 mm (5 μm)) from Phenomenex (Torrance, CA, USA) using a precolumn Phenomenex security guard C18 (4 × 3 mm i.d.).[6] The system was controlled by Analyst v. 1.4.2 software supplied by Applied Biosystems (Foster City, CA, USA).

Procedures. *Extraction of Phenolic Compounds from the Tomato Sauce.* For the extraction of phenolic compounds from the tomato sauce, 5 mL of 80% ethanol acidified with 0.1% HCOOH was added to 0.5 g of sample. The mixture was vortexed for 1 min and then sonicated for 5 min on an ice bath to prevent degradation of the compounds. After centrifugation at 900*g* for 20 min at 4 °C, the supernatant was collected, another 5 mL of the acidified 80% ethanol solution was added to the pellet, and the extraction procedure was repeated. Both supernatants were combined and concentrated to dryness on a sample concentrator (Techne, Duxford, Cambridge, U.K.) at room temperature under a stream of nitrogen gas. The samples were redissolved in 2 mL of H_2O (0.1%HCOOH). After filtration of the aqueous extracts with 4 mm 0.45 μm PTFE syringe filters (Waters Corp.), 20 μL of the resulting filtrate was injected into the LC-MS/MS system, in triplicate.[6,8]

Sample Preparation Method for Plasma and Urine. On the day of the analysis, synthetic urine and plasma samples were defrosted on ice in the dark. For the preparation of the calibration curves, the samples were spiked with increasing concentrations for each phenolic standard. The IS, ethyl gallate, was added at a level of 400 ng mL^{-1}. Twenty-five microliters of H_3PO_4 was added to 2 mL of plasma and 17 μL of HCl to 2 mL of urine, which gave the samples a pH of 2. Before SPE, urine samples were centrifuged at 15000*g* for 4 min at 4 °C.

Solid-Phase Extraction. Solutions of 1.5 M HCOOH, 5% MeOH, MeOH (0.1% HCOOH), and H_2O (0.1% HCOOH) were prepared to be used in the SPE.

HLB plate activation was achieved by adding 2 mL of MeOH and 2 mL of 1.5 M HCOOH, consecutively. After the sample loading into the 96-well plate (2 mL), sample cleanup was performed with 2 mL of 1.5 M HCOOH followed by 2 mL of 5% MeOH solution. Phenolic compounds were then eluted with 2 mL of MeOH acidified with 0.1% HCOOH (v/v). The elution fraction obtained was evaporated to dryness by a sample concentrator (Techne) at room temperature under a stream of nitrogen. Two hundred microliters of water acidified with 0.1% HCOOH (v/v) was added to dissolve the residue to be injected into the LC system.

Chromatographic Conditions. The mobile phase used in LC-MS/MS for the tomato sauce and biological samples was water (A) and MeCN (B) with 0.1% HCOOH in both solvents. For the tomato sauce an increasing linear gradient (v/v) of B was used (t (min), %B), as follows: (0, 5), (10, 18), (13, 100), (14, 100), and (15, 5), followed by a 5 min re-equilibration step, at a constant flow rate of 400 μL min^{-1}. The flow rate for the biological samples was 0.6 mL min^{-1}, and the gradient used (t (min), % B) was (0, 5), (2, 25), (10, 90), (11, 100), and (12, 100), followed by a 5 min re-equilibration step. Twenty microliter aliquots of the extracts were injected in the LC-MS/MS system.

LC-ESI-MS/MS Parameters. The LC-ESI-MS/MS conditions were optimized by direct injection of 50:50 (v/v) water (0.1% HCOOH)/MeCN (0.1% HCOOH) of each phenolic standard individually at a concentration of 1 μg mL^{-1} into the mass spectrometer using a model syringe pump (Harvard Apparatus, Holliston, MA, USA) at a constant flow rate of 5 μL min^{-1}. The turbo ion spray source was in negative mode with the following settings: capillary voltage, −4500 V; nebulizer gas (N_2), 10 (arbitrary units); curtain gas (N_2), 12 (arbitrary units); drying gas (N_2) was heated to 400 °C and introduced at a flow rate of 4500 mL min^{-1}. Table 1 shows the declustering potential (DP), focusing potential (FP), and entrance potential (EP) optimized to detect phenolics with the highest signals.

Full-scan data were acquired by scanning from m/z 100 to 800 in profile mode using a cycle time of 1 s. Multiple reaction monitoring (MRM) experiments in the negative ionization mode were performed using a dwell time of 154 ms. The ions in MRM mode were produced by collision-activated dissociation (CAD) of selected precursor ions in the collision cell of the triple quadrupole and analyzed with the second analyzer of the instrument.[14,19,20] The optimum collision-activated dissociation (N_2) was 4 (arbitrary units). Two transitions were followed for each phenolic compound; one was used for quantification and the other for identification. Table 1 shows these transitions with their optimum collision energy.

To identify the phenolic metabolites, we carried out different experiments such as product ion scan (PIS), neutral loss (NL), and precursor ion scan (PrIS). Only PIS experiments were successfully used for identification purposes due to the lower concentration of the phenolic metabolites investigated and, consequently, the need for greater sensitivity. The PIS experiments were carried out using a cycle time of 2 s.

Quality Parameters. The quality parameters established for the correct validation of the method were recovery, selectivity, limit of detection, limit of quantification, linearity, accuracy, and precision.[11,19,21]

Table 1. Declustering Potential (DP), Focusing Potential (FP), and Entrance Potential (EP) Optimized[a]

	DP (V)	FP (V)	EP (V)	quantification transition	collision energy (V)	identification transition	collision energy (V)
caffeic acid	−40	−170	−11	179 → 135	−20	179 → 107	−30
chlorogenic acid	−40	−180	−11	353 → 191	−20	353 → 179	−30
dihydrocaffeic acid	−40	−170	−11	181 → 137	−20	181 → 121	−30
3- and 4-hydroxyphenylpropionic acid	−30	−200	−10	165 → 121	−20		
ethyl gallate (IS)	−60	−200	−10	197 → 169	−25	197 → 124	−30
ferulic acid	−50	−220	−11	193 → 134	−20	193 → 178	−20
hippuric acid	−40	−170	−10	178 → 134	−20		
isoferulic acid	−40	−170	−11	193 → 178	−20	193 → 134	−20
naringenin	−50	−190	−11	271 → 151	−30	271 → 119	−40
quercetin	−50	−210	−11	301 → 151	−30	301 → 179	−30
4-hydroxyhippuric acid	−40	−170	−10	194 → 100	−20	194 → 150	−30

[a]Quantification and identification transitions with the optimum collision energy.

RESULTS AND DISCUSSION

Chromatographic Separation and Ion Mass Detection. The best compromise between keeping chromatographic run times as short as possible and allowing a convenient resolution of plasma and urine sample extracts was found to be a C18 column applying a gradient elution from 5 to 100% in organic component using LC-MS/MS. Peak resolution was higher than 1.5 except for the separation of hippuric acid, dihydrocaffeic acid, and chlorogenic acid. Peak width was acceptable for all of the compounds, ranging between 5.2 and 13.2 s. The multistep gradient elution yielded an optimum separation of the 11 phenolic compounds in <12 min using LC-MS/MS. Figure 1 shows spiked blank plasma at a level 16 times the LOQ.

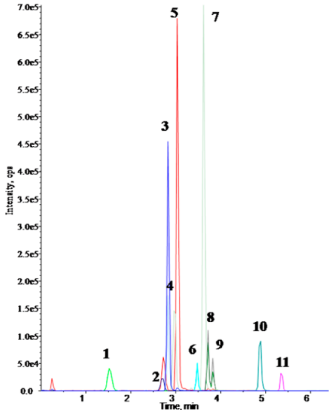

Figure 1. Separation of the 11 phenolics by LC-MS/MS. Peaks: (1) 4-hydroxyhippuric acid; (2) hippuric acid; (3) dihydrocaffeic acid; (4) chlorogenic acid; (5) caffeic acid; (6) ethyl gallate (IS); (7) 3- and 4-hydroxyphenylpropionic acid; (8) ferulic acid; (9) isoferulic acid; (10) quercetin; (11) naringenin.

The coupling of LC with MS is a powerful tool for the identification and quantification of analytes in biological samples.[11,16,17] Optimization of the methods was achieved by selecting the best ionization mode and mass spectrometer parameters. MRM mode exhibited the highest selectivity and sensitivity using LC-ESI-MS/MS.

Method Validation. The described method was fully validated by the criteria of the AOAC International: recovery, selectivity, limit of detection, limit of quantification, linearity, accuracy, and precision.[21]

Recovery. The recovery is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the same concentration of the pure authentic standard. The recovery obtained is illustrated in Tables 3 and 4. The results showed comparable values of recovery in fortified urine and plasma samples, with values being >95% in urine, except for 4-hydroxyhippuric acid and quercetin, which had recoveries of 75 and 65%, respectively, and values >96% in plasma, except for 4-hydroxyhippuric acid, which had 73% recovery. The recoveries of quercetin and 4-hydroxyhippuric acid obtained in the extraction were in agreement with the results of previous studies.[11,22]

Selectivity. Selectivity is the ability of an analytical method to differentiate and quantify the analytes in the presence of other components in the sample. To ascertain selectivity, six

Table 2. Contents of the Quantified Phenolic Acids, Flavanones, and Flavonols in the Administered Sauce

compound	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	concn range (ng mL^{-1})	linear fit	sauce (μg/g FW[a])
protocatechuic acid	8.01	30.4	30−400	0.996	129.2 ± 6.7
3-caffeoylquinic acid	23.9	75.8	75−1600	0.997	60.3 ± 1.1
5-caffeoylquinic acid	23.9	75.8	75−1600	0.997	814.3 ± 15.0
4-caffeoylquinic acid	23.9	75.8	75−1600	0.997	213.7 ± 6.9
dicaffeoylquinic acid	23.9	75.8	75−1600	0.997	54.3 ± 5.1
caffeic acid glucoside II	9.15	40.2	40−800	0.991	437.1 ± 9.4
caffeic acid glucoside IV	9.15	40.2	40−800	0.991	150.7 ± 1.3
caffeic acid glucoside II	9.15	40.2	40−800	0.991	6.1 ± 0.1
ferulic acid glucoside	18.0	50.2	50−2000	0.997	238.8 ± 12.2
naringenin	6.05	28.4	30−2000	0.992	1347 ± 63.6

[a]FW, fresh weight.

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Journal of Agricultural and Food Chemistry

Table 3. Urine Validation: Recovery, LOD, LOQ, Concentration Range, Concentration Added, Accuracy, Intraday, and Interday

					concn added			accuracy (%)			intraday 1 (%RSD)			intraday 2 (%RSD)			intraday 3 (%RSD)			interday (%RSD)		
	recovery (%)	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	concn range (ng mL^{-1}) (r^2)	L[a] (ng mL^{-1})	M[b] (ng mL^{-1})	H[c] (ng mL^{-1})	L[a]	M[b]	H[c]	L[a]	M[b]	H[c]	L[a]	M[b]	H[c]	L[a]	M[b]	H[c]	L[a]	M[b]	H[c]
caffeic acid	97 ± 4	1.5	6	6−3450 (0.990)	12	290	1730	1.7	2.8	0.7	2	3	6	2	2	4	2	5	5	3	15	13
chlorogenic acid	103 ± 2	0.5	2	2−1150 (0.999)	4	96	576	1.9	1.0	0.6	7	2	3	7	3	3	9	3	3	15	4	10
dihydrocaffeic acid	101 ± 2	6.3	25	25−14400 (0.993)	50	1200	7200	0.9	15.0	7.1	3	5	6	3	3	2	9	2	4	12	7	7
3- and 4-hydroxyphenylpropionic acid	97 ± 2	6	20	20−11520 (0.995)	40	960	5720	6.4	1.9	9.1	2	3	4	1	3	2	7	5	5	9	7	7
ferulic acid	95 ± 2	15	50	50−28800 (0.990)	100	2400	14400	9.7	15.0	0.3	6	5	9	6	8	8	9	3	4	11	9	10
hippuric acid	106 ± 2	25	80	80−51840 (0.990)	160	4320	25920	1.4	5.9	1.6	2	6	6	3	4	5	2	5	7	4	9	11
isoferulic acid	99 ± 2	29	90	90−28800 (0.997)	180	2400	14400	0.5	1.1	1.4	4	4	7	2	6	3	8	6	3	9	8	9
naringenin	104 ± 4	0.5	2	2−1150 (0.999)	4	96	576	1.6	0.5	0.6	7	5	8	9	8	7	7	7	6	12	9	11
quercetin	65 ± 3	1.7	6	6−3450 (0.991)	12	290	1730	2.7	7.1	0.5	3	9	3	6	7	8	9	6	9	10	15	14
4-hydroxyhippuric acid	75 ± 3	15	50	50−28800 (0.990)	100	2400	14400	2.2	8.3	3.2	9	8	10	4	3	5	7	8	5	10	14	12

[a]L, low concentration. [b]M, middle concentration. [c]H, high concentration.

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Table 4. Plasma Validation: Recovery, LOD, LOQ, Concentration Range, Concentration Added, Accuracy, Intraday, and Interday

					concn added			accuracy (%)			intraday 1 (%RSD)			intraday 2 (%RSD)			intraday 3 (%RSD)			interday (%RSD)		
	recovery (%)	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	concn range (ng mL^{-1}) (r^2)	L[a] (ng mL^{-1})	M[b] (ng mL^{-1})	H[c] (ng mL^{-1})	L[a]	M[b]	H[c]	L[a]	M[b]	H[c]	L[a]	M[b]	H[c]	L[a]	M[b]	H[c]	L[a]	M[b]	H[c]
caffeic acid	98 ± 3	1.8	6	6−3450 (0.993)	12	290	1730	6.4	0.6	0.7	7	8	7	10	6	4	8	4	4	12	11	8
chlorogenic acid	99 ± 4	0.6	2	2−1150 (0.999)	4	96	576	8.4	1.5	0.7	2	6	5	2	4	3	7	10	5	9	11	7
dihydrocaffeic acid	104 ± 5	4.4	15	15−14400 (0.991)	50	1200	7200	13.5	2.3	0.9	1	7	6	3	7	5	1	7	4	5	15	8
3- and 4-hydroxyphenylpropionic acid	99 ± 5	6	20	20−11520 (0.994)	40	960	5720	0.2	14.5	0.4	9	6	5	8	6	5	8	6	5	11	7	6
ferulic acid	98 ± 4	18	60	60−28800 (0.996)	100	2400	14400	14.3	14.6	0.8	5	5	7	4	2	3	2	3	9	6	9	11
hippuric acid	99 ± 4	25	90	90−51840 (0.992)	160	4320	25920	4.6	15.0	3.0	5	5	7	6	7	4	6	8	4	13	11	9
isoferulic acid	99 ± 4	30	105	105−28800 (0.994)	180	2400	14400	3.7	14.4	0.6	2	2	6	3	7	5	3	9	3	4	12	7
naringenin	96 ± 3	0.5	2	2−1150 (0.995)	4	96	576	12.6	0.5	1.0	8	8	5	6	6	6	5	7	7	9	13	9
quercetin	100 ± 3	1.4	5	5−3450 (0.990)	12	290	1730	5.9	9.7	6.0	5	4	7	3	3	8	5	4	13	6	11	15
4-hydroxyhippuric acid	73 ± 3	15	50	50−28800 (0.992)	100	2400	14400	14.3	2.9	1.6	4	12	3	7	5	5	5	14	7	10	15	11

[a]L, low concentration. [b]M, middle concentration. [c]H, high concentration.

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plasma and urine samples of six volunteers were analyzed at 3 h after ingestion of tomato sauce.

Under the chromatographic and MS/MS conditions used for the assay, analytes were well resolved with resolutions >1.5 for almost all of the compounds, and no interferences from matrix components at the mass transition and retention time of the analytes were observed in plasma and urine, compared with standards dissolved in water.

Limit of Detection (LOD) and Limit of Quantification (LOQ). The LOD was estimated for a signal-to-noise ratio of 3 from the chromatograms of spiked blank plasma and urine samples at the lowest analyte concentration tested. Similarly, the LOQ was determined for a signal-to-noise ratio of 10. Spiked plasma and urine samples at five different concentration levels were prepared in triplicate to establish the LOD and LOQ in the different mass spectrometric systems. Tables 3 and 4 present the LOD and LOQ obtained. These data indicated considerable differences in sensitivity between polyphenols but not variation in the biosamples. Hippuric acid and isoferulic acid presented higher LOD and LOQ than the other compounds in both plasma and urine. The concentrations obtained were in agreement with previous methodology described in the literature,[11,23] and for some compounds such as caffeic acid, chlorogenic acid, and quercetin, the limits were lower than previously reported values.[24]

Linearity. Linearity refers to the response of an instrument to the concentration of an analyte. In our case the response held a linear relationship with the analyte concentration. Linearity was tested by assessing signal responses of target analytes from plasma and urine samples spiked at a concentration ranging from the LOQ for each analyte to 52 μg mL^{-1}, depending on the LOQ of the phenolic compound. Spiked plasma and urine samples at eight different concentrations were prepared in duplicate. Calibration curves were constructed using the internal standard method (analyte/internal standard peak area ratio versus concentration of analyte/internal standard ratio), using 400 ng mL^{-1} of the internal standard. All calibration data are presented in Tables 3 and 4. The analytical procedure was linear over the concentration range tested with the correlation coefficient exceeding 0.990 for all compounds in plasma and urine samples, demonstrating the excellent linearity of the curves.

Accuracy and Precision. Accuracy is the closeness of agreement between the measured value and the accepted "true" or reference value. Experiments were evaluated by repetitively spiking the matrix with known levels of analyte standards. The accuracy of the method was expressed as (mean observed concentration/added concentration) × 100. The accuracy of the method was acceptable at each concentration level: in plasma, 85.7−114.3% for the low, 97.1−115.0% for the medium, and 98.4−103.0% for the high concentration levels; and in urine 90.3−106.4% for the low, 97.2−115.0% for the medium, and 98.4−109.1% for the high concentration levels.

Precision expresses the closeness of agreement among a series of measurements obtained from multiple testing of a homogeneous test sample under the method's established conditions. Precision was determined by independently processing 18 spiked plasma and urine samples at three different levels on three different days (six samples per day and level). The corresponding results of intra- and interday precision variabilities, summarized in Tables 3 and 4, show the good precision of the proposed method, with RSD values lower than 15%.

Analysis of Tomato Sauce. The most abundant type of phenolic compounds found in the studied tomato sauce were phenolic acids, particularly hydroxycinnamic acids and their ester conjugates (mono- and dicaffeoylquinic acids, caffeic acid glucoside, and ferulic acid glucoside), with chlorogenic acid being the most abundant, as previously described by Vallverdú-Queralt et al. The most abundant phenolic compound in the sample was the flavanone naringenin, confirming the high content previously reported in tomato and tomato products.[6] Table 2 shows the contents of the quantified phenolic acids, flavanones, and flavonols in the administered sauce.

Analysis of Plasma and Urine Samples. The developed method was applied to determine the concentration of the studied phenolics and identify their metabolites in plasma and urine samples after human consumption of nutritionally regular doses of tomato sauce. Phenolic compounds in plasma and urine were identified and quantified by comparing their MRM transition, retention time, and product ion scan with those of authentic standards.[11] The phenolic metabolites were identified by a product ion scan, neutral loss, or precursor ion scan in the absence of standards.

The MRM allowed 22 transitions to be monitored (Table 5), corresponding to 11 standard phenolic compounds and their expected metabolites. A chromatogram of urine sample at 4 h after the administration of tomato sauce is shown in Figure 2. Naringenin was detected in urine and plasma, although at very low concentrations. Naringenin was confirmed by the presence of a peak in the transition 271 → 151, at the same retention time as the standard. The compound appeared to a lower extent than its glucuronide metabolite, confirming its rapid metabolism, and was rapidly excreted after ingestion.

Naringenin glucuronide, caffeic acid glucuronide, and ferulic acid glucuronide were confirmed by the presence of peaks in transitions 447 → 271, 355 → 179, and 369 → 193, respectively. To ensure the identity of the conjugated metabolites, PIS was applied as a second experiment. The PIS of naringenin glucuronide shows two characteristic ions, m/z 271 belonging to the aglycone naringenin and m/z 176 belonging to the glucuronyl unit. Another fragmentation ion at m/z 113 is typical of the fragmentation of the glucuronide unit.[6,14] Caffeic

Table 5. Transitions of Phenolic Compounds and Their Metabolites in Plasma and Urine Samples

compound	MRM transitions (m/z, amu)	compound	MRM transitions (m/z, amu)
caffeic acid	179 →135	hippuric acid	178 → 134
caffeic acid glucuronide	355 → 179	hydroferulic acid	195 → 136
caffeic acid sulfate	259 → 179	hydroferulic acid glucuronide	371 → 195
chlorogenic acid	353 → 191	hydroferulic acid sulfate	275 → 195
dihydrocaffeic acid	181 → 137	isoferulic acid	193 → 178
dihydrocaffeic acid glucuronide	357 → 181	naringenin	271 → 151
dihydrocaffeic acid sulfate	261 → 181	naringenin glucuronide	447 → 271
3- and 4-hydroxyphenylpropionic acid	165 → 121	quercetin	301 → 151
ferulic acid	193 → 134	quercetin glucuronide	477 → 301
ferulic acid glucuronide	369 → 193	quercetin sulfate	381 → 301
ferulic acid sulfate	273 → 193	4-hydroxyhippuric acid	194 → 100

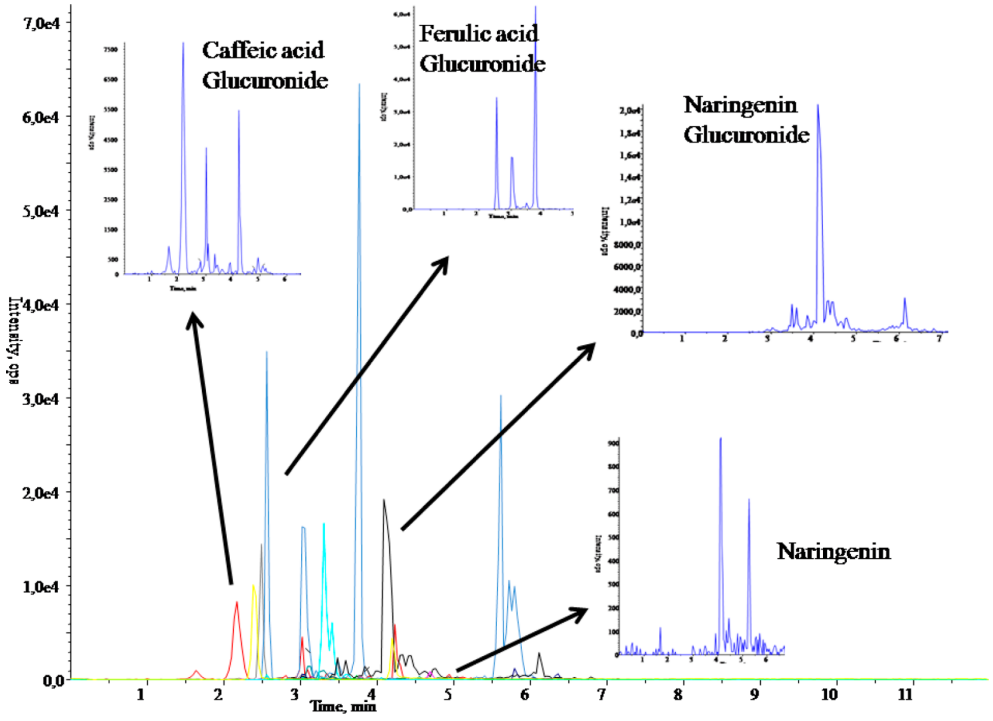

Figure 2. Chromatogram of urine sample at 4 h after the administration of tomato sauce.

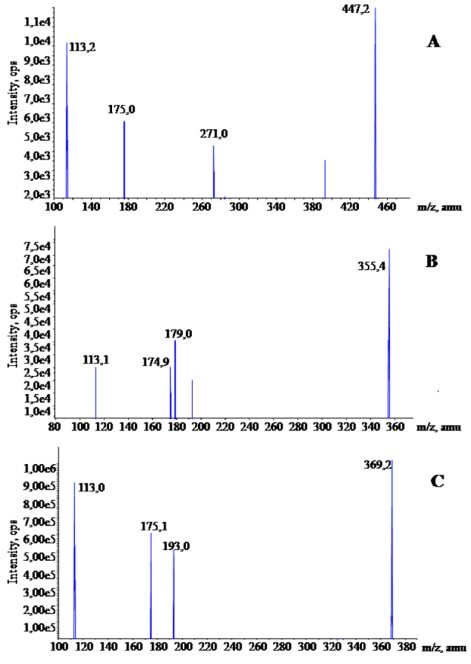

Figure 3. PIS of (A) naringenin glucuronide, (B) caffeic acid glucuronide, and (C) ferulic acid glucuronide.

acid glucuronide and ferulic acid glucuronide were also confirmed by PIS by the presence of m/z 176 amu, corresponding to the glucuronide unit, and by the ions corresponding to the aglycone at m/z 179 for caffeic acid and at m/z 193 for ferulic acid.[6] Figure 3 shows the PIS of naringenin glucuronide, caffeic acid glucuronide, and ferulic acid glucuronide. Ferulic acid glucuronide presented a slower rate of appearance in plasma, which corresponds with the longer and more complex metabolism leading to this compound.[6] On the other hand, the metabolite remained detectable in plasma for a longer period than the other compounds.

We have developed and validated a new LC-MS/MS procedure that determines tomato phenolics and their metabolites in biological samples with an extraction method capable of simultaneously analyzing a high number of samples. To our knowledge, this is the first reported method for the rapid detection and quantification of tomato sauce phenolics and their metabolites in plasma and urine. The procedure offers excellent selectivity and sensitivity. The use of LC-MS/MS to obtain the fragmentation patterns of the phenolics and phenolic metabolites led to a confident assignment of their structural classes. This method can be applied in future clinical and epidemiological studies with a high number of subjects to identify the real active compounds of tomato sauce and, consequently, to study their physiological effects in animal and human populations.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

MeCN, acetonitrile; CVD, cardiovascular disease; CAD, collision-activated dissociation; CE, collision energy; DP, declustering potential; FP, focusing potential; HCOOH, formic acid; FS, full scan; HCl, hydrochloric acid 37%; IS, internal standard; LOD, limit of detection; LOQ, limit of quantification; MeOH, methanol; MRM, multiple reaction monitoring; NL, neutral loss; H_3PO_4, orthophosphoric acid 85%; PrIS, precursor ion scan; PIS, product ion scan; RSD, relative standard deviation; SPE, solid-phase extraction; SEM, standard error; LC-MS/MS, tandem mass spectrometry coupled to liquid chromatography; H_2O, water.

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4.2. Publicación 2. Efectos de una matriz lipídica sobre la exposición plasmática y la excreción urinaria de compuestos fenólicos de salsas de tomate: Evidencia de un estudio piloto humano.

Oil matrix effects on plasma exposure and urinary excretion of phenolic compounds from tomato sauces: Evidence from a human pilot study. Sara Tulipani, **Miriam Martínez-Huélamo**, María Rotchés-Ribalta, Ramón Estruch, Elvira Escribano-Ferrer, Cristina Andrés-Lacueva, Montserrat Illán and Rosa M. Lamuela-Raventós. *Food Chemistry*. 2012, 130(3):581-590.

Los efectos protectores atribuidos a los compuestos fenólicos sobre la salud dependen de su bioaccesibilidad desde la matriz del alimento y su consecuente biodisponibilidad. En esta publicación, hemos llevado a cabo un estudio piloto, prospectivo, aleatorizado, cruzado, abierto y controlado con 5 varones sanos, a los cuales se les administró 100 g de salsa de tomate sin aceite (OF), una segunda elaborada con aceite de oliva virgen extra (VOOE) y una tercera realizada con aceite de oliva refinado (ROOE) para estudiar la biodisponibilidad de los compuestos fenólicos según la matriz lipídica añadida. La salsa fue elaborada en el Campus de la Alimentación de Torribera de la Universidad de Barcelona a partir de tomates comerciales (liso rojo rama).

Los voluntarios no debían tener antecedentes de enfermedades cardiovasculares, ni alteraciones homeostáticas ni ninguna otra enfermedad relevante para ser incluidos en el estudio. Se excluyeron los voluntarios hipertensos, dislipidémicos, fumadores, con alergias o intolerancia al tomate y con dificultad para cambiar los hábitos alimentarios. El esquema del diseño del estudio se detalla en la **Tabla 4**.

Tabla 4. Esquema del diseño del estudio.

Día -3	Dieta sin tomate	
Día -2	Dieta sin tomate	
	No suplementos vitamínicos	
	No medicamentos	
Día -1	Dieta exenta de polifenoles	
	No suplementos vitamínicos	
	No medicamentos	
	Estar en ayuno las 10-12 horas previas al día 0	
Día 0: Estudio	t = 0 h	Extracción de sangre Recogida de orina
	Intervención: 100 g de salsa de tomate con aceite de oliva refinado, salsa de tomate sin aceite de oliva refinado y salsa de tomate con aceite de oliva virgen extra.	
	t = 15 min	Extracción de sangre
	t = 30 min	Extracción de sangre
	t = 1 h	Extracción de sangre
	t = 2 h	Extracción de sangre
	t = 3 h	Extracción de sangre
	t = 4 h	Extracción de sangre Recogida de orina
	t = 5 h	Extracción de sangre
	t = 6 h	Extracción de sangre
	t = 8 h	Extracción de sangre Recogida de orina
	t = 12 h	Extracción de sangre Recogida de orina
Día 1	t = 24 h	Extracción de sangre Recogida de orina

Los tres días previos al estudio, a los sujetos se les restringió el consumo de tomate, igual que el consumo de suplementos vitamínicos y medicamentos. El día anterior al estudio, así como el día del estudio, los voluntarios no podían ingerir alimentos que tuvieran polifenoles (**Tabla 5**) y se les solicitó que siguieran los menús propuestos en la **Tabla 6**.

Tabla 5. Alimentos y bebidas a restringir para seguir la dieta exenta de polifenoles.

Alimentos a restringir	**Bebidas a restringir**
Frutas, verduras, hortalizas, mermeladas, ajo, aceitunas, aceite de oliva, frutos secos, patatas, miel, legumbres, soja y derivados, setas, chocolate y productos que la contengan.	Té, infusiones, café, cerveza, vino, cava, sidra, batidos de cacao, zumos de frutas, batidos de frutas, gazpachos, leche de soja, colas, naranjadas y limonadas y otras bebidas refrescantes con frutas.

Tabla 6. Propuesta de menú suministrada a los voluntarios para las dietas exentas en polifenoles.

Desayuno	Lácteos sin chocolate, ni frutas ni café. Cereales, bollería o galletas no integrales y sin frutas ni chocolate.
Media mañana	Lácteos. Bocadillo con embutido pero sin tomate ni aceite de oliva. Bollería sin chocolate o frutas.
Comida	Pasta carbonara, arroz tres delicias. Carne o pescado a la plancha, hervido o al horno. (Recordar no utilizar aceite). Postre lácteo.
Merienda	Lácteos. Bocadillo con embutido pero sin tomate ni aceite de oliva. Bollería sin chocolate o frutas.
Cena	Sopa de caldo de pollo, carne o pescado pero realizado sin verduras. Tortilla francesa, huevos escaldados. Sepia o calamares a la plancha. Postre lácteo.

Las salsas se analizaron por HPLC-MS/MS tras una extracción líquido-líquido previa y las muestras de plasma y orina recogidas durante el estudio se sometieron a extracción en fase sólida (SPE), seguido del análisis por HPLC-MS/MS. El compuesto mayoritario en las tres salsas fue la flavanona naringenina. También se encontraron ácidos fenólicos, mayoritariamente, ácidos hidroxicinámicos como el ácido 5-cafeoilquínico, y como flavonol se detectó la rutina. No existieron diferencias entre los tres tipos de salsas por lo que la adición de los diferentes aceites al producto parece ser que no aumenta la extractabilidad de los compuestos. En cambio, sí que se observaron diferencias entre el tomate fresco utilizado para la elaboración de las salsas y las salsas. La diferencia fundamental vino marcada por la flavanona naringenina donde su concentración fue muy superior en las salsas respecto al tomate. Este hecho demuestra que el tratamiento térmico y mecánico libera al compuesto de la matriz del alimento y lo hace más bioaccesible.

Seis compuestos fenólicos, tres agliconas (naringenina, ácido ferúlico y ácido cafeico) y sus correspondientes metabolitos glucurónidos, fueron identificados y cuantificados en orina después de la ingesta de las salsas de tomate. Dos de los seis metabolitos urinarios fenólicos también se cuantificaron en muestras de plasma (naringenina glucurónido y ácido ferúlico glucurónido). Sólo tras la ingesta de las salsas de tomate enriquecidas con aceite, el metabolito naringenina glucurónido mostró un perfil bifásico en plasma, lo que sugiere que la matriz lipídica añadida a la salsa puede estimular la aparición de una

reabsorción por vía enterohepática aumentando, de este modo, la vida media plasmática del compuesto.

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Oil matrix effects on plasma exposure and urinary excretion of phenolic compounds from tomato sauces: Evidence from a human pilot study

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ABSTRACT

The health-promoting effects attributable to dietary phenolic compounds strongly depend on their bioaccesibility from the food matrix and their consequent bioavailability. We carried out a pilot randomized controlled cross-over study to evaluate the effect of addition of an oil matrix during tomato sauce processing, on the bioavailability of tomato phenolics. Healthy subjects consumed a single dose of tomato sauce elaborated without oil (OO-F) and with the addition of 5% virgin olive oil (VOO-E) or refined olive oil (ROO-E). Plasma and urine samples were subjected to solid-phase extraction, followed by HPLC–MS/MS analysis. Six phenolic compounds, three aglycones (naringenin, ferulic and caffeic acids) and their corresponding glucuronide metabolites, were identified and quantified in urine after the ingestion of the tomato sauces. Two of the six phenolic urinary metabolites were also quantified in plasma samples. Only after ingestion of the oil-enriched tomato sauces, did the glucuronide metabolites of naringenin show a bi-phasic profile of absorption in plasma, suggesting that the lipid matrix added to the sauce may stimulate the occurrence of re-absorption events by enterohepatic circulation, potentially enhancing the apparent plasma half-life of the flavanone prior to excretion. The interindividual response variability observed underlies the need for further large-scale investigations.

1. Introduction

The Mediterranen diet is an example of a dietary regime associated with a reported low incidence of cancers, CVD, and an overall reduction in mortality (Chrysohoou, Panagiotakos, Pitsavos, Das, & Stefanadis, 2004; Estruch et al., 2006; Knoops et al., 2006). The health-promoting effects of this diet have been mainly attributed to the high consumption of the typical fruit and vegetables of the Mediterranean basin (i.e. leafy green vegetables and tomatoes). Tomato (*Lycopersicon esculentum* Mill., Solanaceae) and tomato processed products (sauce, paste, puree and juice) are typical components, besides being a dietary staple for humans in many other parts of the world. Another major characteristic of the Mediterranean diet is a high consumption of olive oil as the main source of monounsaturated fatty acids, which has long been reported to exhibit numerous biological functions beneficial for the state of health (Cicerale, Lucas, & Keast, 2010). The combination of tomatoes with olive oil, in food preparation, is a typical daily habit, and tomato sauce, the most

Abbreviations: ALA, aldehydic form of ligstroside aglycone; ALA ox, aldehydic form of ligstroside aglycone oxidized; AOA, aldehydic form of oleuropein aglycone; AOA ox, aldehydic form of oleuropein aglycone oxidized; AUC_{last}, area under the plasma concentration-*versus*-time curve from time 0 until the last detectable concentration; CVD, cardiovascular disease; CAD, collision-activated dissociation; CE, collision energy; DP, declustering potential; 3,4-DHPEA, hydroxytyrosol or 3,4-dihydroxyphenylethanol; 3,4-DHPEA-Ac, hydroxytyrosol-acetate; 3,4-DHPEA-EDA, hydroxytyrosol-elenolic acid di-aldehyde; DLA, dialdehydic form of ligstroside aglycone; DOA, dialdehydic form of oleuropein aglycone linked to 3,4-DHPEA-EDA oxidized; FP, focusing potential; FS, full scan; *p*-HPEA, tyrosol or *p*-hydroxyphenylethanol; *p*-HPEA-EDA, tyrosol–elenolic acid di-aldehyde; LOD, limit of detection; C_{max}, maximum plasma concentration; MRT, mean residence time; MI, molecular ion; NL, neutral loss; OO-F, olive oil-free tomato sauce; PFD, polyphenol-free diet; PrIS, precursor ion scan; PIS, product ion scan; ROO-E, refined olive oil-enriched; SPE, solid-phase extraction; SEM, standard error; T_{max}, time to reach the maximum plasma concentration; TFD, tomato-free diet; Q_{∞}, the maximum excreted amount in the 24 h urine collection; VOO-E, virgin olive oil-enriched.

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extensively consumed tomato product worldwide, is commonly elaborated and commercialized, both as an oil-free and oil-enriched product. The protective effects of a regular consumption of raw tomatoes and tomato sauce (Giovannucci, 1999) have long been attributed to the most pronounced bioactives in tomato, such as lycopene and other carotenoids (Agarwal & Rao, 2000). Recently, the high content of phenolic compounds such as flavonoids and hydroxycinnamic acids in tomato has been gaining interest because of the multiple biological effects they seem to express, ranging from free radical-scavenging, metal chelation, inhibition of cellular proliferation, and modulation of enzymatic activity and signal transduction pathways (Crozier, Jaganath, & Clifford, 2009). The flavanone, naringenin (4′,5,7-trihydroxyflavanone) and the hydroxycinnamate, chlorogenic acid (5-caffeoylquinic acid) (Slimestad, Fossen, & Verheul, 2008; Vallverdú-Queralt, Jáuregui, Medina-Remón, Andrés-Lacueva, & Lamuela-Raventós, 2010) are among the most abundant phenolics in tomato, and the most extensively studied.

It is known that the extent of the protective effect of the phenolic compounds *in vivo* strongly depends on their bioaccesibility and their consequent exposition in the organism, on their intestinal absorption and presystemic metabolism, up to their systemic distribution and potential interaction with target tissues. In turn, the food matrix, in which the phenolic compounds are contained, is one of the most important factors governing the *in vivo* bioavailability of these compounds. It plays a crucial role in determining their accessibility and extractability from food, and thus their absorption, metabolism and their final biological action in the human body (Ortuño et al., 2010).

Despite growing knowledge of the effects of the thermic and mechanical treatments involved in tomato sauce processing on carotenoid and vitamin bioavailability (Graziani et al., 2003; Unlu et al., 2007), little is known about the changes in the polyphenol/flavonoid content of tomatoes after their processing to tomato sauce (Bugianesi et al., 2004; Vallverdú-Queralt, Medina-Remón, Andrés-Lacueva, & Lamuela-Raventós, 2011). Furthermore, as the combination of tomatoes and tomato products with a lipid matrix has been reported to favour the extractability and bioaccessibility of tomato carotenoids (Fielding, Rowley, Cooper & O'Dea, 2005; Graziani et al., 2003), recent investigations have also shown that the fat content in a meal may enhance the bioavailability of the flavonol quercetin, due to a better solubility of the relatively lipophilic flavonoid in the intestinal tract in the presence of fat (Lesser, Cermak, & Wolffram, 2004). The typical addition of oil, during tomato sauce processing, may influence the bioavailability of the phenolics contained in tomato by modifying their bioaccessibility from the food matrix, modulating the gastric emptying and/or the intestinal and hepatic metabolism of the absorbed phenolic compounds. However, few studies have yet investigated the absorption and excretion of phenolic compounds from raw tomatoes and tomato sauces, and even less information is currently available on the impact of tomato–olive oil combination, during processing, on the bioaccessibility and human absorption of the phenolic compounds contained in the sauce (Capanoglu, Beekwilder, Boyacioglu, Hall, & de Vos, 2008; Graziani et al., 2003).

The aim of the present study was to investigate whether the absorption and metabolism of the tomato phenolics is influenced by the addition of a lipid matrix during tomato sauce processing, and eventually by the oil typology. For these purposes, we carried out a randomized controlled cross-over study, administering (to human volunteers) a single dose of tomato sauce without oil (OO-F) and tomato sauces elaborated with the addition of virgin olive oil (VOO-E) or refined olive oil (ROO-E) during processing.

2. Materials and methods

2.1. Standards and reagents

Chlorogenic acid, caffeic acid, ferulic acid, isoferulic acid, *p*-hydroxybenzoic acid, protocatechuic acid, *m*- and *p*-coumaric acids, gallic acid, naringenin-7-*O*-glucoside, naringin (naringenin-7-*O*-rhamnoglucoside), quercetin, rutin (quercetin-7-*O*-rutinoside), kaempferol and blank human plasma were purchased from Sigma–Aldrich (St. Louis, MO, USA). Naringenin (4′,5,7-trihydroxyflavanone), the internal standard ethylgallate (IS, added to sample before extraction) and the external standard taxifolin (ES, added to sample after extraction) were purchased from Extrasynthese (Genay, France). HPLC-grade methanol, acetonitrile and formic acid were purchased from Scharlau Chemie S. A. (Barcelona, Spain), and *o*-phosphoric acid (85%) and hydrochloric acid (37%) from Panreac Quimica SA (Barcelona, Spain). Ultrapure water (Milli-Q) was obtained from a Millipore system (Millipore, Bedford, MA, USA).

2.2. Tomato sauce material

A commercial tomato (*L. esculentum* L.) variety, suitable for tomato sauce elaboration, was used for the study. Virgin and refined olive oils were kindly furnished by Juan Ballester Rosés company (S.A., Tortosa, Spain). The OO-F, VOO-E and ROO-E tomato sauces were processed at Torribera campus, University of Barcelona (UB, Barcelona, Spain) by a standardized industrial scale-like making process. Fruits were washed, chopped in a breaker unit and weighed. The VOO and the ROO were rapidly heated up to 110 °C before adding the chopped tomatoes (5% of oil, w/w), and the mixture was cooked at 99 °C for 90 min. The chopped tomatoes were crushed into pulp, which was then separated from seeds and skin, cooled down, aliquotted and stored in vacuum bags at −20 °C until the day of the test. Approximately 1000 g of fresh tomatoes yielded 500 g of tomato sauce. Five percent of water was added to the final OO-F tomato sauce product, in order to standardize the amount of tomato compounds ingested by each intervention.

2.3. Preparation of extracts

For the extraction of phenolic compounds from the starting raw tomatoes and the tomato sauces, 5 ml of 80% ethanol in ultrapure water (v/v), acidified with 0.1% formic acid, were added to 0.5 g of sample. The mixture was vortexed for 1 min and then sonicated for 5 min on ice. After centrifugation at 900*g* for 20 min at 4 °C, the supernatant was collected; a further 5 ml of the acidified 80% ethanol/water solution were added to the pellet, and the extraction procedure repeated. The two supernatants were combined, and the ethanolic component was evaporated to dryness by a sample concentrator (Techne, Duxford, Cambridge, UK) at room temperature under a stream of nitrogen gas. After filtration of the acqueous extracts (~2 ml) with 4 mm, 0.45 μm PTFE syringe filters (Waters Corporation, USA), 50 μl of the resulting filtrate were injected into the LC/MS/MS system, in triplicate.

2.4. Subjects and study design

A total of five healthy men, aged between 25 and 36 (BMI = 25 ± 1.2 kg/m^2), volunteered for this randomised crossover dietary study. The study was explained to subjects through verbal and written instructions, and written informed consent was obtained before participation. Only male subjects were recruited, in order to reduce anthropometric variables and the menstrual cycle phase-related variability in premenopausal women, putatively affecting the absorption, metabolism and excretion of tomato

phenolics. Subjects were non-smokers, had no history of cardiovascular, hepatic or renal disease and had not adhered to any special diets at least 4 weeks prior to participating in the study. To avoid any acute peak in the plasma phenolic concentration and to standardize the baseline point, subjects were asked to follow a tomato-free diet (TFD) during the 3 days preceding the dietary intervention, and a low-polyphenol diet (LPD) during the 24 h immediately preceding the test. A list of acceptable and nonacceptable foods was provided to the subjects, together with two standardised polyphenol-free dinners to be consumed in the evening before the test, and on the day of the test, respectively. On the day of each intervention, the subjects were asked to fill in a 24 h food record to assess their compliance with the recommended LPD. No significant differences were observed in total energy or macronutrient and micronutrient intakes during the 24 h before the interventions, calculated using Food Processor software (data not shown).

Using a computer-generated randomisation number sheet, each volunteer was assigned to receive (in consecutive experiments) the OO-F, the VOO-E and the ROO-E tomato sauce interventions. Between the interventions, after a "washout" period of a minimum 3 days on their usual diet, the subjects repeated the 3 day TFD and the 1 day LPD before being subjected to the following test.

On the day of the intervention, the overnight-fasted subjects had an intravenous catheter inserted, and a baseline blood sample (5 ml) was collected. Then, subjects consumed 100 g of one of the three interventions (OO-F, VOO-E and ROO-E tomato sauces) within a space of 5 min, and refrained from consuming other foods for the following 6 h. A standardized phenolic-free lunch was distributed to the volunteers 6 h after the ingestion of the test sauces. Blood samples (5 ml) were collected into lithium heparin tubes at 15, 30 min and 1, 2, 3, 4, 5, 6, 8, 12 and 24 h after the ingestion of the tomato sauces, and immediately centrifuged at 1500*g* for 20 min at 4 °C. All plasma samples were subsequently aliquotted and stored in Eppendorf tubes at −80 °C prior to analysis. Urine was collected in plastic bottles 10–20 min before consumption of the tomato sauces (baseline, 0 h) and the 24 h-urine after the ingestion of the interventions was collected in four separate fractions (0–4, 4–8, 8–12 and 12–24 h), the participants being instructed to empty their bladders before a new fraction was collected. The urine bottles were stored at 4 °C and, immediately after the participants had started, to collect the next fraction; the amount of urine in each fraction was measured, acidified with 0.2 M HCl and stored at −80 °C prior to analysis. The study protocol was approved by the Ethics Committee of Clinical Investigation of the University of Barcelona (Spain), and the clinical trial was registered with the International Standard Randomized Controlled Trial Number (ISRCTN20409295). Informed consent was obtained from all participants.

2.5. Plasma and urine extraction procedure for tomato phenolic metabolites

Tomato sauce phenolic metabolites in plasma and urine were extracted by a solid-phase extraction (SPE) procedure, previously described and optimized by our work group (Mata-Bilbao et al., 2007), using a Waters Oasis HLB 96-well plate of 30 μm (30 mg) (Waters Oasis, Milford, MA, USA).

On the day of the analysis, the samples were defrosted on ice in the dark. Prior to SPE, urine samples (1 ml) were centrifuged at 15,000*g* for 4 min, at 4 °C, while plasma (1 ml) was acidified by adding 25 μl of *o*-phosphoric acid, to break down protein–polyphenol interactions and reach the optimum pH for phenolic retention in the solid-phase during the extraction procedure (Mata-Bilbao et al., 2007). One millilitre aliquots of plasma and urine samples were spiked with 100 μl of 2nM ethyl gallate as internal standard. After 2 min of vortex-mixing, samples were diluted 1:1 (v/v) with water. Plate activation was achieved by consecutively adding 1 ml of methanol and 1 ml of 1.5 M formic acid in water. After the sample loading into the 96-well plate, sample clean-up was performed with 1 ml of 1.5 M formic acid in water, followed by 1 ml of 5% methanol aqueous solution (v/v). Phenolic metabolites were then eluted with 1 ml of methanol acidified with 0.1% formic acid (v/v). The eluted fraction was evaporated to dryness by a sample concentrator (Techne, Duxford, Cambridge, UK), at room temperature under a stream of nitrogen gas, and reconstituted with 100 μl of water containing 0.1% formic acid and IS2 (500 ppb). The 96-well plate was then vortexed for 5 min and the reconstituted extracts transferred into a 96-well plate suitable for LC–MS/MS analysis.

For calibration, a standard mixture of commercially available naringenin, chlorogenic acid, caffeic acid, ferulic acid and quercetin was serially diluted, in both water and blank human plasma (Sigma–Aldrich, USA) (1–500 ppb). The standard solutions were treated exactly in the same way as the samples and subjected to the extraction procedure; therefore, the results could be read directly from the standard curve and no corrections due to the recovery losses had to be made.

Standard preparation and sample treatments were performed in a dark room with a red safety light to avoid oxidation of the analytes.

2.6. LC–MS/MS analysis

The native phenolic compounds present in raw tomatoes and in OO-F, VOO-E and ROO-E tomato sauces, as well as the corresponding phenolic metabolites putatively expected in urine and plasma samples, were identified and quantified by LC–MS/MS analysis.

Liquid chromatography separations were performed on an HP Agilent Technologies 1100 LC system (Waldbronn, Germany) equipped with a quaternary pump and a refrigerated autosampler. Coupled to the HPLC system, an Applied Biosystems API 3000 triple quadrupole mass spectrometer (PE Sciex, Concord, Ontario, Canada), equipped with a TurboIon spray source, was used in negative mode, to obtain mass spectrometry (MS) and MS/MS data. TurboIon spray source settings were as follows: capillary voltage, −4000 V; nebulizer gas (N_2), 10 (arbitrary units); curtain gas (N_2), 12 (arbitrary units); collision gas (N_2), 10 (arbitrary units); entrance potential, 10 V; drying gas (N_2), heated to 400 °C and introduced at a flow rate of 8000 cm^3/min. The system was controlled by the software analyst v. 1.4.2 supplied by Applied Biosystems (Foster City, CA, USA).

For both the analysis of the plant and the biological sample extracts, Phenomenex (Torrance, CA, USA) Luna C18 (50 × 2.0 mm i.d., 5 μm) and Phenomenex securityguard C18 (4 × 3 mm i.d.) columns were used at 30 °C, with a sample injection volume of 50 μl. Gradient elution was performed with a binary system consisting of [A] 0.1% acqueous formic acid and [B] 0.1% formic acid in acetonitrile. An increasing linear gradient (v/v) of [B] was used [*t*(min), %B], as follows: (a) (0, 5), (10, 18), (13, 100), (14, 100) and (15, 5), followed by a 5 min re-equilibration step, at a constant flow-rate of 400 μl/min; (b) (0, 5), (2, 25), (10, 90), (11, 100) and (12, 100), followed by a 5 min re-equilibration step, at a constant flow-rate of 600 μl/min, respectively, for the analysis of the tomato/tomato sauce extracts and the plasma/urine extracts.

Prior to the HPLC–MS/MS analysis of the samples, direct infusion experiments were carried out with each individual standard compound available. Briefly, a 50:50 (v/v) [A, B] solution of each standard compound (10 μg/ml) was injected into the mass spectrometer, using a model syringe pump (Harvard Apparatus, Holliston, MA, USA), at a constant flow rate of 5 μl/min, and manual tuning was performed in negative mode. For each standard com-

Table 1
List of the phenolic compounds identified in the three tomato sauces in study.

Peak number	Compound	Rt (min)	Molecular Formula	Ions full scan MS		MS/MS experiments			Comparison with standard
				$[M-H]^-$	Fragments	PIS	NL	PrIS	
1	Protocatechuic acid	2.08	$C_7H_6O_4$	153	109	–	–	–	Yes
2	3-Caffeoylquinic acid	2.94	$C_{16}H_{18}O_9$	353	191, 179, 135	353	162	–	No
3	Caffeic hexose II	3.27	$C_{15}H_{18}O_9$	341	179, 135	341	162	179	No
4	Caffeic hexose IV	4.19	$C_{15}H_{18}O_9$	341	281, 251, 221, 179, 161, 135	341	162	179	No
5	Homovanillic hexose 1	4.51	$C_{15}H_{20}O_9$	343	181, 137, 121	343	162	–	No
6	Ferulic hexose	4.75	$C_{16}H_{20}O_9$	355	193, 149, 134	355	–	193	No
7	Caffeic hexose II	4.94	$C_{15}H_{18}O_9$	341	179, 135	341	162	179	No
8	Homovanillic hexose 2	5.04	$C_{15}H_{20}O_9$	343	181, 1371	343	162	–	No
9	Coumaric hexose	5.25	$C_{15}H_{18}O8$	325	163, 119	–	162	–	No
10	5-Caffeoylquinic acid	5.38	$C_{16}H_{18}O_9$	353	191, 179, 161	353	162	–	Yes
11	3,4-Dihydroferulic hexose	5.77	$C_{16}H_{22}O_9$	357	195 , 135, 151	357	162	–	No
12	4-Caffeoylquinic acid	6.15	$C_{16}H_{18}O_9$	353	191, 179, 173, 155, 135	353	162	179	No
13	Quercetin dihexose deoxyhexose	7.08	$C_{33}H_{40}O_{21}$	771	609, 300	771	–	–	No
14	Rutin	11.40	$C_{27}H_{30}O_{16}$	609	301	609	–	–	Yes
15	Naringenin chalcone 3′,5′-di-C-hexose	12.09	$C_{27}H_{34}O_{15}$	597	477, 417, 387, 357	597	–	–	No
16	Dicaffeoylquinic acid	13.20	$C_{25}H_{24}O_{12}$	515	353, 179, 173	515, 353	162	353	No
17	Naringenin	13.87	$C_{15}H_{12}O_5$	271	151, 107, 119	–	–	–	Yes

Table 2
Quantification of the main identified phenolic compounds in the raw tomatoes and in OO-F, VOO-E and ROO-E tomato sauces in study. Values are means ± SEM. In each row, different letters correspond to significantly different concentrations ($p < 0.05$).

Compound	Rt (min)	MRM (m/z)	Raw Fruit (μg/g FW[A])	OO-F sauce (μg/g FW[A])	ROO-E sauce (μg/g FW[A])	VOO-E sauce (μg/g FW[A])
Protocateichuic acid		153 → 109	–	129 ± 6.7	144 ± 5.6	128 ± 14.4
3-Caffeoylquinic acid	2.94	353 → 191	4.3 ± 0.6b	60.3 ± 1.1a	61.2 ± 2.1a	59.8 ± 6.4a
5-Caffeoylquinic acid	5.38	353 → 191	234 ± 46.1c	814 ± 15.0a	552 ± 21.8b	652 ± 34.9b
4-Caffeoylquinic acid	6.15	353 → 191	149 ± 0.5 c	214 ± 6.9a	178 ± 0.3b	197 ± 21.8a,b
Dicaffeoylquinic acid	13.20	515 → 353	36.0 ± 7.9	54.3 ± 5.1	61.1 ± 1.4	48.4 ± 8.5
Caffeic hexose II	3.27	341 → 179	235 ± 44.9b	437 ± 9.4a	280 ± 4.0b	287 ± 34.0b
Caffeic hexose IV	4.19	341 → 179	98.1 ± 15.0b	151 ± 1.3a	106 ± 4.2b	107 ± 12.2b
Caffeic hexose II	4.94	341 → 179	5.5 ± 0.8	6.1 ± 0.1	7.4 ± 2.9	7.7 ± 0.7
Ferulic -hexose	4.75	355 → 193	104 ± 15.1b	239 ± 12.2a	242 ± 45.6a	157 ± 2.9a,b
	Total phenolic acids orally administered (mg)[B]			210.45	163.27	164.36
Naringenin	13.87	271 → 151	164 ± 12.1 b	1347 ± 63.6a	1296 ± 212a	1305 ± 238a
	Total flavanones orally administered (mg)[B]			134.7	129.6	130.5
Rutin	11.40	609 → 301	1.40 ± 0.19 c	3.01 ± 0.04a	2.16 ± 0.04b	2.23 ± 0.30b
	Total flavonols orally administered (mg)[B]			0.301	0.216	0.223

[A] FW: fresh weight.
[B] Corresponding to the dose orally administered (100 g) to the subjects.

pound, the molecular ion (MI) $[M-H]^-$ and the most abundant fragment ions (m/z) were easily detected, and the optimal focusing potential (FP), declustering potential (DP) and collision energy (CE) for the MI fragmentation were noted.

Phenolic compounds were identified by (a) chromatographic retention times, (b) full scan (FS) data, and (c) MS/MS experiments. In the plant extracts, the identity of the phenolic compounds was confirmed by combining product ion scan (PIS), neutral loss (NL), and precursor ion scan (PrIS) MS/MS experiments. In plasma and urine samples, due to the lower concentration of the phenolic metabolites investigated and consequently the need for greatest sensitivity, only PIS experiments were successfully used for identification purposes.

The PIS experiments were carried out at optimal and at double the CE values suggested by the direct infusion experiments in order to confirm the fragmentation pattern of each metabolite, using a cycle time of 2 s. MS/MS product ions were produced by collision-activated dissociation (CAD) of the selected precursor ions in the collision cell of the triple-quadrupole mass spectrometer, and the mass analysed using the second analyser of the instrument.

In parallel, the phenolic compounds in tomatoes/tomato sauce extracts, and the metabolites putatively expected in the biological fluids were quantified by multiple reaction monitoring (MRM), using suitable transitions (Jaganath, Mullen, Edwards, & Crozier, 2006). Phenolic standard curves were constructed with the standard solutions previously described, and conjugated metabolites of the available standard phenolic compounds were quantified using the calibration curves of the corresponding native unconjugated molecules.

The mean recovery of analytes ranged from 96% to 100% in plasma and 65–104% in urine, and the limit of detection (LOD) varied between 0.7 and 30 ng/ml, in plasma and urine. Precision met acceptance criteria (<8% RSD in plasma and <7% RSD in urine).

2.7. Calculation of pharmacokinetic parameters

The pharmacokinetic parameters were calculated by a non-compartmental analysis, using the WinNonlin Professional software version 2.0 (Pharsight Corporation, California, USA). For each phenolic metabolite detected and quantified, the maximum plasma concentration (C_{max}) and the time to reach it (T_{max}) were

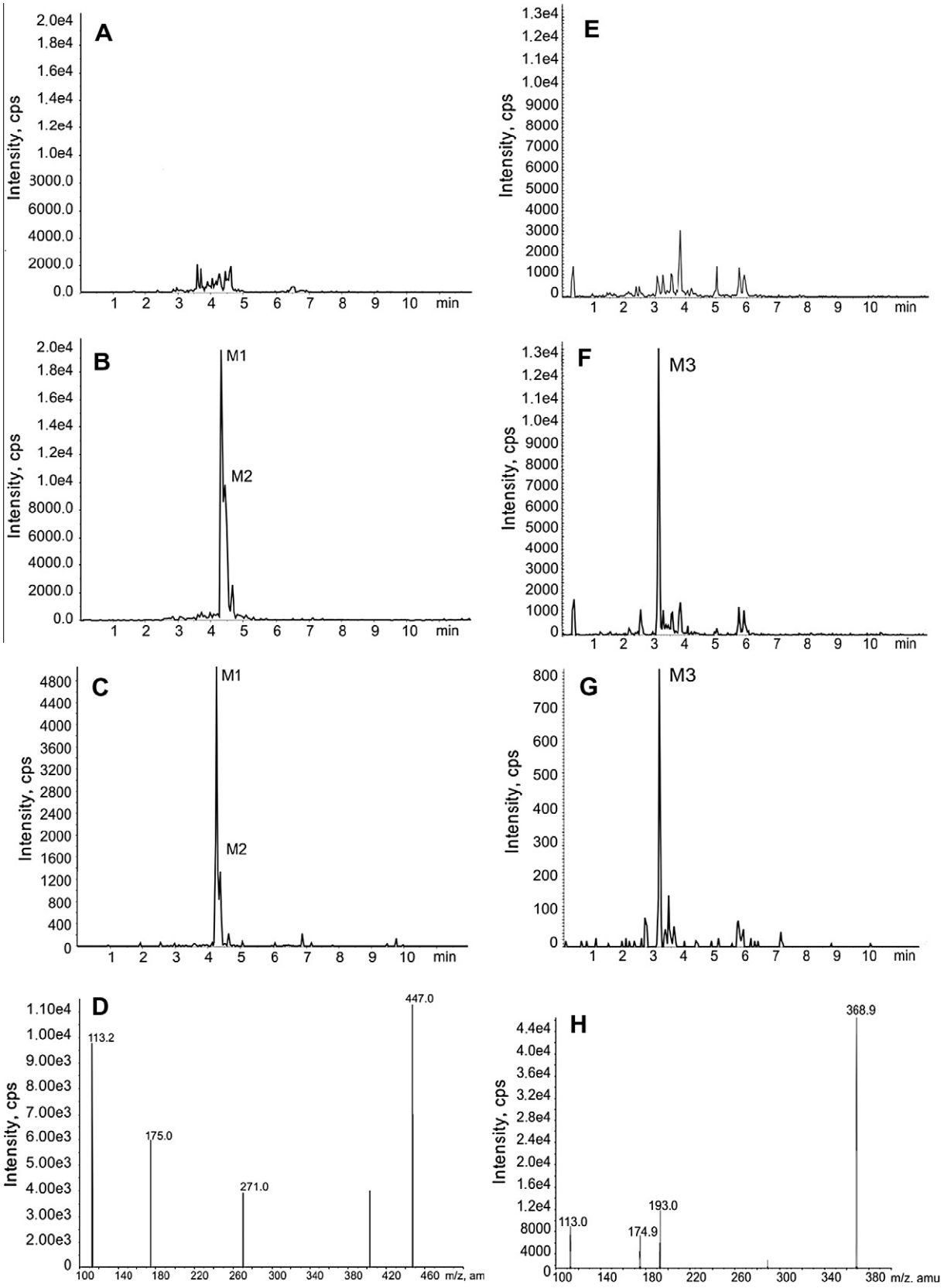

Fig. 1. MRM trace chromatograms of naringenin-7-*O*-glucuronide (M1) and naringenin-4′-*O*-glucuronide (M2) in urine at baseline (0 h) (A), at 4 h (B) and in plasma at 1 h (C) after tomato sauce consumption. and product ion spectra of M1 or M2 in urine (D). MRM trace chromatograms of (iso)ferulic glucuronide (M3) in urine at baseline (0 h) (E), at 4 h (F) and in plasma at 2 h (G) after tomato sauce consumption, and corresponding product ion spectra of M3 in urine (H).

taken directly from the observed data. The area under the plasma concentration-*versus*-time curve from time 0 until the last detectable concentration (AUC_{last}) was calculated using the linear trapezoidal method. The mean residence time (MRT_{last}) was estimated by means of the ratio AUMC/AUC, where AUMC is the first moment curve. The C_{max}-to-AUC_{last} ratio and the maximum excreted amount in the 24 h urine collection (Q_∞) were also calculated for each metabolite.

2.8. Statistical analysis

The statistical analysis of all data was performed using SPSS software (Version 17.0, Japan Inc., Tokyo, Japan). Differences at $p < 0.05$ were considered statistically significant. The phenolic composition of the three tomato sauces was subjected to one-way ANOVA analysis of variance for mean comparison. Data are reported as means ± standard deviation (SD). For each phenolic metabolite quantified in plasma and urine samples, the pharmacokinetic indices obtained, following the three dietary interventions, were compared and subjected to statistical analysis. All data are expressed as means ± standard error (SEM). C_{max}, AUC_{last}, MRT_{last} and C_{max}/AUC values were log-transformed and subjected to one-way ANOVA analysis of variance, while T_{max} data were compared by a non-parametric Kruskal–Wallis test.

3. Results and discussion

3.1. Phenolic composition of tomatoes and tomato sauces

The phenolic compounds identified in tomatoes and tomato sauces administered to the subjects are listed in Table 1, and the contents of the main quantified phenolic acids, flavanones and flavonols are listed in Table 2. Phenolic acids were the most abundant phenolic compounds found in all extracts, particularly hydroxycinnamic acids and their ester conjugates (mono- and di-caffeoylquinic acids, caffeic hexoses, ferulic and dihydroferulic hexoses, coumaric hexose). Chlorogenic acid (5-caffeoylquinic acid) was the most abundant phenolic acid in all three tomato sauces (Slimestad et al., 2008), while the flavanone naringenin (4′,5,7-trihydroxyflavanone) was the most abundant phenolic compound in all the samples, confirming its high content in tomato and tomato products.

When comparing the phenolic profile of the raw tomato fruits and the final tomato sauces, no qualitative differences were observed among them. However, from a quantitative point of view, the phenolic composition of the raw tomatoes significantly differed from the sauces in their lower contents of chlorogenic (5-caffeoylquinic acid) and neochlorogenic acids (3-caffeoylquinic acid), rutin (the major quercetin-glycoside in tomato), and especially in the 8-fold lower concentration of naringenin (Table 2). These data confirmed that heat and mechanical treatments, during the tomato sauce-making process) may induce biochemical changes in the food matrix, positively affecting the bioaccessibility of phenolics from the processed products. In addition, these findings seem to confirm that, as for carotenoids, naringenin bioaccessibility is particularly limited by interactions with the food matrix and improved by mechanical and heat treatments. Data on tomato fruit physiology are consistent with this consideration, since naringenin is trapped in the cutin matrix of the membrane of the ripe fruit where it strongly interacts with the insoluble polyesters of tomato fibre (Laguna, Casado, & Heredia, 1999). Mechanical and heat treatments may provide the energy necessary to break the interactions, potentially improving naringenin bioaccessibility *in vivo*.

When comparing the phenolic content of the tomato sauces, however, a quite homogeneous composition was observed among the three processed products (Table 2). The addition of olive oil during processing, did not seem to increase the extractability and accessibility of the tomato phenolic compounds. On the contrary, the OO-F tomato sauce revealed itself to be significantly richer in chlorogenic acid and caffeic hexose derivatives than the oil-enriched sauces, suggesting that the tomato-olive oil combination, during sauce elaboration, may not necesarily increase the phenolic content of the final product. For completeness, the oils added to the sauces were analyzed for their phenolic contents. The extraction and characterization of the phenolic fraction were achieved by HPLC with a diode array detector, as previously described (Boselli, Di Lecce, Strabbioli, Pieralisi, & Frega, 2009). As expected, no detectable phenolic compounds were observed in the ROO. In VOO, the phenylethyl alcohols typical of olive oil, *p*-HPEA (tyrosol), 3,4-DHPEA (hydroxytyrosol) and its acetate derivative (3,4-DHPEA-Ac), the flavones luteolin and apigenin, the aldehyde vanillin and eight secoiridoids, exclusively present in VOO and extra-VOO (3,4-DHPEA-EDA, DOA, *p*-HPEA-EDA, DLA, AOA, AOA ox, ALA, ALA ox), were present in the range of concentrations usually observed in VOO (0.9–22 ppm) (Boselli et al., 2009) (data not shown) . As expected, none of the phenolic compounds observed in the tomatoes/tomato sauces were identified in the olive oils added to the sauce.

3.2. Identification of phase II phenolic metabolites in human plasma and urine

In total 134.7, 129.6 and 130.5 mg of flavanones (naringenin), 210.5, 163.3 and 164.4 mg of phenolic acids (predominantly caffeoylquinic acids) and 0.30, 0.21 and 0.22 mg of flavonols (rutin) were orally administered to the subjects through the 100 g dose of OO-F, VOO-E and ROO-E, respectively (Table 2). The presence of the most abundant phenolic compounds identified in the tomato sauces (naringenin, caffeoylquinic acids, caffeic acid, ferulic acid and rutin) and their most probably expected phase II conjugated forms (with methyl, glucuronic acid and sulfate groups) were investigated in both biological fluids.

With regard to the flavanone naringenin, the unconjugated native compound was detected only in urine samples. By contrast, two mass signals at m/z 447 → 271 were detected in both plasma and urine samples, indicating the presence of two partially co-eluting glucuronide (+176 amu) derivatives of naringenin (M1 and M2, Fig. 1A–C). These two metabolites were confirmed to be two isomeric naringenin glucuronides by their MS fragmentation patterns (MS2 ions at m/z 271, 175 and 113), as shown in Fig. 1D. It is known that naringenin has three possible sites for conjugation: 7-,4′- and 5-OH, with the 5-OH being the least reactive due to its low acidity (Zhang & Brodbelt, 2004). In view of the identification of naringenin-7-*O*-glucuronide and naringenin-4′-*O*-glucuronide in rat urine after the administration of naringenin chalcone from tomato skin extracts, and considering that the 7-*O*-glucuronide elutes from a reversed phase HPLC column before its 4′-isomer (Davis, Needs, Kroon, & Brodbelt, 2006), M1 was tentatively identified as naringenin-7-*O*-glucuronide and M2 as naringenin-4'-*O*-glucuronide. Nevertheless, the quantification of naringenin glucuronides was expressed as the sum of M1 and M2 in LC/MS/MS.

No mass signals from chrologenic acid (m/z 353 → 191) were detected in either plasma or urine samples, indicating the virtual absence of unmetabolized caffeoylquinic acids in the fluids. These findings are in keeping with previous studies on the urinary recovery of chlorogenic acid in humans and rats (Cremin, Kasim-Karakas, & Waterhouse, 2001), which failed to detect the native compound in urine after an acute dose, suggesting that it may undergo a more complete metabolism than the unesterified hydroxycinnamates. In fact, the gut microflora of animals and humans seem to be able to hydrolyze chlorogenic acid into caffeic acid and quinic acid (Plumb et al., 1999); thus, a combination of phase II conjugated metabolites

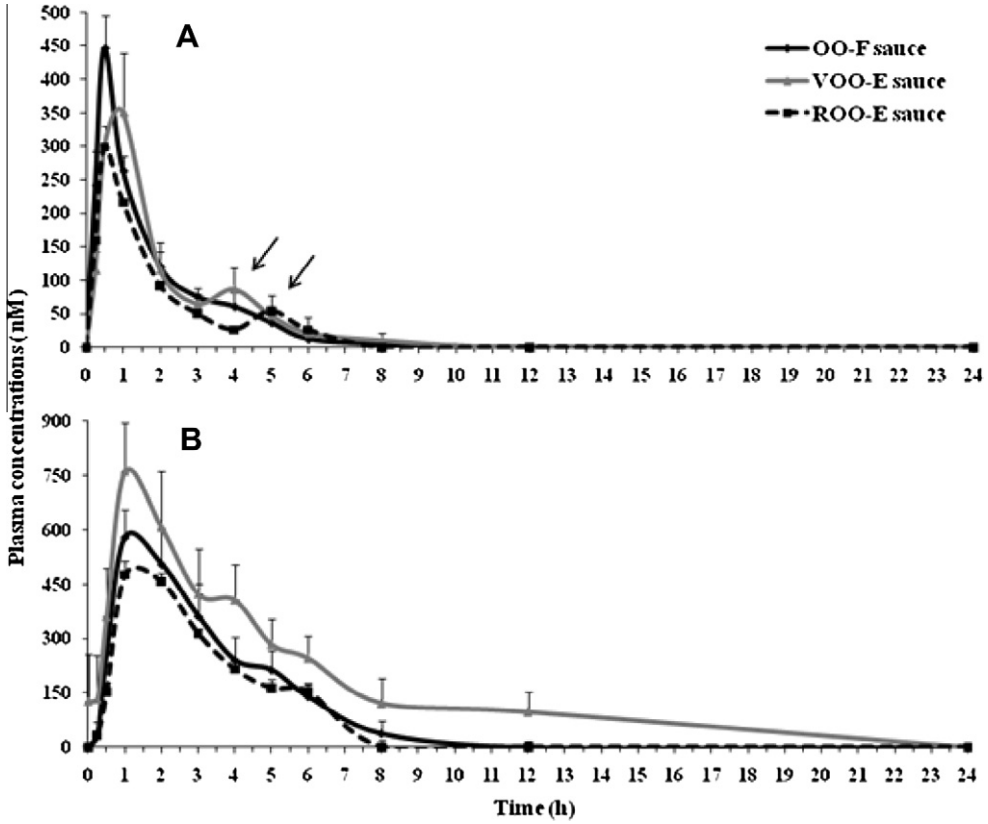

Fig. 2. Plasma naringenin (A) and ferulic (B) glucuronide concentrations in healthy subjects (n = 5) after single dose ingestions of OO-F, VOO-E and ROO-E tomato sauces. Values are means ± SEM.

of caffeic and ferulic acids (i.e. glucuronides and/or sulfates) would be identified in biological fluids after administration of chlorogenic acid (Azuma et al., 2000). In line with these findings, free caffeic (m/z 179 → 135) and ferulic (m/z 193 → 134) acids were clearly detected in urine samples after the ingestion of the tomato sauces, and peaks identification was confirmed by co-chromatography and fragmentation data with respect to the commercial standards. A mass signal at m/z 369 → 193 (Fig. 1E–G) and one at m/z 355 → 179 were also detected, both indicating the loss of a glucuronide unit (−176 amu) within the two molecules. The respective MS fragmentation pattern confirmed their identification as glucuronide derivatives of (iso)ferulic acid (Fig. 1H) and caffeic acid, respectively. Besides, being possible metabolites of the caffeoylquinic acids present in the sauces, these compounds may also originate from the metabolism of caffeic or ferulic hexoses originally contained in the tomato sauces (Stalmach et al., 2009). The mass signal for the ferulic glucuronide was also observed in plasma, and the metabolite peak was consistent with that found in urine. By contrast, the virtual absence of free naringenin, ferulic and caffeic acids and caffeic glucuronide in plasma samples might indicate that these compounds should be present at concentrations below the limit of detection of the instrument, probably because of their extensive first-pass metabolism via phase II conjugation, and their quick excretion via the kidney.

Rutin (quercetin-3-*O*-rutinoside) was the only flavonol found in the tomato sauces administered. As recently described (Jaganath et al., 2006), rutin is extensively metabolised and made bioavailable. Following the release of the aglycone, quercetin would in fact undergo glucuronidation and methylation, with quercetin-3-glucuronide and its methylated form isorhamnetin-3-glucuronide appearing in the circulatory and the excretory system. Further phase II metabolism could also occur in the liver and/or kidneys, resulting in the formation of quercetin-4′-glucuronide, quercetin-3′-glucuronide and combined conjugated forms, such as quercetin diglucuronide and methylquercetin diglucuronide. In the present study, all the above-cited phase II metabolites of rutin were investigated in plasma and urine of the subjects, but no mass signals from flavonols were detected after consumption of the three tomato sauces. The virtual absence of these compounds, expected mainly in urine samples, may be explained by the much lower amount of rutin ingested by the subjects in our study (an average of 246 μg among the three tomato sauces administered) compared to the previous investigation (176 μmoles, corresponding to 107.4 mg of rutin), which probably compromised the possibility of observing any metabolite peaks above the limit of detection of the HPLC–MS system. Additionally, it is known that derivatives containing rhamnose, such as rutinosides, are absorbed ∼10 times less than are glucosides in the small intestine but, once transferred to the large intestine, they are degraded by the rhamnosidases of the intestinal microbiota (Manach et al., 1997). For these reasons, future research will include the study of the phenolic metabolites putatively originating from the intestinal microbiota, in order to fully evaluate the bioavailability of tomato and tomato sauce phenolics after oral ingestion.

Finally, no evidence of sulfation was detected in the present investigation. These findings confirmed previous observations that glucuronidation is the preferential conjugation for naringenin and rutin, although, until now, it has not been reported for the metabolism of chlorogenic, caffeic and ferulic acids.

3.3. Pharmacokinetics of naringenin glucuronides after ingestion of the three tomato sauces

The mean plasma concentration-*versus*-time curves for naringenin (A) and ferulic (B) glucuronides from the three tomato sauces

Table 3
Pharmacokinetic indices for naringenin and ferulic glucuronides after single ingestion (100 g) of OO-F, VOO-E and ROO-E tomato sauces by healthy subjects ($n = 5$). Values are means ± SEM.

		C_{max} (nmol/l)	T_{max} (min)	AUC_{last} (nmol/x min)	MRT (min)	C_{max}-to-AUC_{last} (ratio)
Naringenin glucuronide	OO-F Sauce	446 ± 48.8	30.0 ± 0.0	43559 ± 5630	99.6 ± 16.3	0.0105 ± 0.0011
	VOO-E Sauce	398 ± 86.8	45.0 ± 7.7	37649 ± 6794	91.7 ± 12.7	0.0106 ± 0.0013
	ROO-E Sauce	299 ± 30.1	30.0 ± 0.0	32009 ± 6705	106 ± 12.5	0.0113 ± 0.0025
Ferulic glucuronide	OO-F Sauce	580 ± 85.4	72.0 ± 12.0	118949 ± 26786	158 ± 10.6	0.0056 ± 0.0008
	VOO-E Sauce	727 ± 165	75.0 ± 13.4	197702 ± 53354	218 ± 56.6	0.0056 ± 0.0019
	ROO-E Sauce	544 ± 101	84.0 ± 14.7	96531 ± 20455	149 ± 14.0	0.0061 ± 0.0008

are shown in Fig. 2. The pharmacokinetic parameters for the two metabolites encountered in plasma are shown in Table 3.

None of the subjects had measurable concentrations of glucuronide metabolites of naringenin before ingestion of the sauces. By contrast, the two naringenin glucuronides identified already appeared in plasma 15 min after consumption (Fig. 2A), and their plasma levels decreased below the LOD within 12 h. The short T_{max} for naringenin glucuronides (Table 3) confirmed the rapid intestinal absorption and metabolism of naringenin to these phase II conjugates (Bredsdorff et al., 2010), and stressed the importance of an early collection of plasma samples during acute consumption studies, in order not to subestimate the plasma pharmacokinetics of these metabolites. When comparing the pharmacokinetic parameters obtained with the three interventions, higher C_{max} and AUC_{last} values were observed after the ingestion of the sauce without olive oil. However, the differences were not supported by the statistical analysis ($p > 0.05$, see Table 3). The absence of statistical significance in the differences observed among the three interventions was probably influenced by the great interindividual variability observed in the C_{max} and AUC_{last} values among the subjects (see SEM in Table 3). Also the apparent delay in the T_{max}, observed after the ingestion of VOO-E sauce, was only ascribed to two of the five subjects, resulting without statistical significance.

Interestingly, when comparing the plasma concentration-*versus*-time curves of the naringenin glucuronides in respect to the intervention administered, a typical biphasic absorption profile was only observed following the ingestion of the oil-enriched tomato sauces, with a first peak at 30 min and a second one at 4–5 h (Fig. 2A, see the arrows). These findings may suggest the occurrence of re-absorption events by enterohepatic circulation, specifically stimulated by the tomato–oil combination. It is known that polyphenols undergo repeated recycling through the digestive system, because of the enterohepatic and enteric recycling, which afford their apparent plasma half-life much longer than would be predicted from their intrinsic clearance value. A longer half-life may facilitate the pharmacological functions of polyphenols, since conjugated types may still be good bioactive compounds and target tissues often possess hydrolases (e.g. β-glucosidase or sulfatases) that can reconvert conjugates back to parent compounds. A recent dispositional study (Xu et al., 2006) revealed that large

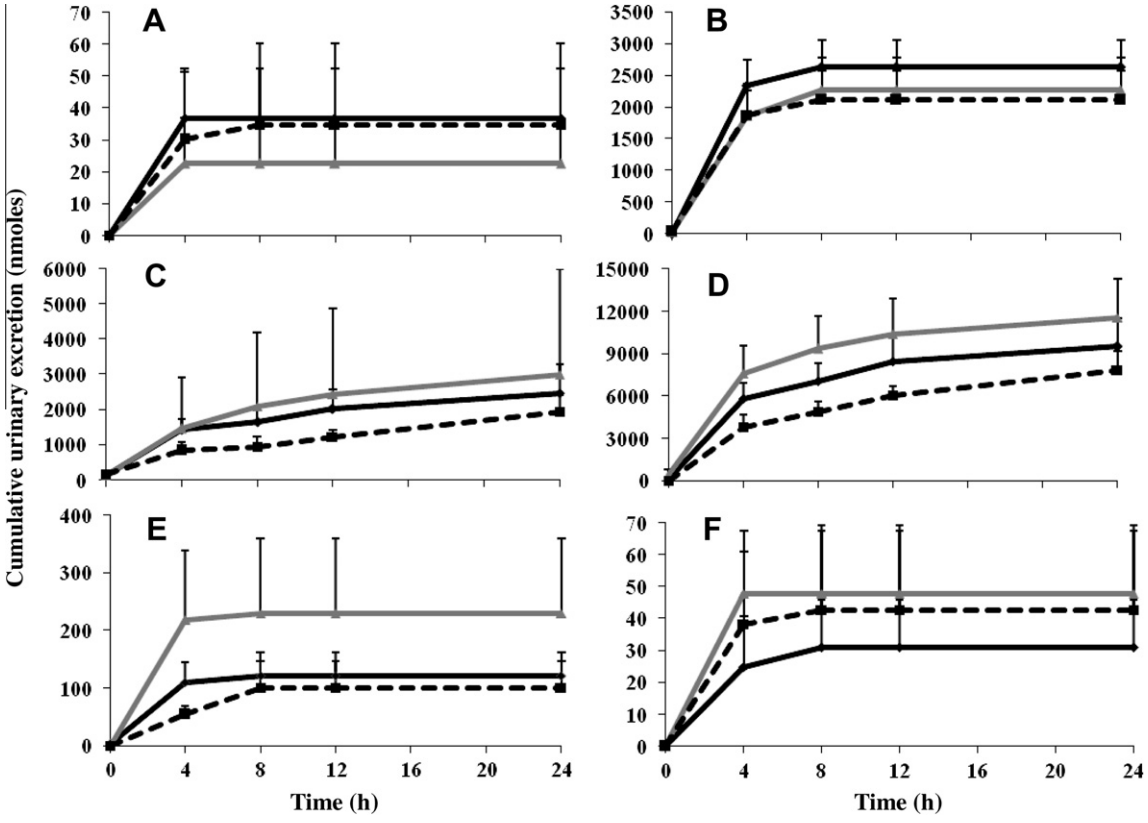

Fig. 3. Cumulative urinary excretion curves of healthy subjects ($n = 5$) for naringenin (A), naringenin glucuronides, (B) ferulic acid (C), ferulic glucuronide (D), caffeic acid (E) and caffeic glucuronide (F) after single dose ingestions of OO-F (black line), VOO-E (grey line) and ROO-E (black dashed line) tomato sauces. Values are means ± SEM.

amounts of one of the naringenin glucuronides (putatively M2) are found in the bile, suggesting that both intestine and liver organs are important contributors to the naringenin first pass metabolism. However, to our knowledge, no literature data are currently available on the extent and mechanisms of hepatic biotransformation of naringenin, particularly in the presence of a lipid matrix in the meal.

3.4. Pharmacokinetics of ferulic glucuronide after ingestion of the three tomato sauces

Higher C_{max}, T_{max}, AUC_{last} and MRT were observed for the ferulic glucuronide in respect to the naringenin glucuronides in plasma. The higher T_{max} and lower C_{max}-to-AUC_{last} ratio confirmed the slower rate of appearance in plasma of ferulic glucuronide (Fig. 2B), which is in agreement with the longer and more complex metabolism leading to the compound. On the other hand, the metabolite remains detectable in plasma for a longer period (MRT 2.5 h, in respect to 1.5 h of the naringenin glucuronide). When comparing the kinetic behaviour of ferulic glucuronide dependently on the sauce administered, apparently higher C_{max}, AUC_{last} and MRT were observed after the ingestion of VOO-E tomato sauce (Table 3). However, the differences were not statistically significant since, as for the plasma kinetics of naringenin glucuronides, high interindividual variations in the C_{max} and AUC_{last} were remarkable. The C_{max}-to-AUC_{last} ratio index did not reveal relevant variations among the three interventions, confirming no significant differences in the rate of appearance of ferulic glucuronide.

3.5. Urinary excretion of phenolic metabolites after ingestion of the three tomato sauces

The cumulative urinary excretion curves for the phenolic metabolites detected in urine are shown in Fig. 3. With the exceptions of ferulic acid and ferulic glucuronide (Fig. 3C and D), the phenolic compounds were mainly excreted into the 0- to 4 h urinary fraction collected after ingestion of the three tomato sauces (ranging from 54% excretion of ferulic acid derivatives up to 91%, for naringenin and naringenin glucuronides). The parent compound, naringenin, that was not observed in plasma after the tomato sauce ingestions, appeared in the 24 h urine to an extremely lower extent (Q_∞ = 37 nmoles) in respect to its glucuronide metabolites (Q_∞ = 2628 nmoles), confirming the rapid metabolism of the compound, and was rapidly excreted after ingestion (Fig. 3A and B). The free ferulic acid, not detected in plasma after the three interventions, was also excreted to a lesser extent than its glucuronide metabolite (Fig. 3C and D). However, both the free acid and its phase II conjugate showed slower urinary excretions than did the flavanone compounds (Fig. 3C and D), in keeping with the slower metabolism of ferulic acid and the longer plasma residence time of the glucuronide conjugate (Fig. 2B). Finally, both free caffeic acid and its glucuronide metabolite, not detected in plasma samples, appeared in urine at very low concentrations and were almost completely excreted within the first fraction of urine collected after ingestion (0–4 h). When comparing the mean urinary excretion curves, once again, no statistically different excretive behaviour was observed in respect to the intervention administered.

4. Conclusions

The present study confirmed that the flavanone naringenin and hydroxycinnamates, such as chlorogenic acid, caffeic and ferulic acid derivatives, are among the most abundant phenolics in tomato sauces. The phenolic content of the sauces in this study significantly differed from the starting raw tomatoes in the higher content of caffeoylquinic acid, rutin, but particularly in the 8-fold higher concentration of naringenin. These data confirmed how heat and/or mechanical treatments, during tomato sauce processing, may increase the phenolic compositions of the processed products.

Six phenolic compounds, namely three aglycones (naringenin, ferulic and caffeic acids) and their corresponding monoglucuronide metabolites, were detected in urine after the single ingestion of all three tomato sauces, reaching the maximum rate of excretion within 4 h from the intervention. Only two of the six urinary phenolic metabolites were also identified in plasma. The naringenin glucuronides rapidly appeared in plasma 15 min after consumption, while higher C_{max}, T_{max}, AUC_{last} and MRT were observed for the ferulic glucuronide, confirming the slower metabolism and urinary excretion of ferulic acid.

The pharmacokinetics of the naringenin glucuronides after ingestion of the oil-enriched tomato sauces let us to hypothesize that the lipid matrix in the sauces may stimulate the occurrence of re-absorption events by enterohepatic circulation, thus potentially affecting the apparent plasma half-life of these compounds. Nevertheless, the great interindividual response variability observed underlies the need for further large-scale investigations ideally involving a larger number of subjects, to confirm the hypothesis formulated through the present work.

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4.3. Publicación 3. El proceso de fabricación de la salsa de tomate afecta a la bioaccesibilidad y la biodisponibilidad de los compuestos fenólicos del tomate: un estudio farmacocinético.

The tomato sauce making process affects the bioaccessibility and bioavailability of tomato phenolics: A pharmacokinetic study. **Miriam Martínez-Huélamo**, Sara Tulipani, Ramón Estruch, Elvira Escribano, Montserrat Illán, Dolores Corella and Rosa M. Lamuela-Raventós. *Food Chemistry*. 2015, 173(15):864-872.

De entre todos los productos procesados de tomate, la salsa es la más consumida en todo el mundo; sin embargo, muy poco se sabe sobre cómo el proceso de fabricación puede afectar la composición fenólica y la biodisponibilidad después de su consumo. En el presente trabajo, hemos llevado a cabo un estudio piloto, prospectivo, aleatorizado, cruzado, abierto y controlado con 8 voluntarios sanos donde se les suministró 250 g de salsa de tomate sin aceite (OF), una segunda elaborada con aceite de oliva refinado (ROOE) y una tercera de 500 g de tomate fresco por cada 70 Kg de peso del voluntario. La salsa fue elaborada a partir de tomates frescos (liso rojo rama) comerciales en el Campus de Torribera de la Universidad de Barcelona.

La principal novedad con el estudio anterior fue la introducción de tomate fresco en el ensayo para estudiar el efecto que tendría el procesado de tomate a salsa sobre la biodisponibilidad de los compuestos fenólicos y, a la vez, estudiar el efecto de la adición de una matriz lipídica. Otro punto distintivo, fue la dosis de las intervenciones que se suministró según el peso del voluntario, así como incluir en el estudio indistintamente hombres como mujeres. El diseño del estudio se muestra a continuación (**Tabla 7**):

Tabla 7. Diseño del estudio.

Día -3	Dieta sin tomate	
Día -2	Dieta sin tomate	
	No suplementos vitamínicos	
	No medicamentos	
Día -1	Dieta exenta de polifenoles	
	No suplementos vitamínicos	
	No medicamentos	
	Estar en ayuno las 10-12 horas previas al día 0	
Día 0: Estudio	t = 0 h	Extracción de sangre Recogida de orina
	Intervención: 250 g de salsa de tomate con aceite de oliva refinado, salsa de tomate sin aceite de oliva refinado y 500 g de tomate fresco por cada 70 Kg de peso del voluntario.	
	t = 15 min	Extracción de sangre
	t = 30 min	Extracción de sangre
	t = 1 h	Extracción de sangre
	t = 2 h	Extracción de sangre
	t = 3 h	Extracción de sangre
	t = 4 h	Extracción de sangre Recogida de orina
	t = 6 h	Extracción de sangre
	t = 8 h	Extracción de sangre Recogida de orina
	t = 12 h	Recogida de orina
Día 1	t = 24 h	Extracción de sangre Recogida de orina

Los resultados al analizar las salsas y el tomate demuestran cómo no existen diferencias entre los dos tipos de salsas pero sí con el tomate obteniéndose concentraciones superiores en las salsas para la naringeina y la rutina. Este hecho vuelve a demostrar como el tratamiento mecánico ayuda a la liberación de ciertos compuestos fenólicos de la matriz del alimento, pero la adición de una matriz lipídica no parece ayudar a que estos sean más bioaccesibles.

Se analizaron los niveles de los compuestos fenólicos del tomate y sus metabolitos en plasma y orina después del consumo agudo de las salsas y el tomate por HPLC-MS/MS. Once y cuatro metabolitos fenólicos fueron encontrados en las muestras de orina y plasma, respectivamente. Se estudió tanto la diferencia entre intervenciones de todos los voluntarios, como la diferencia entre intervenciones de voluntarios de diferente sexo. No se observaron diferencias significativas en este último estudio en ninguna de las dos muestras biológicas. Los compuestos fenólicos naringenina, ácido cafeico y ácido ferúlico, se encontraron intactos en las muestras, así como sus metabolitos de fase

II. Las concentraciones en plasma y la excreción urinaria de la naringenina glucurónido fueron significativamente mayores después del consumo de la salsa de tomate en comparación con las observadas tras el consumo de tomate fresco. También se encontró un comportamiento bifásico en el caso de la naringenina glucurónido y naringenina tras las intervenciones con salsas, mientras que en el tomate fresco dicho comportamiento no se manifestó. Los resultados sugieren que los tratamientos mecánicos y térmicos durante la elaboración de la salsa de tomate pueden ayudar a liberar estos compuestos fenólicos, potencialmente bioactivos, de la matriz del alimento con más eficacia que la adición de un componente lipídico aumentando así su biodisponibilidad.

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The tomato sauce making process affects the bioaccessibility and bioavailability of tomato phenolics: A pharmacokinetic study

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ABSTRACT

Tomato sauce is the most commonly consumed processed tomato product worldwide, but very little is known about how the manufacturing process may affect the phenolic composition and bioavailability after consumption. In a prospective randomised, cross-over intervention study, we analysed the plasma and urinary levels of tomato phenolic compounds and their metabolites after acute consumption of raw tomatoes and tomato sauce, enriched or not with refined olive oil during production.

Respectively, eleven and four phenolic metabolites were found in urine and plasma samples. The plasma concentration and urinary excretion of naringenin glucuronide were both significantly higher after the consumption of tomato sauce than raw tomatoes. The results suggest that the mechanical and thermal treatments during tomato sauce manufacture may help to deliver these potentially bioactive phenolics from the food matrix more effectively than the addition of an oil component, thus increasing their bioavailability.

1. Introduction

It is generally recognised that part of the health-promoting effects of the Mediterranean diet may be attributed to the high content of phytochemical constituents in fruit and vegetables, which are among the food items at the bottom of the Mediterranean diet pyramid (Estruch et al., 2013). Phytochemical phenolic compounds have recently attracted increasing attention (Scoditti et al., 2012) and epidemiologic data have shown an inverse association between the risk of overall mortality or cardiovascular diseases and the consumption of polyphenol-rich foods (Cassidy et al., 2013).

Several *in vitro* and human intervention studies have been carried out to unveil the mechanisms of action underlying the health-promoting properties of polyphenols, which include the induction of antioxidant defenses (Wan et al., 2001), blood pressure lowering

Abbreviations: AUC_{last}, area under the plasma concentration-*versus*-time curve from time 0 until the last detectable concentration; BMI, body mass index; BW, body weight; CAD, collision-activated dissociation; CE, collision energy; DP, declustering potential; EP, entrance potential; AUMC, first moment curve; FP, focusing potential; FS, full scan; HCl, hydrochloric acid; HPLC–ESI-QqQ-MS/MS, high performance liquid chromatography coupled to tandem mass spectrometry; IS, internal standard; LPD, low polyphenol diet; $Q_{u\infty}$, maximum excreted amount in the 24 h urine collection; C_{max}, maximum plasma concentration; MRT_{last}, mean residence time; MRM, multiple reaction monitoring; NL, neutral loss; N_2, nitrogen; OF, olive oil-free tomato sauce; PFD, polyphenol-free diet; PTFE, polytetrafluoroethylene; PrIS, precursor ion scan; PIS, product ion scan; ROOE, refined olive oil-enriched tomato sauce; CL_{ren}, renal clearance; SPE, solid-phase extraction; SEM, standard error; t_{max}, time to reach the maximum plasma concentration; TFD, tomato-free diet.

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effects (Desch et al., 2010), the improvement of endothelial function (Widlansky et al., 2007), the inhibition of platelet aggregation and LDL oxidation, and the modulation of inflammatory response (Zhu et al., 2013).

Tomatoes (*Solanum lycopersicum*, formerly *Lycopersicum esculentum*) are one of the key components of the Mediterranean diet, and their regular consumption has been consistently associated with a lower risk of several types of cancer and coronary heart disease (Paran, Novack, Nir Engelhard, & Hazan-Halevy, 2009). Tomatoes are widely consumed both as fresh fruit and as processed products. Tomato sauce is the most commonly consumed tomato product worldwide and particularly in Spain, where it represents 40.8% of all sauce consumption.

The processing of raw tomatoes into tomato sauces involves several thermal and mechanical treatments and may include the addition of a lipid matrix (commonly up to 5% of olive oil) during production (oil-enriched *versus* oil-free sauce) (Chanforan, Loonis, Mora, Caris-Veyrat, & Dufour, 2012). All these steps may affect the final phenolic composition of the end product. The addition of a lipid matrix during tomato sauce preparation, as well as the simultaneous consumption of fats and triglycerides, have been reported to favour the extractability and bioavailability of tomato carotenoids (Fielding, Rowley, Cooper, & O'Dea, 2005). The combined intake of tomato and a lipid component may increase the absorption of fat-soluble compounds, since dietary fat is important for micelle formation in the small intestine, thus contributing to the solubilisation of fat-soluble compounds in the bile salts and their incorporation into the micelles (Hornero-Méndez & Mínguez-Mosquera, 2007). However, to date few studies have investigated the absorption and excretion of phenolic compounds from raw tomatoes and tomato sauces (Tulipani et al., 2012), and even less is known about the impact of a tomato-olive oil combination during processing on the phenolic bioavailability and bioefficacy in humans.

The working hypothesis of the present study was that domestic or industrial-scale processing in tomato sauce production may produce changes in phenolic extractability due to the disruption of the plant cell wall and thus result in an easier release of bound polyphenolic and flavonoid compounds (van het Hof, West, Weststrate, & Hautvast, 2000). As already hypothesised for tomato carotenoids, the addition of oil during tomato sauce processing may also influence the bioavailability of the relatively lipophilic phenolics in tomato, by modifying their bioaccessibility from the food matrix, and modulating the gastric emptying or hepatic metabolism of the absorbed phenolic compounds. The aim of the present study was to investigate whether the human bioavailability of the phenolic compounds found in tomatoes is influenced by the sauce making process and the addition of refined olive oil during manufacture.

2. Materials and methods

2.1. Standards and reagents

Caffeic acid, chlorogenic acid, dihydrocaffeic acid, ferulic acid, gallic acid, isoferulic acid, kaempferol, *m*-coumaric acid, naringenin-7-*O*-glucoside, *p*-coumaric acid, *p*-hydroxybenzoic acid, protocatechuic acid, quercetin, rutin and human plasma were purchased from Sigma–Aldrich (St. Louis, MO, USA); and naringenin and ethylgallate (internal standard (IS)) from Extrasynthese (Genay, France). HPLC-grade ethanol, acetonitrile, methanol and formic acid were purchased from Scharlau Chemie S.A. (Barcelona, Spain), while hydrochloric acid 37% (HCl) and *o*-phosphoric acid 85% were supplied by Panreac Quimica S.A. (Barcelona, Spain). Ultrapure water (Milli-Q) was obtained from a Millipore system (Millipore, Bedford, MA, USA).

2.2. Tomato and tomato sauce

Commercial tomatoes, "liso rojo rama" variety, used for both the elaboration of the two sauces to the intervention of raw tomato, were purchased from a local market (Barcelona, Spain). Refined olive oil was kindly furnished by Juan Ballester Rosés Company S.A. (Tortosa, Spain). To ensure that the refined olive oil added did not introduce any phenolic compounds to the sauce, a liquid–liquid extraction was used and the extract was analysed by HPLC with a diode array detector (Boselli, Di Lecce, Strabbioli, Pieralisi, & Frega, 2009) obtaining a polyphenol-free profile. Raw tomato sauce ("oil-free": OF) and a refined olive oil-enriched tomato sauce (ROOE) were processed at the Torribera Campus, University of Barcelona (Santa Coloma de Gramanet, Spain) by a standardised industrial scale-like manufacturing process, as previously described by Tulipani et al. (2012). Briefly, tomatoes were cleaned, cleaved, mixed and weighted. For the elaboration of ROOE sauce, 5% of oil was added and the same amount of water was aggregated to obtain the OF sauce. The mixture was cooked for 60 min at 99 °C and finally crushed. The obtained sauces were vacuum-packed and stored in the freezer at −20 °C until the day of the study when were thawed in the refrigerator and administered at room temperature. Considering that 1000 g of fresh tomatoes yielded approximately 500 g of tomato sauce, 3.5 g of sauce per kg of body weight (BW) (3.5 g kg^{-1} BW) and 7 g kg^{-1} BW of raw tomatoes were administered to each volunteer across the three interventions, to standardise the intake.

2.3. Phenolic extraction of the dietary interventions

0.5 g of raw tomato or tomato sauce was weighed and 5 mL of 80% ethanol acidified with 0.1% formic acid was added to the samples. The mixture was vortexed for 1 min and then sonicated for 5 min on ice to prevent the degradation of the phenolic compounds. After centrifugation at 4000 rpm for 20 min at 4 °C, the supernatant was collected and another 5 mL of the acidified 80% ethanol solution was added to the pellet, and the extraction procedure repeated. Both supernatants were combined, and the ethanolic component was evaporated to dryness by a sample concentrator (Techne, Duxford, Cambridge, UK) at room temperature under a stream of nitrogen gas. 400 ng mL^{-1} of ethylgallate (IS) was added to the aqueous extracts (2 mL), which were filtered with 4 mm 0.45 μm polytetrafluoroethylene (PTFE) syringe filters (Waters Corporation, USA), and injected (20 μL) in triplicate into the high performance liquid chromatography coupled to tandem mass spectrometry (HPLC–ESI-QqQ-MS/MS) (Vallverdú-Queralt, Jáuregui, Medina-Remón, & Lamuela-Raventós, 2012).

2.4. Subjects and study design

Eight healthy nonsmoking subjects (50% women, 27–46 years, BMI = 23 ± 0.93 kg m^{-2}) with no history of cardiovascular, hepatic or renal disease and no adherence to any special diets at least during the 4 weeks prior to the trial, were recruited for the randomised crossover dietary study. The study was explained to participants through verbal and written instructions, and written informed consent was obtained before participation. To standardise the baseline point, subjects were asked to follow a tomato-free diet (TFD) during the 3 days preceding the dietary intervention, and a low polyphenol diet (LPD) in the 24 h immediately preceding the test. The subjects were provided with a list of acceptable and unacceptable foods, together with two standardised low polyphenol meals for the evening preceding the test, and the day of the test. On the day of each intervention, all the subjects consumed together a standardised lunch, and were asked to fill in a 24-h food

record to assess their compliance with the dietary recommendations. Total energy, macronutrient and micronutrient contents of the LPD were calculated using Food Processor software.

Each volunteer received the tomato, OF and ROOE sauce interventions in a randomly assigned order and separated by a 3-day washout period (usual diet) followed by a 3-day TFD and 1-day LPD.

On the day of the intervention, the overnight fasted subjects had an intravenous catheter inserted, and a baseline blood sample (5 mL) was collected. Subjects then consumed, in turn, one of the three interventions within a space of 5–10 min, and refrained from consuming other foods for the following 6 h. Blood samples (5 mL) were collected into lithium heparin tubes at 15 and 30 min and 1, 2, 3, 4, 6, 8 and 24 h after the ingestion of the tomato sauces, and immediately centrifuged at 1500 *g* for 20 min at 4 °C. All plasma samples were subsequently aliquoted and stored in eppendorf tubes at −80 °C until analysis. Urine was collected in separate fractions at baseline (0 h) and during 24 h after the dietary interventions ("0–4", "4–8", "8–12" and "12–24" h collection periods). The urine bottles were stored at 4 °C, and immediately after the participants had started to collect the next fraction, the amount of urine in each fraction was measured, acidified with 0.2 M HCl and stored at −80 °C until analysis. The study protocol was approved by the Ethics Committee of Clinical Investigation of the University of Barcelona (Spain), and the clinical trial was registered at the International Standard Randomised Controlled Trial Number (ISRCTN20409295). Informed consent was obtained from all participants.

2.5. Extraction of tomato phenolic metabolites from plasma and urine

Plasma and urine were subjected to the solid-phase extraction (SPE) procedure previously described by our working group using Waters Oasis HLB 96-well plate 30 μm (30 mg) (Waters Oasis, Milford, MA, USA) (Martínez-Huélamo, Tulipani, Torrado, Estruch, & Lamuela-Raventós, 2012; Tulipani et al., 2012).

Briefly, on the day of the analysis, the samples were defrosted on ice in the dark. Prior to SPE, urine samples were centrifuged at 15,000 *g* for 4 min at 4 °C, while plasma samples were acidified by adding *o*-phosphoric acid (2.5%) to break possible polyphenol-plasmatic protein linkages and reach the optimum pH for phenolic retention in the solid-phase during the extraction procedure. Then, 400 ng mL^{-1} of ethylgallate (IS) was added to the samples, and after 2 min of vortex-mixing, the samples were diluted 1:1 (v/v) with water. Plate activation was achieved by consecutively adding 2 mL of methanol and 2 mL of 1.5 M formic acid. After loading into the 96-well plate, sample clean-up was performed with 2 mL of 1.5 M formic acid followed by 2 mL of 5% methanol solution. Phenolic metabolites were then eluted with 2 mL methanol acidified with 0.1% formic acid. The eluted fraction was evaporated to dryness by a sample concentrator (Techne, Duxford, Cambridge, UK) at room temperature under a stream of nitrogen gas, and reconstituted with 200 μL of 0.1% formic acid. The 96-wellplate was then vortexed for 5 min and 20 μL of the reconstituted extracts was injected into the LC–MS system.

For the preparation of the calibration curves, synthetic urine and commercial human plasma were spiked with an increasing concentration of a mixture of 8 phenolic standards (1–500 ng mL^{-1}), before being processed and subjected to the extraction procedure exactly in the same way as the samples.

2.6. HPLC–ESI-QqQ-MS/MS analysis of food and biological samples

The identification and quantification of the phenolic compounds present in tomato, OF and ROOE sauces and the corresponding phenolic metabolites expected in urine and plasma samples were carried out by HPLC–ESI-QqQ-MS/MS analysis. The system used was an HP Agilent Technologies 1100 equipped with a refrigerated auto sampler and a column oven set to 30 °C, coupled to an API 3000 triple-quadrupole mass spectrometer (PE Sciex, Concord, Ontario, Canada) with a TurboIon spray source used in negative mode. Chromatographic separation was achieved on a Luna C_{18} (50 × 2.0 mm, 5 μm) from Phenomenex (Torrance, CA, USA) using a precolumn Phenomenex security guard C_{18} (4 × 3 mm i.d.). The system was controlled by Analyst v. 1.4.2 software supplied by Applied Biosystems (Foster City, CA, USA). The chromatographic conditions were set as described in our previous publications (Martínez-Huélamo et al., 2012; Vallverdú-Queralt et al., 2012).

A direct infusion experiment was carried out with each individual standard compound available (1 μg mL^{-1} at a constant flow rate of 5 μL min^{-1}). TurboIon spray source settings were as follows: capillary voltage, −4000 V; nebuliser gas (N_2), 10 (arbitrary units); curtain gas (N_2), 12 (arbitrary units); drying gas (N_2) heated to 400 °C and introduced at a flow rate of 8000 cm^3 min^{-1}. The declustering potential, focusing potential and entrance potential were optimised for each standard. Multiple reaction monitoring experiment was used for the quantification of the compounds (Martínez-Huélamo et al., 2012).

Compounds lacking the corresponding commercial standard (i.e. sulphate and glucuronide derivatives) were tentatively identified by full scan (FS), product ion scan (PIS), neutral loss (NL) and precursor ion scan (PrIS) experiments, and the calibration curves of the corresponding native unconjugated molecules were used for quantification.

The PrIS experiment is able to identify compounds belonging to a group of substances. NL allows identification with losses of 80 *m*/*z* and 176 *m*/*z* corresponding to sulphates and glucuronides, respectively. Finally, PIS permits the identification of aglycones by comparison of the MS/MS spectra with those corresponding to the standards after typical fragmentations.

To confirm the fragmentation pattern of each metabolite, PIS experiments were carried out at optimal and double collision energy values than suggested by the direct infusion experiments, using a cycle time of 2 s. MS/MS product ions were produced by collision-activated dissociation (CAD) of the selected precursor ions in the collision cell of the triple-quadrupole mass spectrometer, and mass analysed using the second analyser of the instrument.

2.7. Pharmacokinetic analysis

WinNonlin Professional software version 3.3 (Pharsight Corporation, USA) was used to determine the pharmacokinetic parameters by a non-compartmental analysis. The maximum plasma concentration (C_{max}) and the time needed to reach it (t_{max}) were obtained directly from the experimental plasma concentration time data for each phenolic compound and phase II metabolite found in the samples. The linear trapezoidal method was used to calculate the area under the plasma concentration-*versus*-time curve from time 0 until the last detectable concentration (AUC_{last}). The mean residence time (MRT_{last}) was estimated by means of the ratio AUMC/AUC, where AUMC was the first moment curve. The C_{max}/AUC_{last} and cumulative amounts of phenolic compounds excreted in urine ($Q_{u\infty}$) were also calculated.

2.8. Statistical analysis

SPSS software (Version 19.0, Japan Inc., Tokyo, Japan) was used to compare the energy and nutrient intake of the 24 h preceding the interventions; the phenolic composition of the three dietary interventions; the pharmacokinetic parameters after the interven-

tions, and the differences between men and women in the pharmacokinetic parameters within the same intervention and inter-interventions. All the results were subjected to a normality test prior to the statistical analysis and then to one-way analysis of variance (ANOVA) with the Bonferroni post hoc test in the case of homogeneity of variances and T3 Dunnett when the variances were not homogenous. The results were reported as mean ± standard error (SEM). Differences at $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Phenolic composition of the dietary interventions

Table 1 shows the phenolic compounds quantified in the tomatoes and tomato sauces administered to the volunteers. The quantified compounds were divided into phenolic acids, flavanones and flavonols. The flavanone naringenin and the flavonol rutin were the major phenolic compounds in the sauces, whereas their levels were much lower in the raw tomatoes. Phenolic acids were the second most abundant phenolics administered, principally 5-caffeoylquinic acid, followed by 4-caffeoylquinic acid and caffeic acid hexose.

When qualitatively comparing the phenolic profile, no differences were observed among the three interventions, except for the appearance of naringenin-7-*O*-glucoside in the two sauces, which was not detected in the raw fruits.

In terms of quantification, however, flavonoids were more highly concentrated in the sauces. The greatest increase was observed in the flavanone naringenin (7-fold higher in sauces than raw tomatoes). Sauces also showed significantly higher levels of the flavonol rutin (2-fold higher than in raw tomatoes), protocatechuic acid (almost 4-fold higher) and 3-caffeoylquinic acid (4-fold higher). In contrast, 5-caffeoylquinic acid and dicaffeoylquinic acid were more concentrated in raw fruits than in the processed products. The enhanced bioaccessibility of polyphenols in sauces was in agreement with previous observations, confirming that processing treatments may positively affect the level of polyphenols in sauces or other tomato products (Dewanto, Wu, Adom, & Liu, 2002). The probable explanation for this increase is the release of phenolics from the tomato matrix during the treatment through cell wall and cell membrane disruption (Chanforan et al., 2012).

No qualitative and quantitative differences were observed between the two sauces, except for 5-caffeoylquinic acid, 4-caffeoylquinic acid, caffeic acid hexose and naringenin, whose values were slightly higher in the ROOE tomato sauce. As expected, there was no increase in phenolic compounds with the addition of an oil matrix to the sauce.

3.2. Phenolic metabolites in plasma and urine

The volunteers filled in a 24-h food frequency questionnaire to guarantee compliance with the recommended LPD. As shown in Table 2, no significant differences were observed in the total energy, protein, carbohydrate, dietary fibre, total fat, cholesterol, vitamin, mineral and total polyphenol intake in the 24 h before the three interventions. In terms of phenolic intake, the serving of tomatoes, and OF and ROOE sauces respectively furnished 3822.2 μg, 1650.5 μg, and 1782.7 μg of phenolic acids, 251.6 μg, 868.1 μg, and 1011.3 μg of flavanones, and 1269.1 μg, 1487.6 μg, and 1230.9 μg of flavonols. The tomato phenolic metabolites analysed in plasma and urine are listed in Table 3, including a total of 19 transitions corresponding to the most likely detectable metabolites in biosamples, following the intervention ingestion.

Four phenolic compounds and metabolites were identified in plasma (Table 4) and eleven in urine (Table 5). In the present study, higher levels of metabolites were found in the biological fluids than in the previous human pilot study (Tulipani et al., 2012). This increase could be attributed to the effect of greater homogeneity of dose/weight, as in the previous study the intervention was 100 g per volunteer regardless of weight.

Naringenin, naringenin glucuronide, ferulic acid, ferulic acid glucuronide, caffeic acid, caffeic acid glucuronide, dihydroferulic acid, dihydroferulic acid glucuronide, dihydroferulic acid sulphate, chlorogenic acid, and dihydrocaffeic acid were identified in urine. In the previous study (Tulipani et al., 2012), only naringenin, ferulic acid and caffeic acid metabolites were quantified, while here we observed considerably more metabolites since we also looked for compounds arising from the microbiota metabolism.

Fig. 1 shows the product ion scan of the main metabolites found in urine. Naringenin glucuronide (Fig. 1A) was confirmed by a PIS experiment with the loss of 176 *m*/*z* corresponding to the glucuronide unit (Rubió et al., 2012), obtaining an ion at 271 *m*/*z* (naringenin) and an ion at 113 *m*/*z*, characteristic of the fragmentation of the glucuronyl molecule (Tulipani et al., 2012). The ferulic acid glucuronide fragmentation (Fig. 1B) confirmed the loss of the glucuronide unit and its corresponding ions at 175 *m*/*z* and 113 *m*/*z*, and the presence of ferulic acid with an ion at 193 *m*/*z*. Another compound identified was caffeic acid glucuronide (Fig. 1C). In this case,

Table 1
Compounds identified and quantified in the raw tomatoes and in the two tomato sauces administered to the subjects.

Compound	Raw tomatoes (ng/g FW)	OF sauce (ng/g FW)	ROOE sauce (ng/g FW)
3-Caffeoylquinic acid	113.3 ± 5.9[b]	443.8 ± 5.8[a]	419.6 ± 121.0[a,b]
5-Caffeoylquinic acid	2424.2 ± 98.0[a]	1575.9 ± 48.3[b]	1744.7 ± 65.2[b]
4-Caffeoylquinic acid	1495.4 ± 2.2	1197.3 ± 46.7	1356.5 ± 64.4
Dicaffeoylquinic acid	429.4 ± 15.9[a]	174.2 ± 5.9[b]	177.3 ± 9.8[b]
Caffeic acid	796.6 ± 11.2	762.6 ± 30.9	740.7 ± 21.4
Caffeic acid hexose	1286.3 ± 50.0	1401.4 ± 31.0	1749.8 ± 84.5
Caffeic acid hexose II	568.9 ± 27.3	459.6 ± 16.8	422.2 ± 10.0
Ferulic acid hexose	488.8 ± 25.0	441.1 ± 21.6	435.7 ± 7.6
Protocatechuic acid	41.4 ± 1.0[b]	146.1 ± 3.0[a]	154.4 ± 3.8[a]
Quantified phenolic acids orally administrated (μg)[A]	3822.2	1650.5	1782.7
Naringenin	503.2 ± 32.1[b]	3386.6 ± 122.3[a]	3823.7 ± 216.7[a]
Naringenin-7-O-glucoside	n.d[b]	215.9 ± 6.6[a]	221.3 ± 13.3[a]
Quantified flavanones orally administrated (μg)[A]	251.6	868.1	1011.3
Rutin	2538.1 ± 125.2[b]	5950.5 ± 302.6[a]	4923.7 ± 247.4[a]
Quantified flavonols orally administrated (μg)[A]	1269.1	1487.6	1230.9

Results expressed as mean ± SEM.
Values in a row with different letters are significantly different ($p < 0.05$).
[A] Corresponding to the single dose administrated to the subjects 500 g (tomato) 250 g sauces.

Table 2
Energy and nutrient intake of the 24 h preceding the interventions.

	Intervention			
	Raw tomato	OF	ROOE	p^{A}
Energy (kcal)	1797.6 ± 183.5	1874.5 ± 120.1	1933.8 ± 134.8	0.825
Protein (g)	101.1 ± 22.9	85.7 ± 9.8	116.1 ± 47.4	0.487
Carbohydrates (g)	199.2 ± 32.6	235.9 ± 19.1	194.7 ± 10.6	0.433
Dietary fibre (g)	12.8 ± 3.4	18.8 ± 2.9	7.5 ± 1.0	0.150
Total fat (g)	64.7 ± 8.5	65.2 ± 6.5	74.3 ± 9.0	0.666
Saturated	26.8 ± 3.7	28.9 ± 2.8	31.1 ± 4.2	0.703
Monounsaturated	20.1 ± 3.7	18.6 ± 2.0	24.7 ± 3.9	0.429
Polyunsaturated	9.2 ± 1.8	8.0 ± 1.1	9.8 ± 0.9	0.662
Trans fatty acids (g)	0.9 ± 0.2	0.7 ± 0.2	0.7 ± 0.2	0.785
Cholesterol (mg)	461.6 ± 139.7	257.4 ± 36.6	465.6 ± 87.8	0.256
Pro-vitamin A (RE)	313.5 ± 101.8	289.9 ± 41.5	311.2 ± 58.6	0.969
Vitamin A (RE)	449.9 ± 119.0	431.1 ± 81.2	452.2 ± 61.0	0.985
Vitamin B1 (mg)	1.7 ± 0.2	1.8 ± 0.2	2.3 ± 0.4	0.218
Vitamin B2 (mg)	1.9 ± 0.3	1.7 ± 0.2	2.2 ± 0.3	0.423
Vitamin B3 (mg)	22.8 ± 7.7	18.4 ± 2.0	23.3 ± 3.5	0.770
Vitamin B6 (mg)	1.2 ± 0.4	1.0 ± 0.1	1.2 ± 0.2	0.807
Vitamin B12 (μg)	5.3 ± 1.3	5.7 ± 2.1	4.1 ± 0.6	0.778
Vitamin C (mg)	3.4 ± 0.7	2.9 ± 0.7	3.7 ± 1.0	0.737
Vitamin E (mg)	4.9 ± 0.5	5.2 ± 0.9	6.0 ± 0.4	0.573
Folate (μg)	285.0 ± 44.5	277.0 ± 45.0	312.5 ± 54.8	0.875
Calcium (mg)	971.1 ± 181.9	1013.0 ± 177.0	1059.4 ± 143.9	0.942
Magnesium (mg)	242.2 ± 35.6	286.6 ± 27.4	209.3 ± 13.6	0.210
Phosphorus (mg)	1522.3 ± 188.8	1551.6 ± 159.1	1565.8 ± 140.7	0.983
Potassium (mg)	1658.2 ± 167.8	1630.9 ± 153.5	1795.5 ± 236.9	0.810
Selenium (μg)	103.5 ± 18.0	106.4 ± 13.6	134.9 ± 17.9	0.393
Sodium (mg)	2196.1 ± 257.2	2096.3 ± 220.3	2496.5 ± 368.0	0.602
Zinc (mg)	11.0 ± 1.6	10.6 ± 1.0	13.3 ± 2.2	0.483
Polyphenols (mg)	25.2 ± 6.4	30.3 ± 4.8	16.9 ± 4.5	0.273

Results expressed as mean ± SEM.
[A] Data analysed by one-way analysis of variance (ANOVA) for mean comparisons.

Table 3
Tomato phenolic metabolites searched in plasma and urine.

	MRM (*m*/*z*)	DP (V)	FP (V)	EP (V)	CE (V)
Caffeic acid	179 → 135 (107)	−50	−220	−10	−25
Caffeic acid glucuronide	355 → 179	−50	−220	−10	−25
Dihydrocaffeic acid	181 → 137 (119)	−50	−220	−10	−25
Dihydrocaffeic acid glucuronide	357 → 181 (175, 137, 113)	−50	−220	−10	−25
Dihydrocaffeic acid sulphate	261 → 181 (137, 119)	−50	−220	−10	−25
Chlorogenic acid	353 → 191 (179)	−50	−220	−10	−25
Ferulic acid	193 → 134	−60	−200	−10	−20
Ferulic acid glucuronide	369 → 193	−60	−200	−10	−20
Dihydroferulic acid	195 → 136 (151, 177, 135, 123, 119)	−60	−200	−10	−20
Dihydroferulic acid glucuronide	371 → 195 (175, 113)	−60	−200	−10	−20
Dihydroferulic acid sulphate	275 → 195 (177, 151, 136, 135, 123, 119)	−60	−200	−10	−20
Naringenin	271 → 151 (119)	−80	−200	−10	−25
Naringenin glucuronide	447 → 271	−30	−200	−10	−25
Kaempferol	285 → 151 (257)	−90	−200	−10	−25
Kaempferol glucuronide	461 → 285	−90	−200	−10	−25
Quercetin	301 → 151 (179)	−60	−200	−10	−30
Methylquercetin	315 → 301	−30	−200	−10	−30
Quercetin glucuronide	477 → 301	−30	−200	−10	−30
Ethylgallate (IS)	197 → 169 (124)	−60	−200	−10	−25

MRM (multiple reaction monitoring); DP (declustering potential); FP (focusing potential); EP (entrance potential); CE (collision energy).

an ion at 179 *m*/*z*, corresponding to caffeic acid, was encountered, as well as the typical fragment of a glucuronide unit (175 *m*/*z* and 113 *m*/*z* ions). Finally, two metabolites of dihydroferulic acid were identified in urine samples: dihydroferulic acid glucuronide (Fig. 1D) and dihydroferulic acid sulphate (Fig. 1E). As in the above compounds, the glucuronide unit of dihydroferulic acid glucuronide was identified with the presence of ions at 175 *m*/*z* and 113 *m*/*z*. The mass spectrum also showed the presence of dihydroferulic acid with an ion at 195 *m*/*z*. Finally, dihydroferulic acid sulphate was confirmed with the loss of 80 *m*/*z* (Rubió et al., 2012), obtaining dihydroferulic acid (195 *m*/*z*). The presence of an ion at 136 *m*/*z* confirmed the presence of dihydroferulic acid, being characteristic of the fragmentation pattern of this compound.

3.3. Pharmacokinetics in plasma after the three interventions

Table 4 shows the pharmacokinetic parameters of naringenin, naringenin glucuronide, caffeic acid glucuronide, and ferulic acid glucuronide detected in plasma after the three dietary interventions.

No statistically significant differences among interventions were observed in the pharmacokinetic parameters for caffeic acid

Table 4
Pharmacokinetic parameters in plasma after the three interventions.

Compound	Intervention	C_{max} (nmol/L)	t_{max} (min)	AUC_{last} (nmol/L x min)	MRT_{last} (min)	C_{max}/AUC_{last}
Naringenin	Tomato	n.d[b]	n.d[b]	n.d[b]	n.d[b]	n.d
	OF	11.9 ± 2.7[a]	18.8 ± 3.8[a]	979 ± 360[a,b]	62.4 ± 13.9[a]	0.0157 ± 0.0039
	ROOE	12.8 ± 3.1[a]	45.0 ± 8.7[a]	1459 ± 159[a]	58.5 ± 11.0[a]	0.0140 ± 0.0048
Naringenin glucuronide	Tomato	17.4 ± 3.8[b]	130.0 ± 60.8	5330 ± 1105[b]	221.7 ± 16.4[a]	0.0033 ± 0.0001[c]
	OF	427.9 ± 59.0[a]	38.6 ± 5.5	49,783 ± 7843[a]	102.5 ± 5.7[c]	0.0085 ± 0.0002[a]
	ROOE	353.1 ± 55.3[a]	54.0 ± 6.0	58,642 ± 12,126[a]	147.5 ± 15.5[b]	0.0059 ± 0.0006[b]
Caffeic acid glucuronide	Tomato	6.2 ± 1.5	100.0 ± 40.0	1141 ± 56	160.9 ± 26.1	0.0056 ± 0.0017
	OF	5.0 ± 2.7	150.0 ± 30.0	835 ± 138	162.6 ± 48.1	0.0056 ± 0.0023
	ROOE	4.1 ± 0.5	90.0 ± 30.0	689 ± 122.8	139.6 ± 2.6	0.0060 ± 0.0003
Ferulic acid glucuronide	Tomato	389.0 ± 82.6	183.7 ± 67.7	134,631 ± 32,101	276.2 ± 47.6	0.0034 ± 0.0004
	OF	343.9 ± 77.6	180.0 ± 66.1	184,139 ± 34,258	453.7 ± 83.3	0.0024 ± 0.0006
	ROOE	351.5 ± 72.0	385.0 ± 220.5	139,145 ± 46,811	351.6 ± 100.2	0.0041 ± 0.0014

Results expressed as mean ± SEM.
Values in a column with different letters are significantly different ($p < 0.05$).

Table 5
$Q_{u\infty}$ in urine after the three interventions.

Compound	Intervention	$Q_{u\infty}$ (nmol)
Naringenin	Tomato	9.0 ± 1.4
	OF	141.1 ± 38.6
	ROOE	107.4 ± 20.3
Naringenin glucuronide	Tomato	490.5 ± 116.5[b]
	OF	5127.3 ± 1331.5[a]
	ROOE	4063.9 ± 1427.0[a,b]
Caffeic acid	Tomato	379.7 ± 122.1
	OF	144.5 ± 50.7
	ROOE	195.7 ± 46.3
Caffeic acid glucuronide	Tomato	269.0 ± 89.7
	OF	223.3 ± 90.9
	ROOE	118.8 ± 20.2
Ferulic acid	Tomato	2055.3 ± 1124.9
	OF	1106.2 ± 705.2
	ROOE	1413.7 ± 673.0
Ferulic acid glucuronide	Tomato	25008.6 ± 9778.3
	OF	26580.9 ± 10911.7
	ROOE	8272.2 ± 2170.3
Chlorogenic acid	Tomato	66.8 ± 26.2
	OF	62.6 ± 20.3
	ROOE	20.2 ± 7.2
Dihydrocaffeic acid	Tomato	893.8 ± 152.8
	OF	1277.3 ± 496.0
	ROOE	997.3 ± 347.6
Dihydroferulic acid	Tomato	8878.2 ± 2902.2
	OF	7447.1 ± 1953.0
	ROOE	5103.6 ± 1654.4
Dihydroferulic acid glucuronide	Tomato	15316.2 ± 4007.5
	OF	13174.1 ± 3691.8
	ROOE	9838.3 ± 2422.3
Dihydroferulic acid sulphate	Tomato	194149.3 ± 90537.3[a]
	OF	133132.0 ± 50062.6[a,b]
	ROOE	78169.2 ± 34516.4[b]

Results expressed as mean ± SEM.
Values in a column with different letters are significantly different ($p < 0.05$).

glucuronide and ferulic acid glucuronide, while significant differences were observed for naringenin and naringenin glucuronide in almost all the parameters.

In plasma, naringenin concentration was below the limit of detection in each volunteer after the tomato intervention (n.d.), while it was detectable and quantificable after intake of both sauces. When comparing pharmacokinetic parameters of naringenin in tomato and the two sauces (with and without oil), significant differences were found in C_{max}, t_{max}, AUC_{last}, and MRT_{last}, but not in C_{max}/AUC_{last}. This result was partially in accordance with (Bugianesi et al., 2004), who previously evaluated the effect of domestic cooking on the bioavailability of antioxidant molecules in humans after the administration of a test meal containing cherry tomatoes. Since naringenin strongly interacts with insoluble polyesters, which are constituents of tomato peel fibre (Hunt & Baker, 1980), mechanical and thermal processing could facilitate the release of the compound from the matrix and thus increase its bioaccessibility (Bugianesi et al., 2004). These results also agree with the values shown in Table 1.

One of the most abundant derived metabolites found in plasma was naringenin glucuronide. When comparing naringenin glucuronide and naringenin, independently of the intervention, the glucuronide form was clearly more prevalent than the aglycone. These findings may suggest a rapid absorption of naringenin (t_{max} 18.8 ± 3.8 min OF, 45.0 ± 8.7 min ROOE) and a high first-pass metabolism to this phase II conjugate (Ma et al., 2006). Significant differences in C_{max} and AUC_{last} of naringenin glucuronide were found between raw tomatoes and sauces, once again corroborating the influence of mechanical and thermal processing in the higher absorption of polyphenols found in tomato skin, such as naringenin (Bugianesi et al., 2004). Moreover, the slower rate of appearance of this metabolite in plasma after ingestion of raw tomatoes compared to sauces (t_{max}, C_{max}/AUC_{last}) suggests a slower rate of absorption of naringenin from the former.

Fig. 2 shows the mean plasma concentration *versus* time of naringenin (A) and naringenin glucuronide (B). Both compounds presented a biphasic profile in the sauce intervention (OF and ROOE), but not in tomato, probably due to low plasma levels. Ma et al. (2006) describe that glucuronidation takes place both in the intestine wall and the liver, catalysed by the enzyme UDP-glucuronosyl transferase. The glucuronide form excreted in the intestine could be re-absorbed by enterohepatic circulation (Ma et al., 2006). In the case of naringenin, the biphasic profile appeared more significant in the ROOE sauce. Since naringenin is a relatively lipophilic compound (log partition coefficient 2.52–2.84) (Chabane, Ahmad, Peluso, Muller, & Ubeaud-Séquier, 2009), the co-presence of a fat matrix in the small intestine may increase its bioavailability by favouring its solubilisation – a previous step to its absorption by passive diffusion- and absorption through the intestinal epithelium. However, in our study this possibility was not reflected in the pharmacokinetic parameters (C_{max}, AUC_{last}), probably due to the low amount of oil added and low number of volunteers.

3.4. Phenolic metabolites in urine after the three interventions

Table 5 presents the maximum amount excreted in the 24 h urine collection ($Q_{u\infty}$) of the eleven phenolic compounds and metabolites

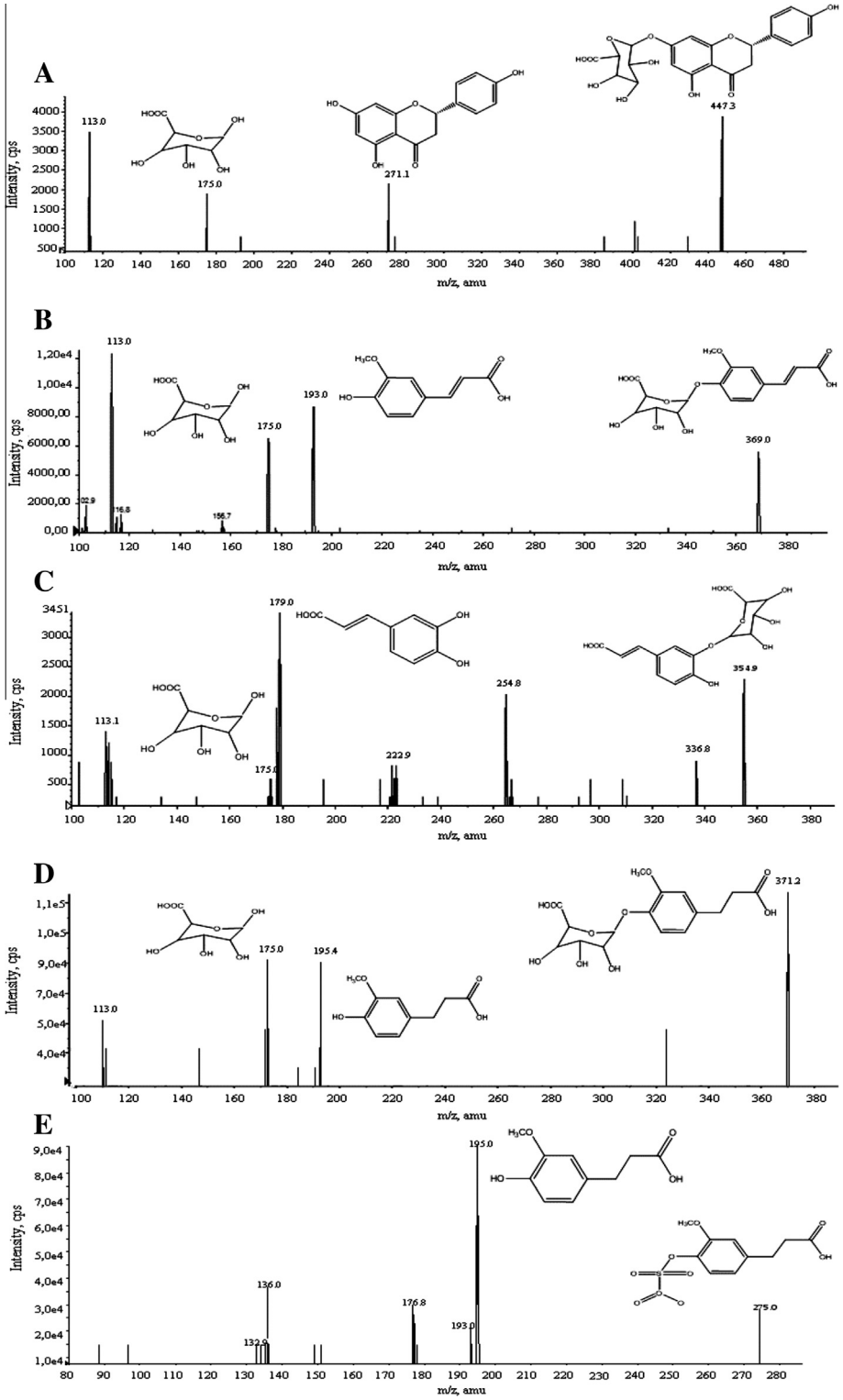

Fig. 1. Product ion scan of the main metabolites found in urine: naringenin glucuronide (A), ferulic acid glucuronide (B), caffeic acid glucuronide (C), dihydroferulic acid glucuronide (D), and dihydroferulic acid sulphate (E).

identified after the three interventions. Only 2 compounds showed significant differences among the three interventions, namely naringenin glucuronide, and dihydroferulic acid sulphate.

Naringenin glucuronide presented a higher $Q_{u\infty}$ after intake of ROOE and OF sauces than raw tomatoes, while the reverse was observed with dihydroferulic acid sulphate. The urinary data were in agreement with the plasma results, confirming the influence of mechanical and thermal processing in the bioavailability of tomato phenolic metabolites. The $Q_{u\infty}$ of naringenin glucuronide after sauce intake was also higher than that of naringenin, which supports the rapid metabolism and excretion of this compound after ingestion.

Chlorogenic acid, found in the plasma of only one volunteer, was excreted unchanged in urine at levels indicative of its absorption

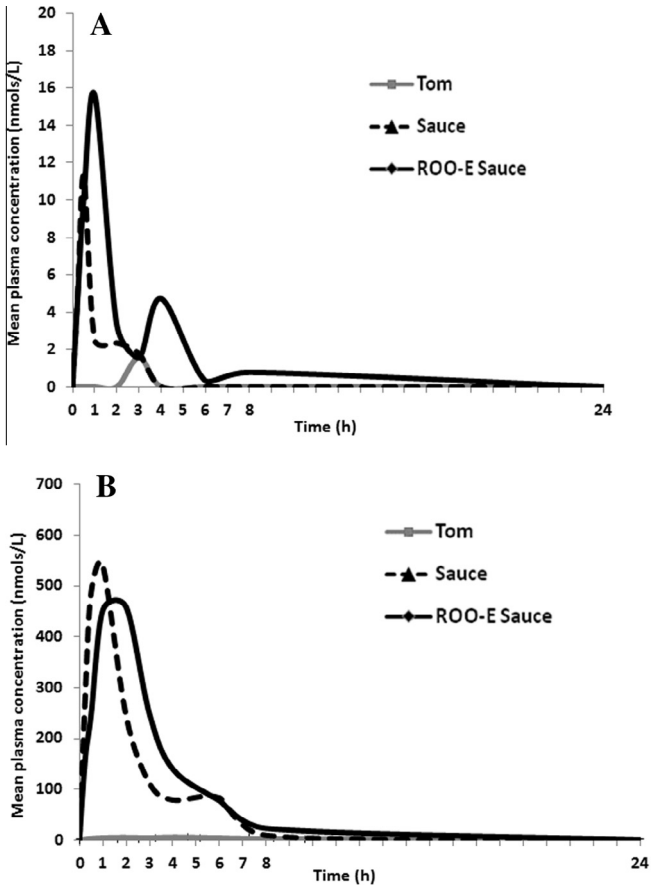

Fig. 2. Mean plasma concentration *versus* time of naringenin (A) and naringenin glucuronide (B) from the three interventions.

after oral administration, which is indicative of its absorption after oral administration. (Lafay, Gil-Izquierdo, et al., 2006; Lafay, Morand, Manach, Besson, & Scalbert, 2006) described its absorption in rats in the intestine (8%) and stomach (16.3%), probably favoured in the latter by the presence of a greater percentage of the un-ionised compound at the acidic pH of the stomach. Absence of chlorogenic acid in the plasma of almost all the volunteers could be explained by a too rapid transit in the organism of the fraction of dose absorbed. (Olthof, Hollman, & Katan, 2001) described absorption of 33 ± 17% of chlorogenic acid in humans after oral ingestion of 2.8 mmol. Taking this percentage as a reference, and considering the mean administered dose was 1922.7 nmol, a small part of the dose absorbed was excreted in urine intact (60 nmol, approximately) and the rest extensively metabolised to caffeic acid, probably in the colon due to microbiota activity (Lafay, Gil-Izquierdo, et al., 2006). The fraction of unabsorbed chlorogenic acid will remain in the intestinal lumen and could provide local biological effects in the intestine.

Ferulic acid, despite having a partition coefficient unfavourable to its permeability through the intestinal epithelium ($P = 0.375$ at pH 3 and $P = 0.489$ at pH 10, (Sohn & Oh, 2003)), presented oral absorption, judging by the levels excreted in urine. Due to its low molecular weight (194 g mol^{-1}) and high hydrophilicity, the mechanism of absorption was probably the paracellular pathway described for other active ingredients with similar characteristics (Rowland & Tozer, 2011). These results are in agreement with Zhao, Egashira, & Sanada, 2004 (Zhao et al., 2004), who showed a percentage of absorption of 74 ± 11% in rat stomach, and (Poquet, Clifford, & Williamson, 2008), the permeation of free ferulic acid by passive diffusion in an *in vitro* model of colonic epithelium. Moreover, since the contribution of ferulic acid to the organism from the administered raw tomato and tomato sauces could arise from different sources (ferulic acid hexose, ferulic acid, chlorogenic acid and caffeic acid metabolism), this could result in quite high and variable plasma levels of ferulic acid glucuronide (see AUClast, Table 4). The variability could also be attributed to the influence of the intestinal microbiota in the hydrolysis of ferulic acid hexose and metabolism of chlorogenic acid to caffeic acid and conversion to ferulic acid.

Regarding caffeic acid, its low molecular weight (180 g mol^{-1}) and partition coefficient ($\log P = 1.2$) facilitate its absorption by passive diffusion through the membrane. (Olthof et al., 2001) described an absorption percentage of 95 ± 4% in humans. In our study we found caffeic acid excreted in urine but none in plasma. The amounts were higher than those of chlorogenic acid, reflecting the greater percentage absorbed, but low considering the amount administered, thus suggesting a high intestinal and/or hepatic first-pass effect.

High amounts of microbiota metabolites (dihydrocaffeic acid, dihydroferulic acid) were excreted in urine, probably due to the unabsorbed chlorogenic acid in the stomach.

Finally, potential gender-dependent differences in $Q_{u\infty}$ for urine and C_{max}, AUC_{last} and C_{max}/AUC_{last} for plasma of naringenin and naringenin glucuronide were tested, within the same intervention and inter-interventions (Table 6). No significant gender-dependent differences were found within the same intervention in either plasma or urine.

Analysing men ($n = 4$) and women ($n = 4$) separately, more significant differences among interventions in flavanone bioavailability in plasma were observed in men than in women. In male volunteers, significant differences were observed in C_{max}, AUC_{last}

Table 6
Pharmacokinetic parameters in plasma (C_{max}, AUC_{last} and C_{max}/AUC_{last}) and in urine ($Q_{u\infty}$) after the three interventions differentiating men and women.

	Compound	Intervention	C_{max} (nmol/L)	AUC_{last} (nmol/L x min)	C_{max}/AUC_{last}	$Q_{u\infty}$ (nmol)
Men	Naringenin	Tomato	n.d^{b}	n.d	n.d	7.6 ± 0.0
		OF	9.8 ± 2.4^{a,b}	704.6 ± 328.1	0.0176 ± 0.0049	230.4 ± 87.5
		ROOE	9.8 ± 1.0^{a}	1626.5 ± 299.9	0.0133 ± 0.0067	167.4 ± 0.0
	Naringenin glucuronide	Tomato	19.9 ± 4.9^{b}	6185.2 ± 1210.5^{b}	0.0032 ± 0.0002^{b}	314.7 ± 172.2
		OF	460.5 ± 72.1^{a}	53104.6 ± 9363.5^{a,b}	0.0088 ± 0.0002^{a}	7788.0 ± 1832.8
		ROOE	438.0 ± 28.4^{a}	75945.5 ± 10638.4^{a}	0.0061 ± 0.0011^{a,b}	8294.5 ± 0.0
Women	Naringenin	Tomato	n.d	n.d	n.d	10.4 ± 0.0
		OF	18.1 ± 0.0	1803.6 ± 0.0	0.0100 ± 0.0000	96.4 ± 21.2
		ROOE	22.0 ± 0.0	1290.5 ± 76.1	0.0161 ± 0.0000	87.4 ± 4.9
	Naringenin glucuronide	Tomato	12.4 ± 0.0	3618.4 ± 0.0	0.0034 ± 0.0000	607.7 ± 134.9^{b}
		OF	384.5 ± 111.0	45353.5 ± 15428.2	0.0081 ± 0.0003	3796.9 ± 1450.1^{a,b}
		ROOE	225.8 ± 131.3	32685.6 ± 2827.5	0.0057 ± 0.0003	2653.7 ± 308.6^{a}

Results expressed as mean ± SEM.
Values in a column with different letters are significantly different ($p < 0.05$).

and C_{max}/AUC_{last} for naringenin glucuronide when comparing tomato with sauce intake, and for naringenin when comparing tomato and ROOE sauce. In the case of urine, naringenin glucuronide presented differences between tomato and ROOE sauce in women, while no differences were found in men. The observed sex-dependent differences may derive from both physiological (differences in body weight, body composition, steroidic hormones, and gastric motility) and molecular causes (differences in the activity or expression of transporters or enzymes involved in biotransformation) (Erlund, 2004). Therefore, further large-scale investigations are required to confirm these preliminary results.

The maximum excreted amount of administered naringenin and derivative compounds in the 24 h urine collection per dose was calculated. 912.1 ± 58.2 nmols of naringenin and naringenin glucoside in 500 g of tomato; 3185.3 ± 114.4 nmols in 250 g of OF sauce; and 3583.6 ± 203.5 nmols in 250 g of ROOE sauce were ingested by the volunteers. The $Q_{u\infty}$/dose obtained in the tomato intervention was 0.55, more than doubling in the sauces: 1.65 for OF and 1.16 for ROOE. Again, the results showed that mechanical and thermal treatments increased the bioavailability of the flavanone naringenin, although there were not significant differences probably due to the large interpersonal variability.

4. Conclusions

In conclusion, the present study confirmed that the mechanical and thermal treatments, as well as the addition of an oil matrix, during tomato sauce processing may increase the bioaccessibility, extractability and bioavailability of phenolics from tomato.

The flavanone naringenin and rutin were the major phenolic compounds in raw tomatoes and tomato sauces, followed by 5-caffeoylquinic acid, 4-caffeoylquinic acid and caffeic acid hexose. The addition of an oil matrix to the sauce (ROOE) did not result in significant differences, although the concentration of naringenin and 5-caffeoylquinic acid was greater in ROOE than OF.

Respectively, eleven and four phenolic compounds were quantified in urine and plasma samples after each intervention. No significant gender-dependent differences were observed within the same intervention in either plasma or urine. Naringenin, caffeic acid, ferulic acid and chlorogenic acid were absorbed in an intact form and were also extensively biotransformed into phase II metabolites.

In plasma and urine, the levels of naringenin glucuronide in sauces were higher than in raw tomato, confirming that food treatment increases the bioavailability of some phenolic compounds.

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4.4. Publicación 4. Desarrollo de un método por UHPLC-QqQ-MS para el análisis de compuestos fenólicos en tomates Cherry, salsa de tomate y zumo de tomate.

Set up of a UHPLC-QqQ-MS method for the analysis of phenolic compounds in cherry tomatoes, tomato sauce and tomato juice.

Giuseppe Di Lecce, **Miriam Martínez-Huélamo**, Sara Tulipani, Anna Vallverdú-Queralt and Rosa M. Lamuela-Raventós. *Journal of Agricultural and Food Chemistry*. 2013, 61(35):8373-8380.

HPLC acoplado a UV o MS es la técnica más habitual para la caracterización de los compuestos fenólicos tanto en alimentos como muestras biológicas. En los últimos años, el UHPLC está ganando relevancia en el análisis de este tipo de muestras, ya que presenta una óptima resolución, sensibilidad y menor tiempo de análisis. Por este motivo se decidió validar un método de extracción líquido-líquido para la determinación de compuestos fenólicos de tomates cherry, zumos y salsas de tomate analizados mediante UHPLC-QqQ-MS. Las principales ventajas del método proporcionan: una alta recuperación de los analitos (90.1 a 115%), bajos LODs (0.008-0.167 mg L^{-1}) y LOQs (0.01-0,83 mg L^{-1}), buena exactitud (85.6 a 115%) y precisión (<15%). La detección de los compuestos fenólicos varió de acuerdo a la naturaleza fisicoquímica de los extractos, a pesar de presentar en los tres alimentos un efecto matriz mínimo. Entre los compuestos fenólicos que se detectaron por separado se incluyen derivados de ácidos hidroxibenzoicos, ácidos hidroxicinámicos, flavonoles y flavanonas. La posibilidad de transferir fácilmente un método existente de HPLC a uno más rápido en UHPLC resulta muy atractiva, y con pequeñas modificaciones, la metodología descrita se puede aplicar a la caracterización fenólica de una amplia gama de alimentos.

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Setup of a UHPLC−QqQ-MS Method for the Analysis of Phenolic Compounds in Cherry Tomatoes, Tomato Sauce, and Tomato Juice

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ABSTRACT: The setup of a novel, rapid, and sensitive UHPLC−QqQ-MS method was described for the determination of phenolic compounds in tomatoes and tomato-based products (tomato sauce and juice). Phenolic compounds including hydroxybenzoic and hydroxycinnamic acid derivatives, flavonols, and flavanones were detected, separated, and quantified in a 3 min chromatographic run. The main advantages of the method include high analyte recovery (90.1−115%), low limit of detection (0.008−0.167 mg L^{-1}) and quantification (0.01−0.83 mg L^{-1}), good accuracy (85.6−115%), and precision (<15%). The detection of the phenolic compounds varied according to the physicochemical nature of the extracts, but generally low matrix-dependent suppression/enhancement effects were observed in all three matrices. The possibility to transfer easily the existing HPLC to the fast UHPLC methods is very attractive, and with minor modifications, the methodology described may be applied to the phenolic characterization of a broad range of plant and food matrices.

KEYWORDS: *tomato, tomato sauce, tomato juice, phenolic compounds, UHPLC−QqQ-MS, matrix effects*

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill. Solanaceae) is one of the most eaten foodstuffs in Mediterranean countries such as Spain and Italy. The extensive use of tomatoes and their processed products (fried or raw canned tomatoes, tomato sauce, tomato paste, tomato juice) as ingredients in prepared foods such as pizza, pasta, snacks, and a variety of vegetable dishes makes their consumption continuous grow worldwide.[1] Overwhelming evidence from nutritional studies indicates that regular consumption of raw tomatoes and tomato-based products is consistently associated with a reduction in the incidence of chronic-degenerative diseases.[2,3] Together with the most extensively studied bioactive compounds contained in tomato such as vitamins (vitamin C, folates) or carotenoids, the health-promoting effects of its (poly)phenolic constituents is attracting interest, including a potential role in lowering risk of cardiovascular diseases, metabolic alterations, and neurodegeneration, as well as in inhibiting cellular proliferation, modulating enzymatic activities, or signal transduction pathways.[4−11]

Phenolic acids are the most representative phenolic compounds found in tomato and processed products, including hydroxybenzoic (C6−C1) and hydroxycinnamic acids (C6−C3) and their ester conjugates, with chlorogenic acid being one of the most abundant.[12,13] A high content of the chalcone and flavanone forms of naringenin (reported as the most abundant phenolic compound in tomato sauce) and the flavonol rutin has also been described in tomato-based products.[13−16] Also more complex phenolic constituents have been described,[16−18] although quantification may be difficult due to the absence of pure reference standard.

High-performance liquid chromatography (HPLC) is the most commonly used technique for the characterization of the phenolic composition of several food matrices, generally coupled to photo diode array[19,20] or mass spectrometry detection.[18−22] Over the past few years, there has been great interest in developing ultra-HPLC (UHPLC) approaches to speed up and increase the resolving power of the analytical separation process. The UHPLC technology arises from the use of small particle size results in higher plate numbers, as well as faster separations, in accordance with the Van Deemter theory.[23] These effects are due to the fact that (a) the chromatographic efficiency is directly proportional to the ratio of column length and particle diameter, and (b) the mobile phase linear velocity is inversely proportional to the particle diameter.[24,25] Coupled to triple quadrupole mass spectrometry (UHPLC−QqQ-MS) it may offer a powerful tool for the quick and quantitative screening of a large number of phytochemical compounds through multiple reaction monitoring (MRM),[16,26,27] thus there is an interest in transferring previous methods performed in conventional HPLC conditions to UHPLC methods. However, it is crucial to verify the efficiency of the new chromatographic methods and evaluate the possible

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Table 1. Phenolic Compounds Analyzed in Cherry Tomatoes, Tomato Sauce, and Tomato Juice[a]

compounds	t_R (min)	$[M - H]^{-1}$	m/z ions	DP (V)	CE (V)	FP (V)	quantification transition
protocatechuic acid	0.61 ± 0.005	153	109	−40	−20	−150	153 → 109
4-hydroxybenzoic acid	0.83 ± 0.003	137	93	−40	−20	−200	137 → 93
chlorogenic acid	0.90 ± 0.010	353	191, 179	−50	−20	−180	353 → 191
caffeic acid	1.16 ± 0.006	179	135	−40	−20	−170	179 → 135
o-coumaric acid	1.53 ± 0.040	163	119	−40	−25	−150	163 → 119
ethyl gallate (IS)	1.61 ± 0.007	197	169	−60	−25	−200	197 → 169
rutin	1.68 ± 0.005	609	300	−60	−50	−230	609 → 300
ferulic acid	1.73 ± 0.010	193	178, 149, 134	−40	−20	−170	193 → 134
naringenin-7-*O*-glucoside	1.96 ± 0.009	433	271	−50	−30	−280	433 → 271
naringenin	2.13 ± 0.010	271	177, 151, 119	−50	−30	−190	271 → 151

[a] t_R, retention time; DP, declustering potential; CE, collision energy; FP, focusing potential.

matrix effects which may increase the level of random errors.[28,29] In the present work, the setup, validation, and application of a novel, rapid, and sensitive UHPLC−QqQ-MS method for the analysis of phenolic compounds (hydroxybenzoic and hydroxycinnamic acid derivatives, flavonols, and flavanones) in tomato fruits, tomato sauce, and tomato juice is reported.

MATERIALS AND METHODS

Standards and Solvents. The standards were handled without exposure to light. Protocatechuic acid, 3- and 4-hydroxybenzoic acid, chlorogenic acid (5-caffeoylquinic acid), gallic acid, caffeic acid, ferulic acid, *o*-, *m*-, and *p*-coumaric acids, naringenin-7-*O*-glucoside, naringin (naringenin-7-*O*-rhamnoglucoside), and rutin (quercetin-3-*O*-rhamnosyl-glucoside) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Naringenin (4′,5,7-trihydroxyflavanone), and kaempferol-3-*O*-glucoside were supplied by Extrasynthese (Genay, France). HPLC-grade acetonitrile, formic acid, and ethanol were purchased from ScharlauChemie S. A. (Barcelona, Spain), while ultrapure water (Milli-Q) was obtained from a Millipore system (Millipore, Bedford, MA, United States).

Commercial Samples. Unprocessed tomato fruits and commercial tomato products were purchased from a local market (Barcelona, Spain). Cherry-type tomatoes were selected to represent the commonly consumed unprocessed tomatoes,[30] whereas tomato sauce and juice were chosen as representative tomato processed products due to their wide consumption worldwide and the different physicochemical properties of the final products (thus matrix effects), as a consequence of the different manufacturing process (i.e., tomato juice contains about 90% of water and is an intermediate product in the making process of the sauce).[31]

Preparation of Standard and Stock Solutions. Individual stock solutions of the standard phenolic compounds (protocatechuic, 4-hydroxybenzoic, chlorogenic, caffeic, ferulic and *o*-coumaric acid, naringenin-7-*O*-glucoside, naringenin, and rutin) were prepared at a concentration of 1 mg mL^{-1} in 80% methanol. A phenolic pool obtained by mixing the individual standard solutions with acidified water (0.1% formic acid) was used as working solution to spike the cherry tomatoes, tomato sauce, and juice extracts. A solution of ethyl gallate at a final concentration of 400 ng mL^{-1} was prepared in acidified water (0.1% formic acid) and used as an internal standard (IS) to check for the extraction efficiency. All standard solutions were stored at −80 °C.

Sample Extraction. Sample extraction was performed in a darkroom with a red safety light as previously described by our research group,[32,33] with some modifications. Aliquots of cherry tomatoes, tomato juice, and tomato sauce (0.3 g each) were homogenized with a blender over an ice bed with 3 mL of 80% ethanol in acidified Milli-Q water (0.1% formic acid), after the addition of the IS and, eventually, of the phenolic standard pool. The homogenate was sonicated for 5 min and centrifuged at 900*g* for 20 min at 4 °C. The supernatant was collected, and the extraction procedure was repeated. Both supernatants were combined and evaporated to dryness on a sample concentrator (Techne, Duxford, Cambridge, U.K.) at room temperature under a stream of nitrogen gas. The residues were reconstituted up to 1.2 mL of water contained 0.1% formic acid, filtered thought a 0.22 μm polytetrafluoroethylene (PTFE) syringe filters (Waters Corporation, United States), and injected into a UHPLC−QqQ system.

UHPLC System. The UHPLC system consisted of an Acquity UHPLC equipped with a Waters binary pump system (Milford, MA, United States), and a Waters BEH C_{18} column (50 mm × 2.1 mm) packed with 1.7 μm particles. The samples were injected into a 10 μL loop, with a mobile-phase flow rate of 400 μL min^{-1}. Gradient elution was carried out with a solvent system of water/formic acid (99.9:0.1 v/v) as mobile phase A and acetonitrile/formic acid (99.9:0.1 v/v) as mobile phase B; the total run time was 3 min, and the gradient elution was as follows: 0.0−1.1 min, B 5−18%; 1.1−1.8 min, B 18−50%; 1.8−2.4 min, B 50−74%; 2.4−2.5 min, B 74−100%; 2.5−2.7 min, B 100−5%; 2.7−3.0 min, B 5%. All the solvents were filtered through 0.22 μm PTFE filters (Waters Corporation, Milford, MA, United States) prior to use. The column was maintained at 30 °C while the autosampler was thermostated at 4 °C. The system was controlled by Analyst v. 1.4.2 software supplied by Applied Biosystems (Foster City, CA, United States).

Mass Spectrometry Conditions. The UHPLC was coupled online with an API 3000 (ABSciex, Concord, Ontario, Canada) triple quadrupole mass spectrometer equipped with a TurboIonSpray source in negative-ion mode to obtain MS/MS data. TurboIonSpray source settings were as follows: capillary voltage −3500 V; nebulizer gas (N_2) 10 (arbitrary units); curtain gas (N_2) 12 (arbitrary units); collision gas (N_2) 4 (arbitrary units); focusing potential −200 V; entrance potential 10 V; drying gas (N_2) heated to 400 °C and introduced to a flow rate of 8000 cm^3 min^{-1}. The declustering potential (DP), collision energy (CE), and focusing potential (FP) were optimized for each compound by infusion experiments of individual standard solutions (10 μg μL^{-1}) dissolved in a 50:50 (v/v) mobile phase at a constant flow rate of 5 μL min^{-1}, using a model syringe pump (Harvard Apparatus, Holliston, MA, United States). Full-scan data acquisition was performed scanning from m/z 100 to 800 in profile mode and using a cycle time of 2 s with a step size of 0.1 u and a pause between each scan of 0.002 s; dwell time was set at 0.016 s. To confirm the identity of some compounds, neutral loss scan and product ion scan experiments were carried out. MS/MS product ions were produced by collision-activated dissociation (CAD) of selected precursor ions in the collision cell of the QqQ mass spectrometer and mass analyzed using the instrument's second analyzer. Additional experimental conditions for MS/MS included collision energy (depending on the compound), CAD gas (nitrogen) at 6 (arbitrary units), and scan range, as necessary for the precursor selected. Neutral loss scan of 162 u was done by scanning within the m/z range from 200 to 800 u, and product ion scan experiments were carried out by scanning within 300 and 800 u. In all the experiments, both quadrupoles (Q1 and Q3) were operated at unit resolution.

The phenolic compounds present in cherry tomatoes and in the tomato-based products were detected and quantified by using the

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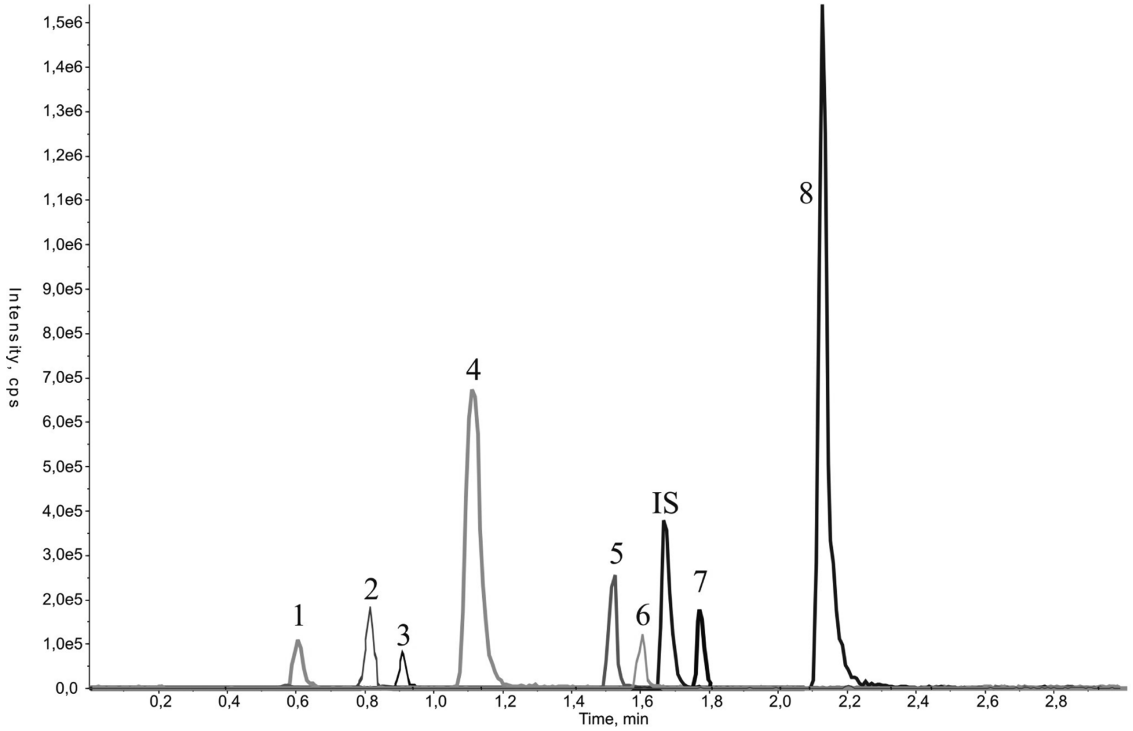

Figure 1. UHPLC chromatogram of spiked and with internal standard tomato juice extract. Peaks: (1) protocatechuic acid, (2) 4-hydroxybenzoic acid, (3) chlorogenic acid, (4) caffeic acid, (5) *o*-coumaric acid, (IS) ethyl gallate, (6) rutin, (7) ferulic acid, and (8) naringenin.

multiple reaction monitoring mode (MRM), tracking the transition of parent and product ions specific to each compound (Table 1).

Method Validation. Quantitative analysis was performed by means of the standard-addition method.[34] In this way, besides estimating the unknown amount of polyphenols occurring in the extracts, it was possible to evaluate sensitivity and linear dynamic range in the different matrices. Recoveries, precision, limits of detection (LODs), and limits of quantitation (LOQs) were calculated after having determined the natural levels of each phenolic compound in the each tomato-based product.

For calibration curve preparation (seven points in duplicate) six of the seven aliquots of tomato, juice, and sauce samples were spiked with different concentrations (50%, 100%, 150%, 250%, 300%, and 400%) of the phenolic standard pool prior to extraction, while the remaining nonspiked aliquot was added with the corresponding volume of Milli-Q water, to maintain the same dilution factor across the samples.

The sensitivity of the method was evaluated by determining the LODs and LOQs. The LOD was calculated as the quantity of analyte able to produce a chromatographic peak three times higher than the noise of the baseline in a chromatogram (S/N = 3) of a nonspiked sample, after having estimated the endogenous amount. The LOQ was set at ten times higher than the noise of the baseline in a chromatogram (S/N = 10). Five replicates were carried out for LOD and LOQ determination.

For analyte recoveries, seven-point calibration curves (0%, 50%, 100%, 150%, 250%, 300%, and 400%) were prepared by spiking tomato and tomato based products (tomato juice and tomato sauce) after and before extraction. Analyte recoveries were determined by subtracting the response obtained from the analyte added and extracted from the matrix, compared to the detector response obtained for the same concentration of the standard added after the extraction. To assess intraday precision and accuracy, six replicates of cherry tomato, tomato sauce, and tomato juice extracts spiked at three different concentration levels (50%, 150%, 300%) were prepared, extracted, and analyzed by UHPLC−QqQ-MS. The procedure was repeated on three different days to determine interday precision. Spiked samples used to assess intra- and interday precision were prepared daily. Accuracy was calculated as the ratio of the mean observed concentration and the known spiked concentration in the matrix and was expressed as [(mean observed concentration)/(added concentration)] × 100. Precision is expressed as the relative standard deviation (% RSD) of all determinations.

Finally, suppression/enhancement effects were also evaluated by calculating the ratio of the peak area of the analytes recorded for the sample spiked with the standards after extraction and the peak area of analytes recorded for the standard solution (expressed in percentage). The described method was fully validated following the criteria of the AOAC International for method validation.[34]

Statistics. The concentration of the phenolic compounds quantified in the extracts was expressed as mean ± SD (μg g^{-1} FW). One-way analysis of variance for mean comparison (SPSS software, Version 17.0 Japan Inc., Tokyo, Japan) was used to assess the observed differences in phenolic content among technical replicates and food matrices. Differences were considered to be statistically significant when the p-value was <0.05.

RESULTS AND DISCUSSION

Until now, HPLC−MS/MS-based methods have been mainly used to study the levels of phenolics in different varieties of tomato,[35] diced tomatoes, and tomato sauces.[36] It was also used to evaluate the effects of storage on phenolic compounds[36] and the effects of pulsed electric fields on tomato polyphenols.[37] The method presented and validated in this work allows quantification of the main phenolic compounds present in tomatoes and tomato-based products (hydroxybenzoic and hydroxycinnamic acid derivatives, flavonols, and flavanones) in a total run time of 3.0 min (Figure 1), thus proposing a useful alternative to conventional HPLC−MS/MS profiling in terms of analysis time, costs, and improved resolution and sensitivity.[1,38,39]

A preliminary screening of the main phenolic compounds present in the dietary matrices in the study was assessed (data not shown). Then, the optimization of the method achieved the best chromatographic (gradient, flow rate, injection volume,

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Table 2. Validation Parameters[a]

compounds	regression eq	linear fit	concn range (mg L^{-1})	LODs (mg L^{-1})	LOQs (mg L^{-1})	recovery (%)
			Cherry Tomato			
protocatechuic acid	$y = 2.1437x + 0.2930$	0.9926	0.04−0.40	0.008	0.020	98.10 ± 7.60
chlorogenic acid	$y = 1.0833x + 1.6091$	0.9957	1.50−12.00	0.045	0.140	99.10 ± 8.70
caffeic acid	$y = 3.1861x + 4.3740$	0.9935	0.50−4.00	0.098	0.390	90.10 ± 3.90
o-coumaric acid	$y = 0.9943x + 1.9596$	0.9901	0.50−4.00	0.034	0.300	96.50 ± 9.80
rutin	$y = 0.1001x + 12.998$	0.9901	14.00−210.00	0.122	0.980	98.60 ± 12.00
ferulic acid	$y = 0.0311x + 0.0724$	0.9907	0.80−6.40	0.129	0.460	110.00 ± 1.90
naringenin-7-*O*-glucoside	$y = 2.4943x + 0.1338$	0.9928	0.10−10.00	0.010	0.080	99.40 ± 10.00
naringenin	$y = 0.4051x + 23.568$	0.9902	12.4−99.20	0.072	0.590	97.80 ± 4.10
			Tomato Sauce			
protocatechuic acid	$y = 1.0273x + 0.5232$	0.9944	0.12−0.96	0.027	0.100	113.00 ± 8.40
chlorogenic acid	$y = 0.6282x + 3.7147$	0.9926	3.80−30.40	0.078	0.260	95.90 ± 13.00
caffeic acid	$y = 1.7994x + 5.2278$	0.9918	0.70−5.60	0.056	0.180	101.00 ± 6.60
o-coumaric acid	$y = 1.0255x + 0.8993$	0.9938	0.50−4.00	0.020	0.300	95.30 ± 14.00
rutin	$y = 0.0820x + 9.3400$	0.9908	11.50 −192.00	0.150	0.830	108.00 ± 12.00
naringenin-7-*O*-glucoside	$y = 0.6687x + 3.3727$	0.9940	1.40−11.20	0.085	0.580	99.20 ± 10.00
naringenin	$y = 0.2047x + 26.325$	0.9915	20.00−220.00	0.154	0.640	105.50 ± 13.00
			Tomato Juice			
protocatechuic acid	$y = 2.2568x + 0.1449$	0.9927	0.20−1.80	0.021	0.120	98.20 ± 3.20
4-hydroxybenzoic acid	$y = 0.5833x + 2.5876$	0.9972	0.30−2.40	0.083	0.260	100.00 ± 6.10
chlorogenic acid	$y = 0.6934x + 0.0605$	0.9900	0.08−0.80	0.031	0.050	101.00 ± 5.60
caffeic acid	$y = 2.5983x + 4.3194$	0.9951	0.40−3.20	0.167	0.310	101.00 ± 6.30
o-coumaric acid	$y = 0.2520x + 0.1709$	0.9922	0.50−10.00	0.087	0.340	99.30 ± 6.20
rutin	$y = 0.2766x + 1.0627$	0.9952	1.50−12.00	0.136	0.650	101.00 ± 0.20
ferulic acid	$y = 0.0345x + 0.0847$	0.9971	1.50−12.00	0.108	0.490	104.00 ± 8.60
naringenin	$y = 0.8636x + 3.7910$	0.9915	1.20−9.60	0.012	0.110	91.40 ± 10.00

[a]Regression equation, linear fit, concentration range, limits of detection (LODs) and quantification (LOQs), and recovery (%) in cherry tomatoes, sauce, and juice extracts.

etc.) and MS conditions parameters (ESI mode, DP, CE, FP, MRM quantitative transition, cycle time, dwell time) for the targeted compounds that were selected during the method optimization phase (Table 1). Finally, the performance validation of the method was carried out by evaluating the quality parameters established for the method validation: linearity, LODs, LOQs, recovery, selectivity, accuracy, precision, and matrix effect (Table 2 and Table 3).

Validation parameters. Linearity and Limits of Detection and Quantification. The linearity of the method was evaluated by analyzing six calibration standards in duplicate over the nominal concentration range (Table 2). A good linearity was obtained, and all correlation coefficients exceeded 0.99. Slopes, which are representative of the method sensitivity, resulted very similar in the different matrices; this suggests that the applied extraction procedure cleaned up the extracts from the various matrices analogously, producing a comparable matrix effect.

The LOD and LOQ values obtained showed a wide range of sensitivity among the different analytes (Table 2). LODs ranged from 0.008 mg L^{-1} to 0.129 mg L^{-1} in cherry tomato, 0.020 to 0.154 mg L^{-1} in tomato sauce, and 0.012 to 0.167 mg L^{-1} in tomato juice. The LOQs varied between 0.02 and 0.98 mg L^{-1} in cherry tomato, 0.10 and 0.83 mg L^{-1} in sauce, and 0.01 and 0.65 mg L^{-1} in juice. The sensitivity of this method represents a significant improvement for most of the analytes when compared to published LC−MS methods in tomatoes and tomato-based products.[14,40,41] Gómez-Romero et al.[40] reported LODs between 0.03 and 1.50 mg L^{-1} and Vallverdú-Queralt et al.[41] higher than 0.0475 mg L^{-1}. The LOD and LOQ obtained are in agreement with data recently proposed in the analysis of low molecular weight polyphenols in several vegetables.[42] Thus, this method offers a strong alternative to conventional HPLC−MS/MS in terms of improved resolution and sensitivity.

Recovery, Accuracy, Precision, and Matrix Effects. The results showed comparable levels of recovery in spiked cherry tomato, and tomato sauce and juice extracts, obtaining values higher than 95% except for caffeic acid, which had recoveries between 90.1 and 94.5% in cherry tomato and juice extracts, respectively. On the other hand, high values of recovery were obtained for protocatechuic acid in tomato sauce extracts (113 ± 8.4) and ferulic acid in cherry tomato (110 ± 1.9) and juice extracts (115 ± 14), respectively (Table 2). Recovery values resulted independent by the applied fortification level. These values are similar to those reported by other authors in tomatoes and tomatoes based products.[13,41]

The precision of the method, expressed as % RSD, met acceptance criteria, since % RSD was lower than 15% at each tested concentration level for intra- and interday precision. These results are in agreement with those reported in a study carried out by Vallverdú-Queralt et al., who reported levels of % RSD lower than 15% in raw tomatoes.[41] The accuracy of phenolic compounds for tomatoes, tomato sauce, and tomato juice ranged between 85.6% and 113%, between 82.8% and 115%, and between 86% and 113%, respectively. These values were acceptable for the low, medium, and high concentration levels (Table 3).

In the evaluation of matrix effects, values lower than 100% indicate that matrix-dependent signal suppression occurs, while values higher than 100% indicate matrix-dependent signal enhancement.[43] The eventual suppression and enhancement

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Table 3. Cherry Tomato and Tomato-Based Product Validation: Accuracy and Intraday and Interday Precision[a]

	low concentration (mg L^{-1})						medium concentration (mg L^{-1})						high concentration (mg L^{-1})					
			intraday (% RSD)						intraday (% RSD)						intraday (% RSD)			
analyte	added	accuracy (%)	1	2	3	interday (% RSD)	added	accuracy (%)	1	2	3	interday (% RSD)	added	accuracy (%)	1	2	3	interday (% RSD)
Cherry Tomato																		
protocatechuic acid	0.04	92.50	7.40	6.90	4.90	12.50	0.12	91.60	3.00	4.20	3.70	7.62	0.24	92.90	12.00	5.20	4.10	8.04
chlorogenic acid	1.50	86.60	7.20	0.70	4.70	2.83	4.50	97.10	4.50	1.00	3.10	3.39	9.00	89.10	4.50	1.90	1.50	7.99
caffeic acid	0.50	92.20	3.50	3.70	6.00	2.66	1.50	91.50	3.60	1.20	6.90	9.43	3.00	89.30	3.50	1.10	3.60	8.17
o-coumaric acid	0.50	94.00	2.70	7.30	3.50	3.62	1.50	85.60	3.20	8.60	3.70	12.50	3.00	113.00	4.70	2.20	3.00	3.32
rutin	14.0	89.40	2.90	2.40	5.40	1.18	42.0	85.90	2.20	1.00	5.30	4.94	84.0	87.40	1.80	0.70	3.20	8.43
ferulic acid	0.80	92.70	3.30	2.30	2.40	1.72	2.40	88.10	2.20	9.00	6.00	9.33	4.80	99.90	2.20	3.20	9.20	7.25
naringenin-7-*O*-glucoside	0.10	87.00	7.70	4.40	6.70	6.15	0.30	86.30	6.40	1.30	7.80	8.47	0.60	86.60	13.00	1.60	5.10	7.81
naringenin	12.4	95.50	6.60	13.10	7.40	14.00	37.2	90.00	5.90	2.60	7.10	5.12	74.40	87.90	2.40	3.70	7.10	9.50
Tomato Sauce																		
protocatechuic acid	0.12	114.00	1.80	2.90	2.00	5.86	0.36	112.00	1.30	2.70	2.50	3.37	0.72	82.80	12.00	2.00	6.50	7.30
chlorogenic acid	3.80	101.00	8.40	3.40	4.30	5.87	11.4	98.00	12.00	0.80	5.60	14.90	22.80	96.60	2.00	4.20	5.90	6.44
caffeic acid	0.70	97.40	1.70	1.50	5.10	10.7	2.10	115.00	4.60	3.10	3.40	13.50	4.20	98.20	3.20	1.10	2.40	6.02
o-coumaric acid	0.50	102.00	12.00	7.20	6.20	6.54	1.50	112.00	7.20	3.20	4.40	5.02	3.00	115.00	8.50	1.60	6.10	12.50
rutin	11.5	113.00	9.00	4.90	4.80	8.43	34.5	91.20	10.00	9.50	12.50	8.55	69.00	96.40	12.00	3.10	2.10	5.98
naringenin-7-*O*-glucoside	1.40	92.50	4.20	2.80	4.40	7.82	4.20	97.20	2.00	3.20	6.10	10.00	8.40	92.10	2.70	2.60	9.50	3.96
naringenin	20.0	96.40	7.80	8.70	2.50	7.51	60.0	90.70	1.90	1.10	1.10	1.15	120.00	88.00	2.50	3.00	2.70	12.20
Tomato Juice																		
protocatechuic acid	0.60	96.60	13.00	10.00	5.40	5.29	0.12	109.00	5.20	8.90	1.90	6.02	0.36	90.30	14.00	11.00	3.00	6.41
4-hydroxybenzoic acid	0.30	87.30	4.00	5.00	2.20	8.87	0.90	87.00	14.00	13.00	7.60	7.24	1.80	105.00	12.00	7.20	3.00	2.02
chlorogenic acid	0.08	113.00	10.00	8.10	5.80	11.8	0.24	98.70	13.00	8.10	6.10	3.04	0.48	95.40	11.00	4.90	6.80	4.13
caffeic acid	0.40	94.20	4.60	3.80	3.50	4.39	1.20	111.00	5.20	8.40	5.60	6.91	2.40	108.00	5.60	10.00	6.30	8.87
o-coumaric acid	0.50	99.20	9.10	9.50	4.50	12.10	1.50	86.00	8.10	6.50	8.60	10.10	3.00	88.60	3.50	2.50	5.90	3.48
rutin	1.50	104.00	4.70	11.00	3.10	7.06	4.50	92.80	14.00	5.10	2.50	7.95	90.0	89.20	9.40	4.30	4.20	13.50
ferulic acid	1.50	110.00	13.60	8.90	3.70	4.64	4.50	94.00	3.30	7.60	5.30	1.49	9.00	86.70	14.00	9.20	5.80	4.18
naringenin	1.20	108.00	3.70	11.00	3.50	4.09	3.60	99.70	12.00	4.90	5.60	3.26	7.20	103.00	4.40	11.00	3.90	3.90

[a]Accuracy and intraday precision: 6 replicates of cherry tomato, tomato sauce, and tomato juice extracts spiked at 3 different concentration levels (50%, 150%, 300%). Interday precision: data obtained in 3 different days.

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Table 4. Phenolic Content and Matrix Effect in Cherry Tomato, Sauce, and Juice Extracts[a]

	content (μg g^{-1} FW)			suppression/enhancement effect (%)		
compound	cherry	sauce	juice	cherry	sauce	juice
protocatechuic acid	0.29 a ± 0.00	0.81 a ± 0.00	0.50 a ± 0.00	101.00 a ± 6.70	95.70 a ± 6.30	98.20 a ± 3.20
4-hydroxybenzoic acid			1.48 ± 0.10			100.00 ± 6.10
chlorogenic acid	2.38 b ± 0.20	9.46 a ± 0.80	0.30 b ± 0.00	99.10 a ± 5.30	92.30 b ± 7.00	101.00 a ± 5.60
caffeic acid	2.20 b ± 0.10	4.65 a ± 0.30	2.66 b ± 0.10	100.00 a ± 13.00	96.40 a ± 2.60	101.00 a ± 6.30
o-coumaric acid	3.15 b ± 0.20	1.40 b ± 0.00	7.10 a ± 0.50	101.00 a ± 5.00	101.00 a ± 7.90	99.30 a ± 6.20
rutin	208.00 a ± 13.00	182.00 a ± 9.10	6.15 b ± 0.30	100.00 a ± 9.40	95.30 a ± 10.50	101.00 a ± 0.20
ferulic acid	3.72 a ± 0.10		3.93 a ± 0.20	96.10 b ± 0.10		104.00 a ± 8.60
naringenin-7-*O*-glucoside	nq ±0.00	8.08 a ± 0.50		101.00 a ± 3.30	91.50 b ± 12.00	
naringenin	93.10 a ± 5.70	206.00 a ± 14.00	7.04 b ± 0.50	99.90 a ± 7.50	93.80 b ± 14.00	91.40 b ± 10.00

[a]Data are the mean ± standard deviation (n = 3) for each matrix. Different letters in the rows represent significant differences in concentration (columns 2−4) or matrix effect (columns 5−7) observed among matrices ($p < 0.05$); nq = detected compound although below the LOQ.

effects are generally matrix, analyte, LC−MS/MS method, and ion source dependent.[43] The sources of these effects are so extremely diverse that they cannot be attributed to only one cause, while depending on a synergic effect of all the analytical conditions involved. However, especially in the analysis of complex dietary matrices, matrix components of the extracts may play a relevant role in causing either ion suppression or enhancement effects, in a variable extent depending of the compound elution and physical-chemical properties. As a consequence, the evaluation of matrix effects in the analysis of food item composition is essential for method assessment. And the generalized use of the external standard calibration plot often gives biased results. In our study, the data obtained did not seem to fit a common rule for all compounds. However, as shown in Table 4, no significant matrix-dependent suppression/enhancement effects were generally observed in the three matrices under the proposed LC−MS conditions (ratio between 91.4% and 104%).

Phenolic Composition of the Three Dietary Matrices. The application of the method has allowed the analysis of main phenolic compounds belonging to the hydroxycinnamic acid, flavonol, and flavanone classes. The reported UHPLC−QqQ method could be successfully applied to the phenolic characterization of similar matrices, with minor modifications (gradient elution, flow rate, total chromatographic runtime). The results obtained of the screening of phenolic compounds (hydroxycinnamic acids, flavonols, flavanones, and their derivatives) are schemed in Table 4.

Chromatographic peaks obtained for each run were drawn by data points ranging between 11 (naringenin-7-glucoside) and 14 (chlorogenic acid). The quantitative determination of the target phenolic compounds was carried out selecting the MRM transitions corresponding to the most abundant phenolics in cherry tomato and tomato-based products and was expressed as mean values ± SD of fresh weight (FW) (Table 4). Other principal compounds such as chalconaringenin and quercetin 3-*O*-(2″-*O*-beta-apiofuranosyl-6″-*O*-alpha-rhamnopyranosyl)-beta-glucopyranoside have been found previously in other types of tomato and tomato based-products, but in this study, these last two compounds were not identified and the setup of validation method was performed only for the main and more representative compounds detected in all extracts.

Statistically significant differences in the content of phenolic compounds were found in cherry tomato and tomato-based products.

Hydroxycinnamic Acids. The spectra generated for hydroxycinnamic acids showed the deprotonated molecule $[M - H]^-$, together with additional identifying fragments. A typical loss of CO_2 was observed for protocatechuic, caffeic, ferulic, and *o*-coumaric acids, giving $[M - H - 44]^-$ as a characteristic ion; ferulic acid was also identified by the loss of a methyl group $[M - H - 15]^-$.

In the product ion spectra, chlorogenic acid (m/z 353) gave a fragment at m/z 191 $[M - H - 162]^-$ corresponding to quinic acid. Furthermore, neutral loss experiment of 162 unit confirmed the loss of a caffeic acid $[179 - H_2O]^-$ unit from the chlorogenic acid.[41] The confirmation of chlorogenic acid was performed by matching reference compound.

Flavonols and Flavanones. The peak with mass signals at m/z 609 was attributed to rutin. The product ion scan of m/z 609 showed peaks corresponding to produce a loss of the rutinoside unit, $[M - H - 308]^-$,while the peak at m/z 271 was ascribed to naringenin, that gave as a characteristic ion (m/z 151) corresponding to retro-Diels−Alder fragmentation in the C-ring involving 1,3 scission.

The main phenolic compound in all the samples was rutin, followed by naringenin (Table 4). Naringenin (45%) is reported to be the main flavonoid in tomatoes, followed by quercetin (39%), myricetin (10%), and kaempferol (5%).[41] Other studies report rutin as the major flavonoid in several tomato cultivars.[14,41] In this study, rutin was found at different concentrations ranging from 6.15 μg g^{-1} FW (tomato juice) to 182 μg g^{-1} FW and 208 μg g^{-1} FW (tomato sauce and raw tomatoes, respectively). The same pattern was observed for naringenin (7.04 μg g^{-1} concentration in tomato juice while 93.1 and 206 μg g^{-1} FW respectively in raw fruit and tomato sauce).

Regarding the family of phenolic acids, chlorogenic acid was the main phenolic acid ranging from 0.30 μg g^{-1} (tomato juice) to 9.46 μg g^{-1} (tomato sauce). The differences in other compounds, such as caffeic or ferulic acid, between tomato-based products were less pronounced.

Cherry tomato and sauce extracts showed a higher content of phenolic compounds than tomato juice extracts. Cherry tomatoes showed the highest amount of rutin and *o*-coumaric acid, while the content of caffeic, chlorogenic, naringenin-7-*O*-glucoside, and protocatechuic acid was higher in tomato sauce. The differences in concentration among these tomato-based products may be due to technological processes or due to the high content of water in tomato juice.

As mentioned above, tomato juice is an intermediate product in the processing of tomato paste which contains a higher quantity of water and, thus, the content of phenolic compounds could decrease. Otherwise, thermal processing of tomatoes to

obtained paste involved a number of heating stages which could be expected to have an effect on heat-labile and oxidizable compounds or increasing the bioavailability for certain compounds. The effect of thermal treatment on phenolic compounds is a controversial issue in the literature, with some studies suggesting exposure to heat results in a considerable loss of hydrophilic antioxidants. Crozier et al. found that cooking involving boiling, microwaving, and frying reduced the quercetin content of tomatoes.[44] Similarly, Capanoglu et al. found that rutin decreased after samples were treated in a three-effect evaporator unit, which included heat treatment up to 80 °C.[45] In contrast, other studies have reported an increase in total phenolic content as a result of processing. In experiments carried out by Chang et al., two tomato varieties were air-dried at 80 °C for 2 h and then at 60 °C for 6 h. Analyses showed that the total flavonoid and total phenolic content increased in comparison with fresh tomatoes.[46] Processing also increased the phenolic content in diced tomatoes,[36] and in tomato paste production.[47]

To the best of our knowledge, this paper describes for the first time an easy, fast, and sensitive UHPLC−QqQ-MS method to identify and quantify the most abundant phenolic compounds present in cherry tomato, tomato sauce, and tomato juice extracts under the same conditions. The UHPLC−QqQ-MS method was completely validated and provided a sensitive analysis for phenol detection, showing satisfactory data for all the parameters tested. Good results were obtained with respect to linearity and recovery as well as precision. The often neglected matrix effects were also taken into account, since each matrix can have a variable influence on analyte determination and consequently on the quality of the results. No significant matrix effects (91.4% < %ME < 104%) were observed in tomatoes, tomato sauces and tomato juices under these chromatographic conditions.

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Notes

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4.5. Publicación 5. Método sensible y rápido por UHPLC-MS/MS para el análisis de compuestos fenólicos de tomate en muestras biológicas humanas.

Sensitive and rapid UHPLC-MS/MS for the analysis of tomato phenolics in human biological samples. **Miriam Martínez-Huélamo**, Sara Tulipani, Olga Jáuregui, Palmira Valderas-Martinez, Anna Vallverdú-Queralt, Ramón Estruch, Xavier Torrado and Rosa M. Lamuela-Raventós. *Molecules.* 2015, 20:20409-20425.

Con la previsión en un futuro de analizar una gran cantidad de muestras biológicas, se optimizó y validó un método por UHPLC-MS/MS para la cuantificación de compuestos fenólicos de tomate en fluidos humanos. UHPLC mejora la separación cromatográfica utilizando columnas de tamaño de partícula muy pequeña (1.7 μm), proporcionando una mayor resolución y una mejor eficacia que los métodos cromatográficos convencionales. Este tipo de columnas también ofrecen compatibilidad en condiciones de alta presión (hasta 15.000 psi), que a su vez aumentan la viscosidad de la fase móvil y la capacidad para disolver los analitos. A mayor resolución del pico y tiempos de ejecución más cortos se traduce en costes de análisis más bajos y, por lo tanto, es más ecológico al reducir la generación de residuos químicos potencialmente peligrosos. Tras la validación, el método fue aplicado a un estudio de intervención dietético con 8 voluntarios sanos. La validación resultó en un aumento de 5 veces en la velocidad (3.5 minutos de tiempo de análisis), aumentó 7 veces en sensibilidad y 2 veces en eficacia (50% de reducción de anchura de pico) en comparación con el anterior método por HPLC-MS/MS (**publicación 1**). Se mejoraron los LODs y LOQs para todos los compuestos fenólicos estudiados en general. Las recuperaciones oscilaron entre 68% y 100% en orina y 61% y 100% en plasma. La exactitud, la precisión intra e interdiaria, y la estabilidad cumplieron con los criterios de aceptación de la AOAC Internacional. Debido a las mejoras en los métodos de análisis, los metabolitos fenólicos detectados en plasma y orina en el estudio piloto de intervención se triplicaron respecto a la metodología por HPLC-MS/MS. En comparación con los métodos tradicionales, que requieren tiempos de análisis más largos, la metodología propuesta es adecuada para el

análisis de compuestos fenólicos en un gran número de muestras de plasma y orina en un marco de tiempo reducido.

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Sensitive and Rapid UHPLC-MS/MS for the Analysis of Tomato Phenolics in Human Biological Samples

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Abstract: An UHPLC-MS/MS method for the quantification of tomato phenolic metabolites in human fluids was optimized and validated, and then applied in a pilot dietary intervention study with healthy volunteers. A 5-fold gain in speed (3.5 min of total run); 7-fold increase in MS sensitivity and 2-fold greater efficiency (50% peak width reduction) were observed when comparing the proposed method with the reference-quality HPLC-MS/MS system, whose assay performance has been previously documented. The UHPLC-MS/MS method led to an overall improvement in the limits of detection (LOD) and quantification (LOQ) for all the phenolic compounds studied. The recoveries ranged between 68% and 100% in urine and 61% and 100% in plasma. The accuracy; intra- and interday precision; and stability met with the acceptance criteria of the AOAC International norms. Due to the improvements in the analytical method; the total phenolic metabolites detected in plasma and urine in the pilot intervention study were 3 times higher than those detected by HPLC-MS/MS. Comparing with traditional methods; which require longer time of analysis; the methodology described is suitable for the analysis of phenolic compounds in a large number of plasma and urine samples in a reduced time frame.

Keywords: polyphenol metabolites; validation; tomato; sauce; microbiota; metabolites; biosamples; blood; urine

1. Introduction

Tomato (*Solanum lycopersicum*) is a food very rich in bioactive compounds such as vitamins or carotenoids and it contains a variety of phenolic compounds [1]. Phenolics play an important protective role in human health, decreasing mortality [2], cardiovascular disease [3], and DNA oxidation [4], and counteracting age-related cognitive decline [5]. The most important phenolics described in tomato and tomato by-products belong to hydroxycinnamic acids, flavanones and

Molecules **2015**, *20*, 20409–20425

flavonols, specifically, naringenin, rutin or 5-caffeoylquinic acid [6,7]. After ingestion, polyphenols are metabolized in the small intestine and liver producing a series of metabolites (methyl, glucuronide, and sulfate), which may pass into the blood stream, accumulate in tissues, and then excreted in urine [8].

An exhaustive identification of polyphenols in food and biological samples is of great interest due to their health-promoting effects. Although a wide range of methods have been reported for the detection of phenolic compounds in food, beverages, or biological samples (*i.e.*, spectrophotometry, capillary electrophoresis, near-infrared spectroscopy, HPLC-UV-DAD) [9–12], liquid chromatography coupled to mass spectrometry (LC-MS) [13–18] is the most commonly used technique due to its high sensitivity and selectivity.

In particular, ultra-high performance liquid chromatography (UHPLC) enhances chromatographic separation by using columns packed with smaller particles (1.7 μm), which provides higher resolution and better efficiency than conventional chromatography [19]. It also offers compatibility with high back-pressure operating conditions (up to 15,000 psi), which in turn increases mobile phase viscosity and the capacity to dissolve analytes [12]. The resulting higher peak resolution and shorter run times translates into lower analytical costs, and is more "environmentally friendly" in terms of reduced generation of hazardous chemical waste [20].

The aim of this work was to improve the efficiency and resolution of the HPLC-ESI-QqQ MS/MS method validated previously by our research group [21] for the detection and quantification of 5 hydroxycinnamic acids, 4 hydroxyphenylacetic acids, 2 hydroxybenzoic acids, 1 hydroxyphenylpropionic acid, 1 flavanone and 2 flavonols. Thus a new UHPLC-MS/MS-driven method was developed which involved the optimization of the main LC (column, elution solvents and gradients) and MS operating conditions (declustering potential (DP), focusing potential (FP) and collision energy (CE)), followed by its application in a pilot tomato sauce dietary intervention study.

2. Results

2.1. UHPLC-MS/MS Method Development

Table 1 shows the LOD and LOQ obtained for each compound when the study of the optimum mobile phase was achieved. Homovanillic acid, phenylacetic acid, quercetin and quercetin-3-*O*-glucuronide up to 100 ng/mL were not detected using 0.1% and 0.05% formic acid, whereas 0.025% formic acid allowed the detection and quantification of all the target compounds. H_2O (0.025% formic acid)/MeCN (0.025% formic acid) improved the LOD of 3-hydroxyphenylacetic acid, dihydrocaffeic acid, ferulic acid, and above all isoferulic acid.

Declustering potential (DP), focusing potential (FP), entrance potential (EP), quantification and confirmation transitions with their corresponding collision energy (CE) were shown in Table 2 obtaining the optimum value for the mass conditions.

The flow rate, which allowed a correct resolution of the compounds, was achieved with 600 μL/min of H_2O (0.025% formic acid)/MeCN (0.025% formic acid) and the best volume injection was obtained with 10 μL.

Comparing a standard mix in the HPLC-MS/MS (Figure 1A) and UHPLC-MS/MS (Figure 1B) systems, the latter provided adequate resolution in less time. The UHPLC equipment allowed the quantification of 16 phenolic compounds in 3.5 min in comparison with 12 min needed for the HPLC analysis.

Table 1. LOD and LOQ of an aqueous mix of 15 phenolic compounds (100 ng/mL) in the study of different mobile phases by UHPLC-MS/MS.

Compounds	LOD [a] (ng/mL) (n = 3) [c]			LOQ [b] (ng/mL) (n = 3) [c]		
	Formic Acid (0.1%)	Formic Acid (0.05%)	Formic Acid (0.025%)	Formic Acid (0.1%)	Formic Acid (0.05%)	Formic Acid (0.025%)
Caffeic acid	1.7 ± 0.1	2.2 ± 0.1	2.1 ± 0.4	5.6 ± 0.5	7.4 ± 0.1	6.8 ± 1.3
5-Caffeoylquinic acid	0.8 ± 0.3	1.8 ± 0.3	5.3 ± 0.4	2.7 ± 0.9	6.1 ± 0.8	17.6 ± 1.3
Dihydrocaffeic acid	10.7 ± 0.6	6.9 ± 0.4	6.0 ± 0.2	35.7 ± 1.6	23.1 ± 0.9	19.8 ± 0.7
3,4-Dihydroxyphenylacetic acid	2.8 ± 0.3	8.2 ± 0.3	2.3 ± 0.4	9.5 ± 0.8	27.3 ± 1.1	7.5 ± 1.5
Ferulic acid	5.5 ± 0.6	1.8 ± 0.2	1.9 ± 0.4	18.3 ± 1.8	5.9 ± 0.7	6.2 ± 1.1
Hippuric acid	12.5 ± 1.7	9.6 ± 2.5	9.4 ± 0.9	41.5 ± 5.8	32.0 ± 2.1	31.5 ± 2.5
Homovanillic acid	n.d. [d]	n.d. [d]	49.2 ± 2.4	n.d. [d]	n.d. [d]	163.9 ± 6.8
4-Hydroxyhippuric acid	0.6 ± 0.1	1.3 ± 0.2	2.5 ± 0.4	2.1 ± 0.6	4.2 ± 0.8	8.3 ± 1.4
3-Hydroxyphenylacetic acid	12.0 ± 1.3	7.1 ± 0.3	6.6 ± 0.6	39.8 ± 3.6	23.7 ± 0.7	21.8 ± 2.1
3-(3-Hydroxyphenyl)propionic acid	5.7 ± 0.9	6.6 ± 0.2	3.4 ± 0.1	18.9 ± 2.7	22.0 ± 0.5	11.2 ± 0.5
Isoferulic acid	54.6 ± 2.2	5.9 ± 0.1	8.9 ± 0.9	181.8 ± 8.4	19.6 ± 0.4	29.7 ± 3.3
Naringenin	0.5 ± 0.1	0.4 ± 0.2	0.7 ± 0.4	1.6 ± 0.4	1.2 ± 0.8	2.4 ± 1.1
Phenylacetic acid	n.d. [d]	n.d. [d]	35.3 ± 3.0	n.d. [d]	n.d. [d]	117.7 ± 8.9
Quercetin	n.d. [d]	n.d. [d]	21.9 ± 0.9	n.d. [d]	n.d. [d]	73.0 ± 2.3
Quercetin-3-*O*-glucuronide	n.d. [d]	n.d. [d]	22.4 ± 0.2	n.d. [d]	n.d. [d]	74.6 ± 0.2

Values are means ± standard deviation; LOD [a]: limit of detection; LOQ [b]: limit of quantification; (n = 3) [c]: replicates; n.d. [d]: no detected.

Table 2. Rt (Retention time), Declustering potential (DP), focusing potential (FP) and entrance potential (EP) optimized. Quantification and confirmation transitions of the phenolic compounds with the optimum collision energy (V).

Compounds	Rt (Min)	DP (V)	FP (V)	EP (V)	Quantification Transition	Collision Energy (V)	Confirmation Transition	Collision Energy (V)
Caffeic acid	1.18	−40	−170	−10	179 → 135	−20	179 → 107	−30
5-Caffeoylquinic acid	1.01	−50	−180	−10	353 → 191	−20	353 → 179	−30
Dihydrocaffeic acid	1.12	−50	−170	−10	181 → 137	−20	181 → 121	−30
3.4-Dihydroxyphenylacetic acid	0.83	−40	−170	−10	167 → 123	−10		
Ethylgallate (IS)	1.56	−60	−200	−10	197 → 169	−25	197 → 124	−40
Ferulic acid	1.70	−40	−170	−10	193 → 134	−20	193 → 178	−30
Hippuric acid	1.10	−40	−170	−10	178 → 134	−20		
Homovanillic acid	1.27	−40	−170	−10	181→ 137	−20		
4-Hydroxyhippuric acid	0.68	−40	−170	−10	194 → 100	−20	194 → 150	−30
3-Hydroxyphenylacetic acid	1.28	−40	−170	−10	151 → 107	−10		
3-(3-Hydroxyphenyl)propionic acid	1.67	−40	−170	−10	165 → 121	−20	165 → 119	−35
Isoferulic acid	1.80	−50	−220	−10	193 → 178	−20	193 → 134	−35
Naringenin	2.51	−50	−190	−10	271 → 151	−30	271 → 119	−40
Phenylacetic acid	1.10	−50	−170	−10	135 → 91	−30		
Quercetin	2.46	−60	−210	−10	301 → 151	−30	301 → 179	−40
Quercetin-3-*O*-glucuronide	1.76	−60	−210	−10	477 → 301	−30	477 → 151	−40

Molecules **2015**, *20*, 20409–20425

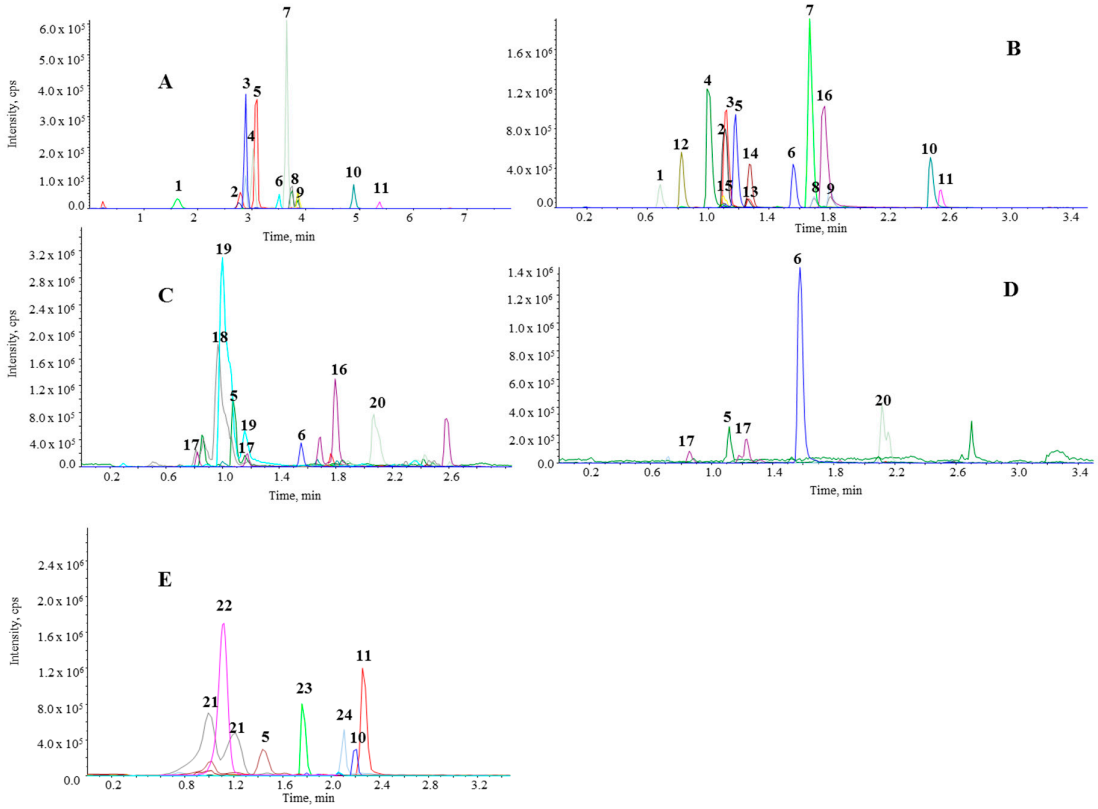

Figure 1. Chromatograms of standards solution obtained by HPLC-MS/MS (**A**) and UHPLC-MS/MS (**B**) analysis. Chromatograms of urine at 6 h (**C**), plasma at 1 h (**D**) and tomato sauce (**E**). Peaks: (1) 4-hydroxyhippuric acid; (2) hippuric acid; (3) dihydrocaffeic acid; (4) 5-caffeoylquinic acid; (5) caffeic acid; (6) ethylgallate (IS); (7) 3-(3-hydroxyphenyl)propionic acid; (8) ferulic acid; (9) isoferulic acid; (10) quercetin; (11) naringenin; (12) 3,4-dihydroxyphenylacetic acid; (13) homovanillic acid; (14) 3-hydroxyphenylacetic acid; (15) phenylacetic acid; (16) quercetin-3-*O*-glucuronide; (17) ferulic acid glucuronide; (18) caffeic acid sulfate; (19) ferulic acid sulfate; (20) naringenin glucuronide; (21) caffeic acid hexoside; (22) homovanillic acid hexoside; (23) rutin; (24) hydroxybenzoic acid.

2.2. Validation Parameters

2.2.1. Limits of Detection (LOD) and Quantification (LOQ)

The sensitivity of the method was evaluated by determining the LOD and LOQ. There were no differences in LOD and LOQ values between the two biosamples (urine and plasma) (Table 3). Nevertheless, the results showed a wide range of sensitivity according to the analyte. LOD were established between 0.5 and 62.5 ng/mL in urine and 0.3 and 44.1 ng/mL in plasma. In the case of LOQ, values ranged from 1.8 to 203.4 ng/mL in urine and 1.6 to 145.8 ng/mL in plasma. The most notable improvement was for 4-hydroxyhippuric acid and isoferulic acid, whose LOD decreased 25-fold and 15-fold in urine and 11-fold and 9-fold in plasma, respectively if HPLC and UHPLC were compared. The LOD for ferulic and hippuric acids also decreased, particularly in plasma, but to a lesser extent.

Table 3. LOD, LOQ, recovery, concentration range and correlation coefficient in urine and plasma samples by HPLC-MS/MS and UHPLC-MS/MS.

Compounds	HPLC (n = 3) [a]				UHPLC (n = 3) [a]				HPLC (n = 3) [a]		UHPLC (n = 3) [a]		HPLC		UHPLC	
	URINE		PLASMA		URINE		PLASMA		URINE	PLASMA	URINE	PLASMA	URINE	PLASMA	URINE	PLASMA
	LOD [b] (ng/mL)	LOQ [c] (ng/mL)	LOD [b] (ng/mL)	LOQ [c] (ng/mL)	LOD [b] (ng/mL)	LOQ [c] (ng/mL)	LOD [b] (ng/mL)	LOQ [c] (ng/mL)	Rec. [e] (%)	Rec. [e] (%)	Rec. [e] (%)	Rec. [e] (%)	Conc. Range [f] (ng/mL) (r^2) [g]	Conc. Range [f] (ng/mL) (r^2) [g]	Conc. Range [f] (ng/mL) (r^2) [g]	Conc. Range [f] (ng/mL) (r^2) [g]
Caffeic acid	1.5 ± 0.1	6.0 ± 0.1	1.8 ± 0.1	6.0 ± 0.4	0.5 ± 0.2	2.0 ± 0.4	0.7 ± 0.1	2.8 ± 0.1	97 ± 4	98 ± 3	100 ± 2	91 ± 2	6–3450 (0.990)	6–3450 (0.993)	2–1152 (0.994)	3–1728 (0.995)
5-Caffeoylquinic acid	0.5 ± 0.1	2.0 ± 0.2	0.6 ± 0.1	2.0 ± 0.1	1.1 ± 0.1	4.3 ± 0.1	0.5 ± 0.1	1.7 ± 0.1	103 ± 2	99 ± 4	99 ± 4	92 ± 2	2–1150 (0.999)	2–1150 (0.999)	4–2304 (0.992)	2–1152 (0.998)
Dihydrocaffeic acid	6.3 ± 0.2	25 ± 0.9	4.4 ± 0.1	15 ± 0.3	5.7 ± 0.6	20.2 ± 2.1	5.3 ± 0.3	18.0 ± 1.0	101 ± 2	104 ± 5	97 ± 5	90 ± 4	25–14,400 (0.993)	15–14,400 (0.991)	20–11,520 (0.996)	18–10,368 (0.992)
3,4-Dihydroxyphenylacetic acid	n.s. [d]	n.s. [d]	n.s. [d]	n.s. [d]	1.1 ± 0.1	3.5 ± 0.2	1.6 ± 0.1	5.4±0.2	n.s. [d]	n.s. [d]	78 ± 3	69 ± 2	n.s. [d]	n.s. [d]	4–2304 (0.994)	5–2880 (0.998)
Ferulic acid	15 ± 0.7	50 ± 2.7	18 ± 2.1	60 ± 7.0	1.9 ± 0.1	6.6 ± 0.2	1.6 ± 0.1	5.6 ± 0.2	95 ± 2	98 ± 4	100 ± 4	100 ± 3	50–28,800 (0.990)	60–28,800 (0.996)	7–4032 (0.998)	6–3456 (0.990)
Hippuric acid	25 ± 1.6	80 ± 3.2	25 ± 0.8	90 ± 2.1	4.4 ± 0.2	15.0 ± 0.6	2.1 ± 0.3	6.5 ± 0.9	106 ± 2	99 ± 4	97 ± 2	99 ± 4	80–51,840 (0.990)	90–51,840 (0.992)	15–8640 (0.999)	7–4032 (0.991)
Homovanillic acid	n.s. [d]	n.s. [d]	n.s. [d]	n.s. [d]	4.5 ± 0.1	17.3 ± 0.4	8.5 ± 0.3	29.2 ± 1.0	n.s. [d]	n.s. [d]	97 ± 4	92 ± 4	n.s. [d]	n.s. [d]	17–9792 (0.996)	29–16,704 (0.991)
4-Hydroxyhippuric acid	15 ± 1.2	50 ± 2.6	15 ± 0.7	50 ± 3.2	0.6 ± 0.1	1.8 ± 0.2	1.4 ± 0.1	4.6 ± 0.3	75 ± 3	73 ± 3	68 ± 3	61 ± 4	50–28,800 (0.990)	50–28,800 (0.992)	2–1152 (0.999)	5–2880 (0.999)
3-Hydroxyphenylacetic acid	n.s. [d]	n.s. [d]	n.s. [d]	n.s. [d]	0.9 ± 0.1	2.8 ± 0.2	1.6 ± 0.3	5.3 ± 0.9	n.s. [d]	n.s. [d]	99 ± 2	98 ± 2	n.s. [d]	n.s. [d]	3–1728 (0.999)	5–2880 (0.998)
3-(3-Hydroxyphenyl)propionic acid	6.0 ± 0.8	20 ± 1.4	6.0 ± 1.2	20 ± 2.9	2.5 ± 0.1	9.0 ± 0.2	2.4 ± 0.2	8.6 ± 0.5	97 ± 2	99 ± 5	98 ± 4	94 ± 3	20–11,520 (0.995)	20–11,520 (0.994)	9–5184 (0.991)	9–5184 (0.994)
Isoferulic acid	29 ± 4.3	90 ± 13.6	30 ± 2.4	105 ± 8.0	2.0 ± 0.3	6.2 ± 1.0	3.4 ± 0.2	12.0 ± 0.5	99 ± 2	99 ± 4	100 ± 3	97 ± 4	90–28,800 (0.997)	105–28,800 (0.994)	6–3456 (0.993)	12–6912 (0.993)
Naringenin	0.5 ± 0.1	2.0 ± 0.1	0.5 ± 0.1	2.0 ± 0.1	0.6 ± 0.1	2.8 ± 0.3	0.3 ± 0.1	1.6 ± 0.1	104 ± 4	96 ± 3	99 ± 2	96 ± 2	2–1150 (0.999)	2–1150 (0.995)	3–1728 (0.999)	2–1152 (0.999)
Phenylacetic acid	n.s. [d]	n.s. [d]	n.s. [d]	n.s. [d]	62.5 ± 3.0	203.4 ± 9.9	44.1 ± 5.4	145.8 ± 17.8	n.s. [d]	n.s. [d]	95 ± 3	99 ± 4	n.s. [d]	n.s. [d]	203–116,929 (0.992)	146–84,096 (0.994)
Quercetin	1.7 ± 0.1	6.0 ± 0.3	1.4 ± 0.1	5.0 ± 0.4	2.3 ± 1.4	8.5 ± 4.8	4.5 ± 0.1	15.8 ± 0.3	65 ± 3	100 ± 3	85 ± 4	100 ± 2	6–3450 (0.991)	5–3450 (0.990)	9–5184 (0.998)	16–9216 (0.998)
Quercetin-3-*O*-glucuronide	n.s. [d]	n.s. [d]	n.s. [d]	n.s. [d]	1.4 ± 0.1	4.4 ± 0.1	1.9 ± 0.1	6.1 ± 0.2	n.s. [d]	n.s. [d]	99 ± 2	95 ± 2	n.s. [d]	n.s. [d]	5–2880 (0.997)	6–3456 (0.993)

Values are means ± standard deviation; (n = 3) [a]: replicates; LOD [b]: limit of detection; LOQ [c]: limit of quantification; n.s. [d]: not studied; Rec. [e]: recovery; Conc. Range [f]: concentration range; (r^2) [g]: correlation coefficient.

Molecules **2015**, *20*, 20409–20425

2.2.2. Linearity

Calibration curves showed linear responses between LOQ and 576 times LOQ for each analyte, establishing ranges from 2 ng/mL to 116,929 ng/mL in urine, and 2 ng/mL to 84,096 ng/mL in plasma. A weighting factor was necessary for almost all the phenolic compounds studied to achieve accuracy between 85%–115%. The correlation coefficient (r^2) between 0.990 and 0.999 for plasma and 0.991 and 0.999 for urine demonstrate an adequate linearity, similarly of those obtained in the HPLC-MS/MS validation [21].

2.2.3. Recovery

Table 3 shows the recoveries obtained with UHPLC system and the previous HPLC method validation. In the HPLC system, the recovery of the compounds studied ranged from 65% to 106% in urine and 73% to 104% in plasma. The lowest recovery in urine was for quercetin, and 4-hydroxyhippuric acid in plasma. In the case of UHPLC, there was little variation between the two biological matrices, with recoveries greater than 85% except for 3,4-dihydroxyphenylacetic acid and 4-hydroxyhippuric acid (78% in urine and 69% in plasma, and 68% in urine and 61% in plasma, respectively).

2.2.4. Accuracy and Precision

Intra- and interday accuracy and precision were studied by injection of plasma and urine extracts spiked at three different concentrations: low (3-fold the LOQ), medium (48-fold the LOQ), and high (288-fold the LOQ). The accuracy obtained was between 91.3% and 113.9% in urine, and 99.0% and 114.8% in plasma, thereby meeting with the AOAC acceptance criteria. Phenylacetic acid was the phenolic acid in urine with both the highest intra- and interday accuracy. In plasma, the top percentage was given by homovanillic acid. Intra- and interday precision studies gave the same results as for accuracy. No polyphenol in urine or plasma samples exceeded the 15% RSD set by the AOAC. The highest values were 3.6% RSD in urine, corresponding to 4-hydroxyhippuric acid, and 13.5% RSD in plasma, to quercetin.

2.2.5. Stability

The processed sample stability refers to the variation of concentration, since the SPE is performed until the samples are in the autosampler, in this case about 24 h. The results show a reduction in concentration ranging between 3.0% for caffeic acid and 12.9% for phenylacetic acid in urine, and 2.8% for homovanillic acid and 13.3% for quercetin in plasma, but without significant differences between the biological samples and standards. For the freeze/thaw stability, three freeze-thaw cycles were assessed for a mix of the standards used for the validation in plasma and urine samples. Similar results were obtained, with a reduction ranging from 3.1% for dihydrocaffeic acid to 12.6% for quercetin in urine, and 3.8% for 3-hydroxyphenylacetic acid to 9.1% for quercetin in plasma.

2.3. Phenolic Quantification in Tomato Sauce and Biological Samples

Figure 2 shows the concentration of the phenolic compounds in the tomato sauce administered to the eight volunteers. The main polyphenols present in the tomato sauce were from two classes: flavonoid and phenolic acids, the latter represented by a wider range of compounds (mono-, di- and tricaffeoylquinic acids, caffeic acid and two hexosides, ferulic acid hexoside, two coumaric acid hexosides, protocatechuic acid, and homovanillic acid hexoside). Homovanillic acid hexoside was the predominant polyphenol in the sauce (0.140 $\pm$ 0.005 mg/g FW) highlighting above the rest of compounds. Flavanones and flavonols were also found in the tomato sauce being naringenin and rutin the compounds with higher concentration, 6.650 $\pm$ 0.003 and 5.390 $\pm$ 0.015 μg/g FW, respectively.

Molecules **2015**, *20*, 20409–20425

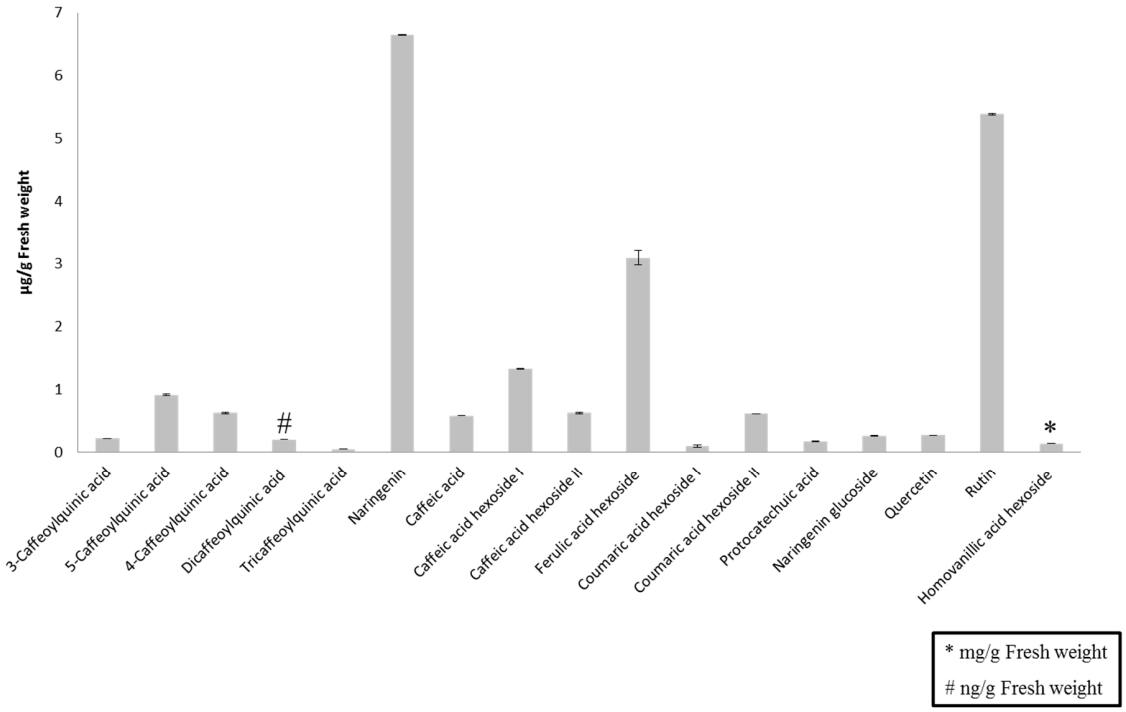

Figure 2. Quantification of phenolic compounds from tomato sauce administered to the volunteers by UHPLC-MS/MS analysis. Values are mean ± standard deviation (µg/g FW). Diccaffeoylquinic acid was expressed as ng/g FW and homovanillic acid hexoside as mg/g FW.

Table 4 shows the concentration of phenolic compounds found in plasma and urine after the tomato sauce intervention. Twelve phenolic compounds were quantified in plasma and twenty-eight in urine by UHPLC-MS/MS. The concentration ranged between 2.7 to 183 ng/mL in plasma for 5-caffeoylquinic acid and caffeic acid sulfate, respectively, and between 17.0 to 33,188 ng/mL for 5-caffeoylquinic acid and 4-hydroxyhippuric acid, respectively. A great variety of metabolites belonging to microbiota were quantified in urine at high concentrations as 16,425 ng/mL for 3-hydroxyphenylacetic acid sulfate or 225,696 ng/mL for ferulic acid sulfate.

Table 4. Phenolic compounds and metabolites quantified in urine and plasma by UHPLC-MS/MS.

Compounds	Product Ions in MS^2 Experiments	Urine (ng/mL)	Plasma (ng/mL)
Caffeic acid *	179,135,107	206 (4.0–822)	n.d. [a]
Caffeic acid glucuronide (CA)	355,179,135,175,113	54.9 (2.0–975)	6.4 (3.0–12.1)
Caffeic acid sulfate (CA)	259,179,135	1396 (10.2–42,286)	183 (15.3–862)
5-Caffeoylquinic acid *	353,191,179	17.0 (7.2–53.6)	2.7 (2.0–3.5)
Dihydrocaffeic acid *	181,137,59	194.7 (30.1–1031.9)	n.d. [a]
Dihydrocaffeic acid glucuronide (DHCA)	357,181,137,175,113	2127 (23–10,071)	n.d. [a]
Dihydrocaffeic acid sulfate (DHCA)	261,181,137	2775 (52–9424)	n.d. [a]
3,4-Dihydroxyphenylacetic acid *	167,123	1095 (33.9–2249)	n.d. [a]
Ferulic acid *	193,134,175	453 (31–2139)	8.1 (6.5–40.3)
Ferulic acid glucuronide (FA)	369,193,134,175,113	2852 (110–69,495)	53.5 (6.7–1881)

Molecules **2015**, *20*, 20409–20425

Table 4. *Cont.*

Compounds	Product Ions in MS^2 Experiments	Urine (ng/mL)	Plasma (ng/mL)
Ferulic acid sulfate (FA)	273,193,134	22,569 (713–536,479)	95.5 (24.5–316)
Homovanillic acid *	181,137	4050 (1073–9387)	n.d. [a]
4-Hydroxyhippuric acid *	194,100	33,188 (691–214,695)	n.d. [a]
3-Hydroxyphenylacetic acid *	151,107	739 (244–1291)	n.d. [a]
3-Hydroxyphenylacetic acid glucuronide (3-HPAA)	327,151,107,175,113	295 (11.0–1608)	n.d. [a]
3-Hydroxyphenylacetic acid sulfate (3-HPAA)	231,151,107	16,425 (175–352,967)	n.d. [a]
3-(3-Hydroxyphenyl)propionic acid *	165,121	1402 (188–4007)	n.d. [a]
3-(3-Hydroxyphenyl)propionic acid glucuronide (3-(3-HPPA))	341,165,121,175,113	258 (15.3–4914)	n.d. [a]
3-(3-Hydroxyphenyl)propionic acid sulfate (3-(3-HPPA))	245,165,121	3156 (27.6–145,199)	n.d. [a]
Isoferulic acid *	193,134,175	1156 (247–3427)	108 (10.6–494)
Naringenin *	271,151,119	39.1 (6.9–400)	11.7 (2.0–52.6)
Naringenin glucuronide (N)		854 (214–1558.5)	73.4 (2.0–830)
Phenylacetic acid *	135,91	1129 (297–6667)	n.d. [a]
Phenylacetic acid glucuronide (PAA)	311,135,91,175,113	318 (206–431)	n.d. [a]
Phenylacetic acid sulfate (PAA)	215,135,91	1378 (110–26,708)	n.d. [a]
Quercetin *	301,151,179	70.8 (9.1–417)	99.0 (23.7–331)
Quercetin glucuronide *	477,301,151,175,113	14.4 (11.9–101)	20.9 (14.3–63.4)
Quercetin sulfate (Q)	381,301,151	32.0 (11.9–489.8)	3.8 (3.5–4.2)

Values are median (minimum-maximum values); *: commercial standard; n.d. [a]: not detected; CA: Caffeic Acid; DHCA: Dihydrocaffeic Acid; FA: Ferulic Acid; 3-HPAA: 3-Hydroxyphenylacetic acid; 3-(3-HPPA): 3-(3-Hydroxyphenyl)propionic acid; N: Naringenin; PAA: Phenylacetic acid; Q: Quercetin.

3. Discussion

3.1. UHPLC-MS/MS Method Development

Before the validation of the method, different mobile phases were tested to obtain the best detection and separation of the compounds (Table 1). Between the three phases studied, 0.1%, 0.05% and 0.025% of formic acid, the mobile phase with less formic acid achieved the best results confirming that low-pH conditions created by high acid concentrations (up to 0.1%) decrease the negative-ion ESI response by damaging the formation of the deprotonated analyte [22].

After the election of the mobile phase, several experiments with 50:50 (v/v) of (0.025% formic acid)/MeCN (0.025% formic acid) were achieved to obtain the optimum DP, FP, EP, CE and the quantification and confirmation transitions which are shown in Table 2. The results were similar as those obtained in a previous validation method developed in HPLC-MS/MS [21].

Different linear gradient were proved to attain a good separation and resolution of the analytes. The best results obtained for the conditions were described in the chromatographic separation section with a total time run of 3.5 min. Diverse flow rates, 400, 600 and 800 µL/min, and injection volumes, 2, 5 and 10 µL were also studied to obtain a good separation and detection of the analytes. The optimum flow rate was achieved in 600 µL/min, as in the HPLC-MS/MS method. 10 µL was demonstrated to be the best injection volume to obtain the greatest detected peak height. The injection volume was halved regarding to the HPLC-MS/MS method allowing more volume sample if various injections were needed.

The time-saving and decrease in the total consumption of mobile phase implies a reduction in analysis cost [20,23]. Compared with HPLC-MS/MS [21], the UHPLC-MS/MS method offered a 5-fold decrease in retention time (RT), up to 7-fold increase in detected peak height, and a 2-fold decrease in peak width thereby enhancing the sensitivity of the method.

Molecules **2015**, *20*, 20409–20425

3.2. Validation Parameters

The AOAC International criteria were consulted as to the validation of the method [24]. A great improvement in terms of LOD and LOQ was achieved using UHPLC compared with HPLC for almost all the studied phenolic compounds, in both plasma and urine (Table 3). In comparison with our work, Rubió *et al.* [25] obtained higher LOD (1–240 ng/mL) and LOQ (3.3–801.5 ng/mL) in the validation of an UHPLC-MS/MS system to detect plasma phenolic metabolites. In particular, our method performed better for caffeic acid, with a LOD of 0.7 ng/mL compared to 64.9 in the study of Rubió *et al.* [25]. 3,4-Dihydroxyphenylacetic acid and 3-(3-hydroxyphenyl)propionic acid followed the same trend as caffeic acid, with lower values in our study (1.6 and 2.4 ng/mL, respectively), compared to the data of Rubió *et al.* [25] (15.1 and 38.2 ng/mL, respectively). Another study, carried out by Magiera *et al.* [16] to determine polyphenols and their metabolites in human urine by UHPLC coupled to a 4000 Q TRAP triple quadrupole linear ion trap mass spectrometer, obtained a LOQ for 3,4-dihydroxyphenylacetic acid comparable to our results, while other metabolites, for example, caffeic acid, presented lower values (1 ng/mL) in comparison with our data (2.0 ng/mL). Lastly, Oliveira *et al.* [26] reported the validation of a method that detected 11 phenolic acids in plasma, urine and liver by an UHPLC system coupled to a single-quadrupole mass spectrometer. The LOQ for caffeic, ferulic and 5-caffeoylquinic acids were 38, 52 and 52 ng/mL, respectively, in plasma, and 50, 96 and 48 ng/mL, respectively, in urine. Our study achieved a greater improvement in LOD in comparison with Oliveira *et al.* [26].

Recovery results were similar to those reported by Rubió *et al.* [25], who found levels of 77% for quercetin and 3,4-dihydroxyphenylacetic acid, and 98% for naringenin. Our data are also in agreement with Magiera *et al.* [16], who reported recoveries between 91% and 100% for caffeic, ferulic, and 3-hydroxyphenylacetic acids and naringenin. In another study on phenolic microbial metabolites in humans and rats, the mean recovery of analytes ranged from 87% to 109% [27]. Our results are therefore in agreement with the reported literature data.

Respect to accuracy and precision, a possible matrix effect may explain the higher values for quercetin and 4-hydroxyhippuric acid, as peaks with good symmetry were obtained without tailing, as can be seen in Figure 1. The proposed method therefore demonstrated good accuracy and precision in both urine and plasma samples. The results were similar to those obtained by HPLC-MS/MS, confirming that the method is applicable for the determination of phenolic compounds in both types of biological samples. The high accuracy for homovanillic acid and phenylacetic acid, although still within the limits established by the AOAC, may be due to their similar elution times, since they practically co-eluted.

Finally, quercetin seems to be the phenolic compound most affected in terms of stability, either in freeze/thaw or post-preparative studies. Ramešová *et al*. [28] reported that quercetin was potentially affected by exposure to atmospheric oxygen conditions. Their study confirmed the presence of four decomposition products by HPLC-DAD and HPLC-MS: 2-(3′,4′-dihydroxybenzoyl)-2,4,6-trihydroxybenzofuran-3(2*H*)-one, 2-(3,4-dihydroxyphenyl)-2-oxoacetic acid, 2,4,6-trihydroxybenzoic acid and 3,4-dihydroxybenzoic acid [28]. Although there was a decrease in the stability of some polyphenols, there were no significant differences in either two stability studies.

3.3. Phenolic Quantification in Tomato Sauce and Biological Samples

Three classes of polyphenols were quantified: 13 phenolic acids (three caffeoylquinic acids and two derivatives; caffeic acid and two hexosides; one ferulic acid hexoside; two coumaric acid hexosides; protocatechuic acid; and homovanillic acid hexoside), two flavanones (naringenin and naringenin glucoside), and two flavonols (quercetin and rutin). Most of the polyphenols belong to phenolic acids, being the homovanillic acid hexoside the major one in the tomato sauce. Naringenin and rutin were the most abundant phenolic compounds preceding homovanillic acid hexoside. Other authors have also described those as the three major polyphenols in tomato and tomato

Molecules **2015**, *20*, 20409–20425

by-products [6,29–31]. Ferulic acid hexoside, caffeic acid hexoside I and 5-caffeoylquinic acid were also present at high concentration, similarly to those obtained by Minoggio *et al.* [32] between 300 and 5800 ng/g FW. The validated method was successfully applied for the analysis of human plasma and urine samples from the intervention study. The biological samples were screened for the phenolic compounds previously analyzed in the validation study, identifying and quantifying the analytes by comparing their MRM transition, RT, and product ion scan with those of the standards. Phase II metabolites (glucuronide and sulfate conjugates) were also monitored to shed more light on the metabolism of the target compounds. In the absence of standards, the phenolic metabolites were identified by PIS, NL or PrIS (Table 4). Samples over the calibration curve were diluted and reinjected in the UHPLC-MS/MS system. Polyphenols, when reached the intestine, are transformed in a wide variety of phenolic metabolites [33,34], that are absorbed by the gut, circulated in the blood and metabolized in the liver to glucuronides or sulfates metabolites [35]. Table 4 confirmed a great metabolism of the phenolic compounds described in the tomato sauce as 12 phenolic compounds were quantified in plasma and 28 in urine. Both, plasma and urine metabolites tripled the compounds determined by HPLC-MS/MS [36]. Notably, isoferulic acid, caffeic acid sulfate, ferulic acid sulfate, dihydrocaffeic acid metabolites and quercetin and its metabolites (glucuronide and sulfate), none of which were identified by HPLC-MS/MS in either plasma or urine, were detected by UHPLC-MS/MS.

We can therefore report an efficient performance by the validated UHPLC method, as it allowed the identification of phenolic compounds or metabolites undetected by the HPLC system, due to the improvement in the limits of detection and quantification for almost all compounds.

4. Experimental Section

4.1. Chemicals

Caffeic acid, 5-caffeoylquinic acid, dihydrocaffeic acid, 3,4-dihydroxyphenylacetic acid, ethylgallate (internal standard (IS)), ferulic acid, hippuric acid, homovanillic acid, 3-hydroxyphenylacetic acid, 3-hydroxybenzoic acid, isoferulic acid, naringenin, naringenin glucoside, *p*-coumaric acid, protocatechuic acid, quercetin-3-*O*-glucuronide and rutin were purchased from Extrasynthese (Genay, France); 3-(3-hydroxyphenyl)propionic acid, human plasma, phenylacetic acid and quercetin were supplied by Sigma-Aldrich (St Louis, MO, USA); and 4-hydroxyhippuric acid was purchased from PhytoLab GmbH & Co. KG. (Vestenbergsgreuth, Germany). The purity of all standards was superior at 90%. All reagents were of HPLC grade: ethanol (EtOH), acetonitrile (MeCN), methanol (MeOH), and *o*-phosphoric acid 85% were purchased from Panreac Quimica S.A. (Barcelona, Spain); and formic acid was from Scharlau Chemie S.A. (Barcelona, Spain). Ultrapure water (Milli-Q) was obtained from a Millipore system (Millipore, Bedford, MA, USA).

4.2. Method Development

4.2.1. UHPLC Column

For the development of a faster chromatographic method, smaller particles and inner column diameters are needed [37,38]. A Waters BEH C_{18} column (50 mm × 2.1 mm) packed with 1.7 μm particles using an Acquity UPLC BEH C_{18} VanGuard pre-column 1.7 μm (2.1 mm × 5 mm) was selected for the development of the UHPLC method instead of the Luna C_{18} (50 mm × 2.0 mm) of 5 μm used for the analysis in the HPLC method [21].

4.2.2. Mobile Phase

The method validated in the HPLC-MS/MS system was adjusted to be used in the UHPLC equipment. To obtain a better separation and resolution of the analytes, an aqueous mix of 15 commercial phenolic compounds (100 ng/mL final concentration) were analyzed, using the

Molecules **2015**, *20*, 20409–20425

following mobile phase combinations: (1) [A] H_2O (0.1% formic acid)/[B] MeCN (0.1% formic acid); (2) [A] H_2O (0.05% formic acid)/[B] MeCN (0.05% formic acid); and (3) [A] H_2O (0.025% formic acid)/[B] MeCN (0.025% formic acid).

4.2.3. MS Conditions

An API 3000 triple-quadrupole mass spectrometer (Sciex, Framingham, MA, USA) with a turbo ion spray source controlled by Analyst v.1.4.2 software supplied by Sciex (Framingham, MA, USA, version 1.4.2) was used for infusion experiments. 50:50 (v/v) of water (0.025% formic acid)/MeCN (0.025% formic acid) was employed for infusion experiments, injecting each phenolic standard individually at a concentration of 1 μg/mL. The Turbo Ion spray source was used in negative mode with the following settings: capillary voltage, −3500 V; nebulizer gas (N_2), 10 (arbitrary units); curtain gas (N_2), 12 (arbitrary units); drying gas (N_2) was heated to 400 °C and introduced at a flow rate of 5000 cm^3/min. Table 2 shows the optimal DP, FP, and EP to enhance the ESI detection of the target phenolics. Multiple reaction monitoring (MRM) experiments in the negative ionization mode were performed using a dwell time of 30 ms, with 434 cycles and between 10 to 14 data points on the chromatographic peaks. The ions in MRM mode were produced by collision-activated dissociation (CAD) of selected precursor ions in the collision cell of the triple quadrupole and analyzed with the second analyzer of the instrument. The optimum collision-activated dissociation (N_2) was 4 (arbitrary units). The transition chosen for the quantification and confirmation are shown in Table 2 with its appropriate CE.

4.2.4. Flow Rate and Volume of Injection

Once obtained the best mobile phase and the optimum mass conditions for each analyte, several flow rates, 400, 600 and 800 μL/min, were studied at the same time that the volume of injection comparing 2, 5 and 10 μL to enhance the separation and detection of the analytes.

4.2.5. Chromatographic Separation

The final mobile phase used was water (A) and MeCN (B) with 0.025% formic acid in both solvents. An increasing linear gradient of B was used (t (min), %B), as follows: (0.0, 5); (2.0, 25); (2.5, 90); (2.65, 100); (2.8, 100); (2.9, 5), and (3.5, 5). The mobile-phase flow rate for the biological samples was 600 μL/min, and 10 μL of the sample was injected into the UHPLC system.

4.3. Quality Parameters

The method was validated following the criteria of AOAC International [24]. The quality parameters established were LOD, LOQ, linearity, recovery, accuracy, precision, and stability.

LOD is the smallest quantity of analyte that can be shown to be significantly greater than the measurement error of the blank at a prescribed level of confidence. The LOD was estimated from the chromatograms of spiked blank plasma and urine samples at the lowest analyte concentration tested for a signal-to-noise ratio of 3. Similarly, LOQ, the smallest amount of analyte in a test sample that can be quantitatively determined with suitable precision and accuracy, was determined for a signal-to-noise ratio of 10. Spiked plasma and urine samples at five different concentration levels, ranged between 0.05 and 300 ng/mL, were prepared in triplicate in order to establish the LOD and LOQ in the different mass spectrometric systems.

The IS method was used for the preparation of the calibration curves using eight different concentrations within the range of the LOQ for each analyte to 576 times the LOQ. In order to obtain the most reliable calibration curve, a 1/x or 1/(x × x) weighting factor, or none, was applied, according to the analyte. The calculated standard concentration was established within 15% deviation from the nominal value except at the LOQ concentration, for which the maximum acceptable deviation was set at 20%.

Molecules **2015**, *20*, 20409–20425

Recovery was assessed by preparing eight-point calibration curves (pre-extracted spiked samples) and eight-point external curves (post-extracted spiked samples). To calculate recovery, concentration must first be computed by interpolating areas obtained from the post-extracted spiked samples into the pre-extracted spiked calibration curve. Then, the ratio analyte concentration/IS concentration was plotted against the calculated concentration explained above and a linear regression model was applied. The slope of the linear regression multiplied by 100 represents the analyte recovery.

Accuracy was determined by spiking blank urine and plasma with three known concentrations: low (3-fold the LOQ), medium (48-fold the LOQ), and high (288-fold the LOQ), with respect to the calibration curves, in five replicates. The results were expressed as the percentage of the ratio of the mean concentration observed and the known spiked concentration in the biological matrices. The mean value should be within 15% of the nominal value. Intra- and interday precision was assessed using five determinations per three concentration levels (low (3-fold the LOQ), medium (48-fold the LOQ), and high (288-fold the LOQ)) in a single analytical run or on three different days, respectively. The precision determined at each concentration level should not exceed 15% of the relative standard deviation (RSD).

The chemical stability of an analyte in a given matrix under specific conditions for given time intervals is assessed in several ways. Stability evaluations should cover the expected sample handling and storage conditions during the length of the study. The factors studied in this method were freeze-thaw cycle stability and processed sample stability.

4.4. Method Application: Pilot Dietary Intervention Study

4.4.1. Biological Material

The optimized method was applied to a small-scale prospective single-arm intervention study conducted in eight volunteers aged between 19 and 38 years (28 $\pm$ 6.9 years) with a mean body mass index of 23 $\pm$ 3.73 kg/m^2. On the day of intervention, the volunteers consumed 250 mL of tomato sauce per 70 kg of body weight. Blood was collected 1 h and urine 3–6 h after the consumption of the intervention and stored at -80 °C until analysis.

Commercial tomatoes (*Lycopersicum esculentum* L.) were used for the elaboration of the tomato sauce at the Torribera Campus, University of Barcelona (Santa Coloma de Gramanet, Barcelona) following a standardized making process [36].

The study protocol was approved by the Ethics Committee of Clinical Investigation of the University of Barcelona (Spain), and the clinical trial was registered at the International Standard Randomized Controlled Trial Number (ISRCTN20409295). Informed consent was obtained from all participants.

4.4.2. Phenolics of Tomato Sauce and Biological Samples Extraction

A liquid-liquid extraction with ethanol/H_2O (0.1% formic acid) (80/20, v/v) was used to extract the phenolic compounds from the tomato sauce, as previously described by Di Lecce *et al.* [7]. Briefly, tomato sauce (0.3 g) was weighed and ethanol/H_2O (0.1% formic acid) (80/20, v/v, 3 mL) added. The homogenate was sonicated for 5 min and centrifuged at 4000 rpm for 20 min at 4 °C. The supernatant was collected, and the extraction procedure was repeated. Both supernatants were combined and the ethanol phase evaporated under a stream of nitrogen gas. The residues were reconstituted up to 1.2 mL with water containing 0.1% formic acid, filtered thought a 0.22 µm polytetrafluoroethylene (PTFE) syringe filters (Waters Corporation, Mildfore, MA, USA), and injected into the UHPLC-MS/MS system. Extractions were performed in triplicate and quantified with the corresponding commercial standards. When standards were not available, as in the case of di-, tricaffeoylquinics and the hexoside isomers, the compounds were quantified based on the free form of the corresponding metabolite.

Molecules **2015**, *20*, 20409–20425

Phenolic compounds were extracted from both plasma and urine samples by solid phase extraction (SPE) as previously described by our research group with minor modifications [21]. Prior to the SPE, plasma and urine samples were acidified with *o*-phosphoric acid and formic acid, respectively, and urine samples were centrifuged at 11,884 rpm for 4 min at 4 °C. Then, MeOH (1 mL) and 1.5 M formic acid (1 mL) was added to activate the HLB plate 30 μm (30 mg). Plasma or urine sample (1 mL), previously acidified and spiked with ethyl gallate (IS), was loaded into the 96-well plate for clean-up with 1.5 M formic acid (1 mL) and 5% MeOH solution (1 mL). The elution was achieved with MeOH (1 mL) acidified with 0.1% formic acid. The elution fraction obtained was evaporated to dryness by a sample concentrator (Techne, Staffordshire, UK) at room temperature under a stream of nitrogen. 100 μL of water acidified with 0.1% formic acid was added to dissolve the residue and filtered through a 0.22 μm polytetrafluoroethylene (PTFE) syringe filters (Waters Corporation).

5. Conclusions

We have validated an UHPLC-MS/MS method to determine tomato phenolics and their metabolites in biological samples with a previous solid phase extraction capable of analyzing a high number of samples in a short period of time. To our knowledge, this is the first method reported for the rapid detection and quantification of tomato sauce phenolics and their microbiota-derived metabolites in plasma and urine samples. The method offers excellent sensitivity, reproducibility and recovery. This procedure, due to its rapidity and simplicity, can be applied in future clinical and epidemiological studies with a high number of blood and urine samples.

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Author Contributions: R.M.L.-R. and R.E. designed the research; X.T. elaborated the tomato sauce; P.V.-M. recollected the plasma and urine of the volunteers; M.M.-H., S.T. and A.V.-Q. conducted the research; O.J. provided technical support; M.M.-H. analyzed the data; and M.M.-H. wrote the paper. All authors read and approved the final manuscript.

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

Abbreviations

Acetonitrile (MeCN); collision-activated dissociation (CAD); declustering potential (DP); electrospray ionization (ESI); entrance potential (EP); ethanol (EtOH); focusing potential (FP); high performance liquid chromatography (HPLC); high performance liquid chromatography coupled to mass spectrometry in tandem mode (HPLC-MS/MS); internal standard (IS); limit of detection (LOD); limit of quantification (LOQ); mass spectrometry (MS); methanol (MeOH); multiple reaction monitoring (MRM); neutral loss (NL); polytetrafluoroethylene (PTFE); precursor ion scan (PrIS); product ion scan (PIS); relative standard deviation (RSD); retention time (RT); solid-phase extraction (SPE); ultra-high performance liquid chromatography (UHPLC); ultra-high performance liquid chromatography coupled to mass spectrometry in tandem mode (UHPLC-MS/MS).

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Sample Availability: Not available.

4.6. Publicación 6. La biodisponibilidad de los polifenoles del tomate se ve reforzada por el procesado y la adición de grasa: evidencia de un ensayo de alimentación aleatorizado.

Bioavailability of tomato polyphenols is enhanced by processing and fat addition: evidence from a randomized feeding trial. **Miriam Martínez-Huélamo**, Anna Vallverdú-Queralt, Giuseppe Di Lecce, Palmira Valderas-Martínez, Sara Tulipani, Olga Jáuregui, Elvira Escribano, Ramón Estruch, Montse Illan and Rosa M. Lamuela-Raventós. *Molecular Nutrition and Food Research* (2015). En revisión.

El tomate contiene una variedad de compuestos fenólicos que han sido asociados a propiedades beneficiosas en la salud. Sin embargo, el efecto del procesado y la adición de aceite durante la preparación de salsas de tomate en su biodisponibilidad todavía no están claros. Los estudios de intervención en humanos presentan una gran variabilidad interindividual, por lo que se necesita un elevado número de individuos para obtener resultados relevantes. Por este motivo, se decidió realizar un ensayo controlado, abierto, aleatorizado y cruzado con cuarenta individuos sanos para analizar en plasma y orina los niveles de los compuestos fenólicos de tomate y sus metabolitos después del consumo de tomate y salsas de tomate, con y sin la presencia de aceite de oliva refinado durante su producción. Las salsas, como en los estudios anteriores, fueron elaboradas a partir de tomate comercial (liso rojo rama) en el Campus de Torribera de la Universidad de Barcelona. Los voluntarios no debían tener antecedentes de enfermedades cardiovasculares, ni alteraciones homeostáticas ni enfermedades relevantes para ser incluidos en el estudio. Se excluyeron los voluntarios hipertensos, dislipidémicos, fumadores, con alergias o intolerancia al tomate y con dificultad para cambiar los hábitos alimentarios. El diseño del estudio, muy similar al de la publicación 4, se muestra en la **Tabla 8**.

Tabla 8. Diseño del estudio

Día -3	Dieta sin tomate	
Día -2	Dieta sin tomate	
	No suplementos vitamínicos	
	No medicamentos	
Día -1	Dieta exenta de polifenoles	
	No suplementos vitamínicos	
	No medicamentos	
	Estar en ayuno las 10-12 horas previas al día 0	
Día 0: Estudio	t = 0 h	Extracción de sangre Recogida de orina
	Intervención: 250 g de salsa de tomate con aceite de oliva refinado, salsa de tomate sin aceite de oliva refinado y 500 g de tomate fresco por cada 70 Kg de peso del voluntario.	
	t = 15 min	Extracción de sangre
	t = 30 min	Extracción de sangre
	t = 1 h	Extracción de sangre
	t = 2 h	Extracción de sangre
	t = 3 h	Extracción de sangre
	t = 4 h	Extracción de sangre Recogida de orina
	t = 6 h	Extracción de sangre
	t = 8 h	Extracción de sangre Recogida de orina
	t = 12 h	Recogida de orina
Día 1	t = 24 h	Extracción de sangre Recogida de orina

Una vez recogidas las muestras biológicas se analizaron por el método previamente validado por UHPLC-MS/MS (**publicación 5**) así como las salsas y el tomate analizados también por UHPLC-MS/MS validado anteriormente (**publicación 4**).

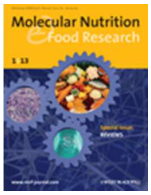
Los resultados, tras el análisis de las intervenciones, confirman lo demostrado en previas publicaciones. Los principales compuestos fenólicos encontrados pertenecen a los ácidos fenólicos, flavanonas y flavonoles, siendo el ácido homovanílico hexósido, la naringenia y rutina los mayoritarios respectivamente. No existen diferencias entre las salsas pero sí entre salsas y tomate, presentando una concentración superior en diferentes compuestos, sobre todo la naringenina y la rutina en las salsas, gracias a la liberación de estos compuestos de la matriz del alimento mediante el tratamiento mecánico.

Se encontraron diez compuestos fenólicos en el plasma y noventa y tres metabolitos en orina. La adición de aceite de oliva refinado a la salsa de tomate se tradujo con una

mejora en la biodisponibilidad de flavanonas, flavanoles y algunos metabolitos de los ácidos hidroxicinámicos mediante el aumento de su vida media plasmática y posiblemente la estimulación de su reabsorción por circulación enterohepática. Una amplia variedad de metabolitos microbianos intestinales también se detectaron después de las tres intervenciones, entre los que destacan las flavanonas, ácidos hidroxicinámicos, flavonoles, ácidos hidroxifenilpropiónicos, ácidos hidroxifenilacéticos y ácidos hidroxibenzoicos. Las flavanonas y flavonoles presentaron mayor biodisponibilidad después de la ingesta de la salsa de tomate con aceite de oliva, lo que sugiere que el tratamiento realizado al tomate fresco mejoró la absorción de la aglicona aumentando así su bioaccesibilidad.

En conclusión, el tratamiento mecánico y la adición de una matriz lipídica a las salsas de tomate implica un incremento en la biodisponibilidad de los compuestos fenólicos naringenina y quercetina contenidos en el fruto, favoreciendo de esta forma los efectos beneficiosos de los compuestos sobre las células y tejidos, impidiendo el desarrollo de enfermedades crónicas. Sería necesario desarrollar un mayor número de investigaciones en el que se evaluara el efecto matriz más extensamente en otras frutas y hortalizas.

Molecular Nutrition and Food Research



Bioavailability of tomato polyphenols is enhanced by processing and fat addition: evidence from a randomized feeding trial

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	Carlos III,
Keywords:	bioavailability, microbiota metabolites, naringenin, olive oil, tomato sauce

Bioavailability of tomato polyphenols is enhanced by processing and fat addition: evidence from a randomized feeding trial.

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ABBREVIATIONS USED

Concentration-*versus*-time curve from time 0 until the last detectable concentration (AUC_{last}); mean body mass index (BMI); body weight (BW); caffeic acid (CA); maximum plasma concentration (C_{max}); m, o, *p*-coumaric acids (CouA); 5-caffeoylquinic acid (5-CQA); dihydrocaffeic acid (DHCA); 3,4-dihydroxyphenylacetic acid (DHPAA); declustering potential (DP); entrance potential (EP); ethanol (EtOH); ferulic acid (FA); focusing potential (FP); glucuronides (G); hippuric acid (HA); 3-hydroxybenzoic acid (3-HBA); 4-hydroxybenzoic acid (4-HBA); hydroferulic acid (HFA); 4-hydroxyhippuric acid (4-HHA); 3-hydroxyphenylacetic acid (3-HPAA); high performance liquid chromatography coupled to Orbitrap mass spectrometry (HPLC-Orbitrap); 3-(3-hydroxyphenyl)propionic acid (3-(3HPPA)); 3-(4-hydroxyphenyl)propionic acid (3-(4HPPA)); homovanillic acid (HVA); isoferulic acid (IFA); isorhamnetin (IR); ethylgallate (internal standard (IS)); acetonitrile (MeCN); methanol (MeOH); Ultrapure water (Milli-Q); mean residence time (MRT_{last}); mass spectrometry (MS); naringenin (N); naringenin-7-*O*-glucuronide (NG); naringenin-7-*O*-glucoside (N7Glu); tomato sauce without refined olive oil (oil free: OF); protocatechuic acid (PA); phenylacetic acid (PAA); polytetrafluoroethylene (PTFE); quercetin (Q); quercetin-3-*O*-glucuronide (Q G); cumulative amounts of compounds excreted in urine ($Q_{u\infty}$); rutin (R); tomato sauce enriched with refined olive oil (ROOE); retention time

(rt); sulfates (S); standard deviation (SD); solid-phase extraction (SPE); time needed to reach the maximum plasma concentration (t_{max}); and ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS).

Keywords: bioavailability; microbiota metabolites; naringenin; olive oil; tomato sauce.

Abstract

Scope: Tomato contains a variety of phenolics associated with health-promoting properties. However, the effects of processing and the addition of oil during tomato sauce preparation on microbial metabolism of phenolics in the small intestine are still unclear.

Methods and results: An open, controlled, randomized and crossover feeding trial with forty healthy volunteers was carried out to analyze the metabolites in plasma and urine after the consumption of tomato and tomato sauces, with (ROOE) and without refined olive oil (oil-free: OF). Ten phenolics in plasma and ninety-three metabolites in urine were quantified. Processing tomatoes into sauce enhanced the bioavailability of flavanones, flavanols and some hydroxycinnamic acids, as reflected by the increase in the area under the plasma *vs.* time curve. An increase in their plasma half-life was also observed, particularly after ingestion of ROOE, possibly favored by enterohepatic circulation. A wide variety of gut microbial metabolites were also detected, namely flavanones, hydroxycinnamic acids, flavonols, hydroxyphenylpropanoic acids, hydroxyphenylacetic acids, and hydroxybenzoic acids.

Conclusions: Flavanones and flavonols in ROOE presented higher bioavailability, suggesting that the processing undergone by the raw tomato improved their absorption.

1. Introduction

Tomatoes, one of the most commonly consumed agricultural commodities, are rich in bioactive compounds like fibre, polyphenols, glycoalkaloids, vitamins C and E, and carotenoids[1–4]. The consumption of polyphenols is related to a decrease in cardiovascular events and mortality[5], but they have to reach the target tissues at an effective concentration to exert a protective effect[6]. The bioavailability of some flavonoids is impaired by their low water solubility, low absorption, rapid excretion, and/or extensive metabolism [6–9] by enzymes and gut microbiota.

There is only scant information available on the impact of mechanical and thermal treatments during tomato sauce processing on the bioavailability of tomato phenolics after consumption[10]. In previous work, our research group has shown that the processing can enhance the bioavilability of some phenolic compounds, so we decided to perform a new study, this time with a significantly higher number of volunteers and targeting an extensive number (19) of previously unstudied phenolics of microbial origin. The starting hypothesis was that the more lipophilic phenolics, such as flavonols and flavanones, would benefit from the mechanical and/or thermal treatments during tomato sauce processing, particularly from the addition of a lipid emulsifier (refined olive oil). In contrast, more hydrophilic compounds, such as hydroxycinnamic acids, would not increase their availability and potential bioefficacy. To verify the hypothesis, in an open, controlled, randomized and crossover feeding trial with forty healthy volunteers, phenolic metabolites were quantified by UHPLC-ESI-QqQ-MS/MS and identified by HPLC-Orbitrap to investigate if the processing of tomatoes, with or without the addition of olive oil, influences phenolic bioavailability and the metabolic profile.

2. Materials and Methods

2.1. Standards and Reagents

Phenolic standards were purchased from Extrasynthèse(Genay, France), Sigma-Aldrich(St Louis, MO, USA), PhytoLab GmbH & Co. KG.(Vestenbergsgreuth, Deutschland) and Cayman Chemical Company (Michigan, USA). Reagents were supplied by Panreac Quimica S.A. (Barcelona, Spain) and Scharlau Chemie S.A. (Barcelona, Spain). More details are given in the Supporting Information.

2.2.Tomato interventions

Commercial tomatoes were used for the tomato intervention and the tomato sauces were prepared following a standardized manufacturing process[10]. In the tomato sauce preparation, 5% of refined olive oil (ROOE), or an equivalent amount of water (oil free: OF) were added.

2.3. Volunteers

Forty volunteers with a mean age of 28±11years and BMI 23.3±3.8kg/m^2 completed the study. All participants reported no cardiovascular, hepatic or renal disease, were non-smokers and were not receiving any nutritional supplements. The study was explained to participants through verbal and written instructions, and written informed consent was obtained before participation.

2.4. Study design

The study was an open, controlled, randomized and crossover feeding trial. Three days before the intervention, the volunteers were asked not to consume tomatoes or tomato derivatives, while polyphenol consumption was avoided 24h before as well as during the day of the study. After 8 hours of fasting, volunteers randomly consumed: 500g of tomato/70kg BW and 250g of OF or ROOE/70kg BW. Blood samples were collected before the intervention (0h), and after the consumption of tomato or tomato sauces at:

15min, 30min, 1h, 2h, 3h, 4h, 6h, and 24h. Samples were collected in plasma EDTA tubes, centrifuged at 1500g for 20min at 4ºC and stored at –80ºC until the analysis. Urine samples were also collected at: 0h, 0-3h, 3-6h, 6-12h, and 12-24h. After measuring the volume of urine excreted, samples were stored in eppendorfs at –80ºC until the analysis.

The study protocol was approved by the Ethics Committee of Clinical Investigation of the UB, and the clinical trial was registered at the International Standard Randomized Controlled Trial Number (ISRCTN20409295).

2.5. Extraction and analysis of tomato, tomato sauces and biological samples

Tomato and tomato sauces administered in the study were extracted by a liquid-liquid extraction and analyzed by UHPLC-MS/MS, as reported before[11]. Plasma and urine samples were extracted by a solid-phase extraction (SPE) validated by our working group using a Waters Oasis HLB 96-well plate 30µm (30mg) (Waters Oasis, Milford, MA, USA) and analyzed by UHPLC-MS/MS and UHPLC-LTQ-Orbitrap[12]. More details of the chromatographic separation and mass conditions are given in the Supporting Information.

2.6. Pharmacokinetic analysis

Pharmacokinetic parameters were determined by a non-compartmental analysis using WinNonlin Professional software version 3.3 (Pharsight Corporation, USA). The maximum plasma concentration (C_{max}) and the time needed to reach it (t_{max}) were obtained directly from the individual maximum values and corresponding time. The area under the plasma concentration-*versus*-time curve from time 0 until the last detectable concentration (AUC_{last}) was calculated by the linear trapezoidal method. The mean residence time (MRT_{last}) was estimated by means of the AUMC/AUC ratio, where AUMC was the first moment curve. The quotient C_{max}/AUC_{last} and maximum

cumulative amounts of phenolic compounds excreted in urine ($Q_{u\infty}$) were also calculated.

2.7. Statistical Analysis

Statistical analysis was performed using the SPSS software (Version 19.0, Japan Inc., Tokyo, Japan). Data are expressed as means±SD. Differences were considered significant at $p<0,05$. The tests applied are explained in the Supporting Information.

3. Results and discussion

3.1. Phenolic compounds of tomato and tomato sauces

Table 1 summarizes the 14 phenolic compounds quantified in the three interventions. According to the literature, naringenin (N), rutin (R) and 5-caffeoylquinic acid (5CQA) are the major flavonoids in tomato fruit[2, 13–15]. In our investigation, homovanillic acid (HVA) hexoside (H), followed by N and R, were the dominant polyphenols quantified in the three interventions. N content was within 4788 and 7483ng/g FW, and R within 2216 and 5389ng/g FW, which closely matches the data of other studies[16, 17]. In contrast, few investigations have identified HVA-H as a predominant phenolic compound in tomato[1, 4], unlike in our analysis, where it was quantified at levels between 93537 and 138340ng/g FW. Besides HVA-H, other phenolic acids found at a high percentage in the three interventions were 5CQA, caffeic acid (CA)-H-I and ferulic acid (FA)-H, with maximum values of 1075 and 1572ng/g FW for 5CQA and CA-H, respectively, in raw tomatoes, and 3091ng/g FW for FA-H in OF. The 5CQA concentration fell within the range reported by Minoggio *et al.*[18], who obtained values between 300 and 5800ng/g FW in tomato.

The processing of tomato into sauces resulted in a quantitative but not qualitative change in the polyphenolic content, with the exception of FA, which appeared only in raw tomato, and protocatechuic acid (PA), which was found only in tomato sauce.

HVA-H, N and R concentrations were higher in the tomato sauces than tomato, but without significant differences ($p>0.05$), and the opposite pattern was observed for 5CQA, 4CQA and coumaric acid (CouA)-H-I. Similarly, Capanoglu *et al.*[3] described an increase of N and R when tomatoes were processed into paste. This increase, which occurred after the fruit was cut, could be induced by the production of enzymes involved in the regeneration of antioxidant compounds[3]. The use of refined olive oil in tomato sauce preparation slightly enhanced the concentration of phenolic compounds in ROOE but without significant differences ($p>0.05$), as reported by Vallverdú-Queralt *et al.*[19].

3.2. Identification of phenolic metabolites in human plasma and urine

In order to obtain a correct identification of phenolic metabolites, especially those lacking a corresponding commercial standard, a UHPLC-LTQ-Orbitrap was used. **Table 2** shows the fragmentation of the targeted compounds (mass errors<1.8mDa). **Figure 1** shows the mass spectra of 3-(4-hydroxyphenylpropionic acid) (HPPA) sulfate (S), *m/z* 245.0119 (A) and dihydrocaffeic acid (DHCA) glucuronide (G), *m/z* 357.0821 (B), as the main fragments. CA-G (*m/z* 355.0665) and CA-S (*m/z* 258.9912) were confirmed by the loss of the glucuronide or sulfate unit, CA appearing with an ion at 179 and 135*m/z*. The ions at 311 and 215*m/z* in CA-G and CA-S, respectively, were due to the loss of CO_2 $[M-H-44]^-$[20]. This typical loss was also found in CouA-S (*m/z* 242.9963), with the appearance of an ion at 199*m/z* as well as 163 and 119m/z, corresponding to CouA and its fragmentation. FA-G (*m/z* 369.0821) and FA-S (m/z 273.0069) were confirmed mainly by the presence of 193 and 134*m/z*, corresponding to FA. The fragmentation of the glucuronide group was confirmed by the characteristic presence of 175 *m/z* and 113*m/z* [21, 22]. The mass spectrum of hydroferulic acid (HFA)-G (*m/z* 371.0978) and -S (*m/z* 275.0225) showed the presence of ions at 195 and

136 corresponding to the fragmentation of the resulting acid. The 175 and 113*m/z* in the HFA-G spectrum confirmed the presence of the G unit. The same was observed for 3-(4-HPPA)-G (*m/z* 341.0872) and 3-(4-HPPA)-S (*m/z* 245.0119), with fragments at 165 and 121*m/z* in both mass spectra corresponding to 3-(4-HPPA) and its fragmentation after the loss of the G and S units, respectively. Finally, 181 and 137*m/z* corroborated the presence of DHCA in the mass spectrum of DHCA-G (*m/z* 357.0821), and 187 and 151*m/z* were attributed to 3-hydroxyphenylacetic acid (3-HPAA) in the case of 3-HPAA-S (*m/z* 230.9963).

3.3. Bioavailability of flavanones and derivatives

As shown in **Figure 2A**, N-G appeared in plasma soon (15 min) after the consumption of tomato, OF or ROOE, indicating a rapid absorption of N, with a subsequent glucuronidation in the intestinal epithelium[23]. Recently, Orrego-Lagarón *et al.*[8] demonstrated in a single-pass intestinal perfusion model in mice that the main absorption and first-metabolism of N occurs in the small intestine. In our study, the rapid appearance of N and N-G in plasma was indicated by the t_{max} and C_{max}-to-AUC_{last} ratio (**Table 3**). Our results are similar to those of Bugianesi *et al.*[24], who reported a t_{max} of 2h after the ingestion of cooked tomato paste in men, and Guo *et al.*[25], who obtained a t_{max} of 0.5h in rats after the administration of Chinese medicine pills, containing N among other compounds.

Comparing interventions, N was more rapidly absorbed after the tomato processing, since the C_{max}-to-AUC_{last} ratio was lower and the t_{max} about 2-fold higher in raw tomato. Specifically, the C_{max}-to-AUC_{last} ratio for N-G was significantly different between tomato and the two sauces. N had a lower C_{max} than its glucuronide, in agreement with previous studies[10, 26], indicating that glucuronidation is an early step in N metabolism of N, accounting for 98% of plasma metabolites[27]. This phenomenon was

not reflected by the AUC, probably due to the high variability in the results and the biphasic shape of the plasma curve, which can in some cases overestimate it.

The C_{max} of both N and N-G was higher after the consumption of the sauces than raw tomatoes, suggesting that the processing improved the absorption of the aglycone and increased its bioaccessibility[10]. The addition of an oil matrix to the sauce also seemed to be associated with the enhanced concentration of N found in biosamples, possibly due to the affinity of the compound to the lipophilic constituents of the olive oil. Ban *et al*.[7] used nanoparticles made up of physiological lipids to protect flavonoids (N, quercetin (Q) and hesperidin) from the harsh conditions in the digestive system until their absorption into enterocytes, thereby improving their bioaccessibility. Similarly, the refined olive oil used to elaborate ROOE may have acted as a lipid nanoparticle system, promoting N absorption. These results were corroborated by AUC_{last} values, which were highest in the ROOE intervention, but without significant differences, probably due to the variability among individual volunteers.

Returning to **Figure 2**, about 2h after the ingestion of ROOE, a double peak appeared in the N-G pharmacokinetics, as in a previous study[10]. This biphasic profile has also been reported in other studies, the second peak being attributed to a glucuronidation in the liver with the participation of UDP–glucuronosyltransferase. The subsequent enterohepatic circulation of N-G would produce the second peak in the plasma[23, 28].

Among the cumulative amounts of compounds excreted in urine ($Q_{u\infty}$), N-G showed higher values than N (**Table 3**), providing further evidence of the extensive metabolism of the aglycone, as observed in plasma. The improvement of N bioavailability after processing was also confirmed by the urine excretion data, with higher values for N and N-G after ingestion of OF and ROOE than raw tomato, the differences for N-G being significant.

3.4. Bioavailability of flavonols and derivatives

As mentioned before, R was one of the major compounds found in tomato and tomato sauces (**Table 1**), yet although its concentration in all the interventions was high, no peak was detected in plasma or urine samples. Intestinal microflora deglycosylate and remove the rutinoside moiety of R, leading to quercetin, which is absorbed, circulated in the blood and excreted into the bile and urine as G and S metabolites[29]. Q was quantified in plasma after the three interventions (**Table 3**), which indicates a greater bioavailability than N, considering the high content of N in tomato. The C_{max} and AUC_{last} of Q after sauce ingestion were high, slightly more so for ROOE, which showed significant differences in AUC_{last} with tomato. As Q is a lipophilic compound[29], the addition of a fatty matrix to the sauce may increase its bioavailability and enhance its presence in plasma. An increase in Q was also observed for OF, although without significant differences, indicating that the mechanical and thermal process enhanced flavonol bioavailability. Wang *et al.*[28] reported that flavanols have slow rates of absorption due to their low solubility in the small intestine. Similarly, in our study the rate of absorption of Q in the three interventions was slow, with a high MRT in plasma. The cumulative amounts of Q excreted in urine after all the interventions were low in comparison with flavanones and hydroxycinnamic acids (**Table 3**). Q-G and Q-S metabolites were found in urine, but in low concentrations, similar to the aglycone. Significant differences in Q concentration were obtained between tomato and ROOE and in Q-S between tomato and OF.

Finally, **Figure 2C** shows the mean plasma concentration of Q versus time in the three interventions. As already reflected by the other pharmacokinetic results, sauce consumption resulted in a higher Q plasma concentration than tomatoes. Double peaks were common among the three interventions, which may be explained by enterohepatic

circulation, as in the case of N-G. Metabolites, excreted in the bile, are secreted into the small intestine where they undergo deconjugation by the intestinal microflora, followed by reabsorption in the gut and portal blood circulation[28, 29]. Another possible explanation may be the aforementioned transformation of R into Q. As reported by Shimoi *et al.*[29], R is absorbed more slowly than Q, so the second peak in the plasma samples might correspond to its conversion.

3.5. Bioavailability of hydroxycinnamic acids and derivatives

Analysis of plasma and urine revealed the presence of 7 hydroxycinnamic acid derivatives in plasma and 31 metabolites in urine. Isoferulic acid (IFA), four FA-G and two FA-S were identified and quantified in plasma samples (**Table 3**). T_{max} values were slightly higher than those obtained for N or N-G, particularly after the ingestion of ROOE. Specifically, FA-G-II and -III presented significant differences between tomato and ROOE. FA was not detected in tomato, OF or ROOE (**Table 3**), but it can be derived from the metabolism of CA, which would explain its delayed appearance in plasma. Moreover, as FA is a hydrophilic compound[30], the presence of a lipid matrix would not enhance its absorption, resulting in an increment of t_{max}. A slower rate of appearance was observed for almost all metabolites except FA-G-II and -III, which had similar rates to flavanones. The metabolites that remained in the organism longest were IFA, FA-G-I, FA-G-IV and FA-S, regardless of intervention. Only in the case of FA-G-II were there significant differences between ROOE and tomato and OF. In general, the AUC_{last} and C_{max} were higher in the sauces than raw tomato for almost all the metabolites but without significant differences. **Figure 2B** shows the mean plasma concentration of the four FA-G *vs.* time for ROOE, the only intervention in which all the metabolites were reabsorbed. As for N-G, a double peak was produced by the four

isomers, which could be attributed to enterohepatic circulation, as reported by Zaho *et al.*[31] in Wister male rats.

Although a large variety of CQA was supplied in each intervention, no presence of those compounds was detected in plasma, and only low concentrations of 5CQA in urine[32]. Stalmach *et al.*[33] studied the bioavailability of 5CQA after the administration of coffee to 11 healthy volunteers, concluding that the compound had a reduced bioavailability associated with the dose ingested, and provided an extensive variety of CA, FA, DHCA and dihydroferulic acid(DHFA) metabolites, as in the present work. Neither CA nor CouA, nor their metabolites, were found in plasma, probably being below the limit of detection of the method, as they appeared in urine samples.

Figure 3 shows the phenolics and the sum of metabolites of each acid in urine in the three interventions. All free forms underwent an extensive metabolism to their G and S forms if the concentration of the aglycone was compared with the resulting metabolites. The compounds with the highest excretion were FA metabolites, probably due to the metabolism of 5CQA and CA[33, 34]. Significant differences were obtained in FA between tomato and OF, but their metabolites showed similar excretion among the three interventions. On the contrary, a clear increasing trend of CA-G excretion occurred after ingestion of ROOE, but due to considerable inter-individual variability, no significant differences were found.

3.6. Microbial metabolites

A wide variety of microbial metabolites of flavanones, hydroxycinnamic acids and flavonols were found in urine samples. **Figure 4** represents the main metabolic pathway tentatively proposed for the microbiota derivatives of the phenolic compounds present in tomato and tomato sauces. The C-ring cleavage of N yielded 3-(4-HPPA), and subsequent 3-(4-HPPA) β-oxidation and dehydroxylation of 4-HPPA led to 3-

phenylpropionic acid (3-PPA). After glycination and β-oxidation of 3-(4-HPPA) in the liver, 4-hydroxyhippuric acid (4-HHA) was formed[8, 9]. 3-(4-HPPA) metabolites (2-G and 6-S) were also found in the three interventions. The sulfation and glucuronidation of 3-(4-HPPA) may occur in the liver after its absorption into the circulatory system[33].

Q presented a clear metabolism, as described in previous studies[9, 35]. The C-ring fission yielded 3,4-DHPA, 3-(3-HPPA) and phenylacetic acid (PAA), and two subsequent dehydroxylations of 3,4-DHPAA led to 3-HPAA, and then PAA[36]. 3-HPAA was absorbed by the colon, where it was β-oxidated and glycinated to 3-HHA. In addition, 6-G and 5-S of 3-HPAA and one S of PAA were also quantified in the samples.

Finally, metabolites of hydroxycinnamic acids were also found in urine samples. After a reduction of the double bond of CA, DHCA was obtained together with 8-G and 7-S metabolites after its passage through the liver. Subsequent dehydroxylation led to 3-(3-HPPA). After a double β-oxidation and posterior glycination in the liver, 3-HHA was formed[9]. HFA and 8-G and 4-S metabolites were also found in urine after the reduction of the double bond of FA. Successive demethylation and dehydroxylation resulted in 3-(4-HPPA). Another compound obtained in high quantities was HVA, which may have arisen directly from the cleavage of the hexoside group in the HVA-H found in tomato and sauces, or through the methylation of 3/4-HPAA[36].

As mentioned above, the sum of the G and S of each phenolic acid in urine after the three interventions is shown in **Figure 3**. In general, a change in the slope of the curves was observed, indicating an increase in the excretion of almost all the compounds 6-12h after the interventions, demonstrating their absorption and metabolism in the colon[33]. On the contrary, hydroxycinnamic acids, flavanones or flavanols were absorbed in the

small intestine. The higher urine concentrations of G and S metabolites than their aglycones indicate an extensive metabolism of phenolic compounds. Comparing interventions, few significant differences were obtained, only for HFA-G, HFA-S, 3,4-DHPAA and PAA-S between tomato and OF. These results could be attributed to individual variability, as different microbiota in each volunteer can result in a distinct transformation and absorption.

4. Conclusions

An extensive identification and quantification of phenolic compounds was achieved after the ingestion of tomato and tomato sauces with and without refined olive oil. Ten phenolics were quantified in plasma and ninety-three in urine, including flavanones, hydroxycinnamic acids, flavonols and microbiota metabolites. The bioavailability of N-G and Q was higher in ROOE than in tomato or OF, suggesting it may be enhanced by tomato processing as well as the addition of an oil matrix. A double peak in the plasma pharmacokinetics profile of N-G, Q and FA-G after the ingestion of ROOE indicated that the addition of a fatty matrix to tomato sauce may favour the reabsorption of the compounds through enterohepatic circulation. 55 microbial metabolites found in urine were derived from the ingestion of tomato and tomato sauces. Higher concentrations were obtained for the glucuronide and sulfate metabolites than for their aglycones but almost without significant differences, which was probably due to variability among the volunteers.

In conclusion, the mechanical treatment and addition of a fatty matrix to the tomato sauce enhanced the bioavailability of the phenolic compounds N and Q contained in the fruit, favouring their beneficial effects in cells and tissues. Further studies in which the matrix effect is extensively evaluated in other fruits and vegetables should be performed.

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FIGURE CAPTIONS

Figure 1.

MS^2 spectra of 3-(4-HPPA)-S(A) and DHCA-G(B).

Figure 2.

Mean plasma concentration *versus* time of N-G in the three interventions(A), FA-G-I, II, III and IV in ROOE(B) and Q in the three interventions(C).

Figure 3.

Cumulative urinary excretion curves for phenolics and the sum of metabolites of each acid in the three interventions.

Figure 4.

Metabolic pathway tentatively proposed for the microbiota derivatives of the phenolic compounds present in raw tomato and tomato sauces.

Table 1. Phenolic compounds in tomatoes and tomato sauces administered to the volunteers.

Compound	RT (min)	MRM (m/z)	TOMATO ng/g FW	OF ng/g FW	ROOE ng/g FW
3-Caffeoylquinic acid	1.07	353→191	290.4±12.1	219.3±3.1	508.9±28.9
5-Caffeoylquinic acid	1.26	353→191	1074.6±111.9	908.4±13.7	770.0±47.0
4-Caffeoylquinic acid	1.49	353→191	1146.9±16.7[a]	625.1±13.7	679.3±4.8[a]
Dicaffeoylquinic acid	1.91	515→353	14.1±0.4	0.2±0.2	3.0±0.8
Tricaffeoylquinic acid	2.06	677→515	99.5±3.8	19.0±0.8	21.0±2.4
Caffeic acid	1.45	179→135	509.6±22.0	582.4±2.7	634.6±23.2
Caffeic acid hexoside I	1.00	341→179	1572.0±23.1	1326.4±8.2	1363.7±42.6
Caffeic acid hexoside II	1.22	341→179	769.9±38.5	624.0±9.6	644.9±22.6
Ferulic acid	1.85	193→134	488.5±27.0	n.d.	n.d.
Ferulic acid hexoside	1.14	355→193	3084.2±90.3	3091.0±115.8	2456.2±29.0
Coumaric acid hexoside I	1.02	325→163	601.1±27.0	96.9±16.6	66.4±12.2
Coumaric acid hexoside II	1.31	325→163	433.3±12.2[a]	610.0±3.5	619.0±2.8[a]
Protocatechuic acid	0.98	153→109	n.d.	170.1±5.0	141.9±2.4

Homovanillic acid hexoside	1.25	343→181	93536.9±2935.6	138339.8±5170.2	127802.2±1760.3
Quantified phenolic acids orally administrated (µg)*			51811	36653	33928
Naringenin	2.26	271→151	4787.7±636.3	6647.1±34.8	7482.5±214.8
Naringenin glucoside	1.94	433→271	85.5±10.3	259.3±3.2	269.7±8.6
Quantified flavanones orally administrated (µg)*			2437	1727	1938
Quercetin	2.13	301→151	254.6±3.2	267.8±0.4	283.4±16.6
Rutin	1.77	609→300	2215.8±93.4	5388.7±15.1	5099.9±60.5
Quantified flavonols orally administrated (µg)*			1235	1414	1346

* Corresponding to the single dose administrated to the volunteers 500g tomato and 250g sauces. Results expressed as mean±SD. Values in a row with the same letters are significantly different(p<0.05). Retention time(RT), Multiple reaction monitoring(MRM), Oil free sauce(OF), Refined Olive Oil Enriched(ROOE), Fresh Weight(FW).

Table 2. Identification of phenolic metabolites through UHPLC-Orbitrap.

Compounds	RT (min)	Molecular Formula	Acc Mass	Error (mDa)	MS^2 ions
CA-G-I	1.45	$C_{15}H_{15}O_{10}$	355.0665	0.067	179,135
CA-G-II	1.56	$C_{15}H_{15}O_{10}$	355.0665	0.853	179,135
CA-G-III	1.84	$C_{15}H_{15}O_{10}$	355.0665	0.393	311,179,135
CA-G-IV	2.07	$C_{15}H_{15}O_{10}$	355.0665	0.633	311,179,135
CA-G-V	2.31	$C_{15}H_{15}O_{10}$	355.0665	0.273	311,179,135
CA-S- I	1.77	$C_9H_7O_7S$	258.9912	0.310	215,179,135
CA-S- II	1.87	$C_9H_7O_7S$	258.9912	0.280	215,179,135
CA-S- III	1.95	$C_9H_7O_7S$	258.9912	0.020	215,179,135
CA-S- IV	2.01	$C_9H_7O_7S$	258.9912	0.210	215,179,135
CouA-S-I	1.79	$C_9H_7O_6S$	242.9963	0.085	199,163,119
CouA-S-II	1.92	$C_9H_7O_6S$	242.9963	0.115	199,163,119
CouA-S-III	2.04	$C_9H_7O_6S$	242.9963	0.275	199,163,119
CouA-S-IV	2.12	$C_9H_7O_6S$	242.9963	0.495	199,163,119
FA-G-I	1.53	$C_{16}H_{17}O_{10}$	369.0821	0.203	193,175,149,134,113
FA-G-II	1.88	$C_{16}H_{17}O_{10}$	369.0821	0.933	351,193,175,134,113
FA-G-III	2.08	$C_{16}H_{17}O_{10}$	369.0821	1.183	351,325,193,175,134,113
FA-G-IV	2.17	$C_{16}H_{17}O_{10}$	369.0821	1.723	351,325,193
FA-S-I	1.87	$C_{10}H_9O_7S$	273.0069	0.950	193,149,134
FA-S-II	1.98	$C_{10}H_9O_7S$	273.0069	0.630	193,149,134
FA-S-III	2.10	$C_{10}H_9O_7S$	273.0069	0.460	193,149,134
DHCA-G-I	0.75	$C_{15}H_{17}O_{10}$	357.0821	0.382	181,137
DHCA-G-II	0.85	$C_{15}H_{17}O_{10}$	357.0821	0.258	181,175,166,113
DHCA-G-III	0.98	$C_{15}H_{17}O_{10}$	357.0821	1.632	181,175
DHCA-G-IV	1.03	$C_{15}H_{17}O_{10}$	357.0821	0.752	295,181,175,113

DHCA-G-V	1.12	$C_{15}H_{17}O_{10}$	357.0821	0.082	339,181,175,166,137,113
DHCA-G-VI	1.32	$C_{15}H_{17}O_{10}$	357.0821	0.038	313,175,137,113
DHCA-G-VII	1.39	$C_{15}H_{17}O_{10}$	357.0821	0.502	313,181,175,137,113
DHCA-G-VIII	1.52	$C_{15}H_{17}O_{10}$	357.0821	1.242	181,175,166,137,113
3-HPAA-S-I	0.69	$C_8H_7O_6S$	230.9963	0.066	187,151,107
3-HPAA-S-II	0.91	$C_8H_7O_6S$	230.9963	0.241	151,107
3-HPAA-S-III	1.00	$C_8H_7O_6S$	230.9963	0.054	187,151,143,133,107
3-HPAA-S-IV	1.12	$C_8H_7O_6S$	230.9963	0.686	213,187,151,107
3-HPAA-S-V	1.18	$C_8H_7O_6S$	230.9963	0.214	213,187,151,107
3-(4-HPPA)-G-I	1.78	$C_{15}H_{17}O_9$	341.0872	0.217	323,297,193,175,165,121,113
3-(4-HPPA)-G-II	1.85	$C_{15}H_{17}O_9$	341.0872	0.987	323,175,165,121,113
3-(4-HPPA)-S-I	0.70	$C_9H_9O_6S$	245.0119	0.414	227,165,121
3-(4-HPPA)-S-II	1.04	$C_9H_9O_6S$	245.0119	0.296	227,201,165,121
3-(4-HPPA)-S-III	1.26	$C_9H_9O_6S$	245.0119	0.414	165,121
3-(4-HPPA)-S-IV	1.32	$C_9H_9O_6S$	245.0119	0.284	227,165,121
3-(4-HPPA)-S-V	1.59	$C_9H_9O_6S$	245.0119	0.574	227,165,121,119
3-(4-HPPA)-S-VI	1.66	$C_9H_9O_6S$	245.0119	0.574	227,165,121,119
HFA-G-I	1.14	$C_{16}H_{19}O_{10}$	371.0978	0.137	353,327,195
HFA-G-II	1.41	$C_{16}H_{19}O_{10}$	371.0978	0.597	356,353,327,195
HFA-G-III	1.58	$C_{16}H_{19}O_{10}$	371.0978	0.223	353,195,175,151,136,113
HFA-G-IV	1.67	$C_{16}H_{19}O_{10}$	371.0978	0.903	353,195,175,113
HFA-G-V	1.75	$C_{16}H_{19}O_{10}$	371.0978	0.357	353,195,175,151,139,136,113
HFA-G-VI	1.83	$C_{16}H_{19}O_{10}$	371.0978	0.443	353,195,175,119,113
HFA-G-VII	1.91	$C_{16}H_{19}O_{10}$	371.0978	0.743	353,195,175,113
HFA-G-VIII	2.14	$C_{16}H_{19}O_{10}$	371.0978	0.713	353,195,175,151,139,113
HFA-S-I	1.44	$C_{10}H_{11}O_7S$	275.0225	0.300	195,136,135,134
HFA-S-II	1.58	$C_{10}H_{11}O_7S$	275.0225	0.090	195,136,135,134

Retention time(RT), Accurate mass(Acc mass), Caffeic acid(CA), Glucuronide(G), Sulfate(S), Coumaric acid(CouA), Ferulic acid(FA), Dihydrocaffeic acid(DHCA), Hydroxyphenylacetic acid(HPAA), Hydroferulic acid(HFA).	HFA-S-III	1.66	$C_{10}H_{11}O_7S$	275.0225	0.158	195,136,119
	HFA-S-IV	1.71	$C_{10}H_{11}O_7S$	275.0225	0.610	195,136,119

Table 3. Pharmacokinetics parameters in plasma and urine after the three interventions.

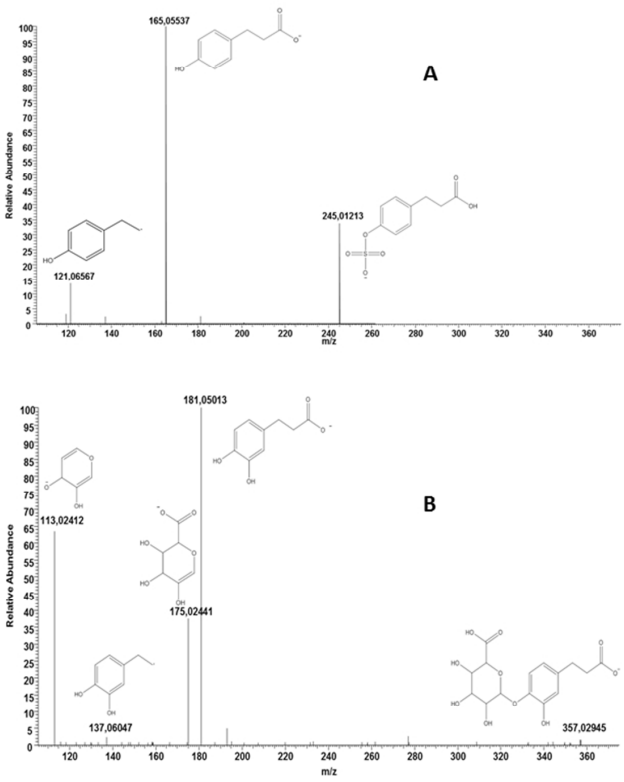
	Intervention	C_{max} nmol/L	t_{max} h	AUC_{last} nmol/L x min	MRT h	C_{max}-to-AUC_{last} ratio h^{-1}	Qu_{∞} µmol
N	Tomato	15.4±5.7[a,b]	1.4±1.8	185.4±143.8	10.7±5.0	0.2±0.4	0.8±1.3
	OF	21.7±17.4[a]	0.5±0.2	212.9±174,0	8.0±6.5	0.6±0.6	2.1±7.3
	ROOE	20.8±15.3[b]	0.6±0.3	223.8±167.1	7.6±5.7	0.4±0.4	4.3±16.0
N-G	Tomato	53.3±64.1[a]	2.1±1.9	109.1±105.5	3.2±0.6[a,b]	0.4±0.2[a,b]	10.5±45.0[a]
	OF	130.7±80.9	0.8±0.4	173.1±139.0	1.3±0.5[b]	1.0±0.6[a]	59.5±300.0[a,b]
	ROOE	150.2±96.7[a]	0.7±0.4	268.9±220.8	1.6±0.7[a]	0.8±0.5[b]	66.7±368.0[b]
FA	Tomato	n.d.	n.d.	n.d.	n.d.	n.d.	7.9±6.7[a]
	OF	n.d.	n.d.	n.d.	n.d.	n.d.	5.5±3.9[a]
	ROOE	n.d.	n.d.	n.d.	n.d.	n.d.	6.8±6.0
IFA	Tomato	194.5±154.4	1.0±0.9	550.2±527.5	5.8±7.1	0.5±0.3[a]	100.3±111.9
	OF	183.3±124.4	1.0±1.0	1066.8±1337.8	8.2±7.7	0.6±0.5	83.8±90.6
	ROOE	245.9±183.8	1.4±1.3	1396.0±1452.3	9.2±7.9	0.3±0.3[a]	105.6±128.1

FA-G-I	Tomato	89.1±78.1	2.6±2.0	875.3±926.5	8.1±4.3	0.2±0.1	46.3±43.1
	OF	102.9±108.8	2.9±2.2	1166.8±1328.3	9.7±4.7	0.1±0.1	33.4±33.7
	ROOE	114.2±102.0	3.6±2.2	1026.4±960.9	7.6±4.0	0.2±0.2	40.3±43.1
FA-G-II	Tomato	144.7±114.0	1.3±0.9[a]	249.4±272.1	1.9±1.2[b]	0.8±0.4	15.4±11.6
	OF	139.8±110.8	1.6±1.0	299.4±253.4	2.6±2.1[a]	0.6±0.3	14.5±10.9
	ROOE	188.4±172.8	2.8±1.6[a]	475.8±587.4	2.8±1.0[a,b]	0.6±0.3	16.3±14.2
FA-G-III	Tomato	51.9±44.2	1.0±0.7[a]	190.2±212.2	6.0±6.3	0.7±0.3	13.7±11.5
	OF	88.0±75.3	1.5±1.1	213.1±222.1	3.1±2.7	0.6±0.3	13.7±10.9
	ROOE	94.4±73.6	2.3±1.7[a]	122.0±84.4	4.3±4.2	0.9±0.5	13.5±11.2
FA-G-IV	Tomato	146.7±154.6	1.6±2.0	1267.3±1580.9	10.3±5.2	0.3±0.3	45.9±34.7
	OF	144.7±124.0	1.4±1.7	1293.8±1399.4	10.2±7.0	0.2±0.2	47.2±40.3
	ROOE	n.d.	n.d.	n.d.	n.d.	n.d.	51.4±49.7
FA-S-I	Tomato	n.d.	n.d.	n.d.	n.d.	n.d.	1153.6±1068.7
	OF	92.1±58.6	2.3±1.5	531.1±611.4	10.0±9.2	0.5±0.4	1130.2±1115.6
	ROOE	84.0±71.8	7.3±11.3	402.9±701.9	6.2±8.0	1.3±0.9	1194.5±1140.1

FA-S-II	Tomato	156.3±212.2	1.4±1.4	181.1±277.8	1.64±0.9	1.2±0.6	139.8±108.8
	OF	186.6±139.6	1.5±1.3	678.7±678.9	10.7±7.5	0.5±0.4	113.8±76.6
	ROOE	82.2±35.6	1.6±0.5	358.4±152.0	8.7±7.9	0.5±0.4	156.4±148.9
FA-S-III	Tomato	n.d.	n.d.	n.d.	n.d.	n.d.	142.6±209.2
	OF	n.d.	n.d.	n.d.	n.d.	n.d.	50.7±66.1
	ROOE	n.d.	n.d.	n.d.	n.d.	n.d.	119.3±166.4
Q	Tomato	150.9±84.2	2.0±1.9	1801.2±1004.8[a]	11.7±3.2	0.1±0.1	0.6±0.6[a]
	OF	163.7±80.7	1.9±1.4	2430.9±1832.5	11.6±2.4	0.1±0.1	0.3±0.3
	ROOE	164.0±83.9	2.0±2.3	2668.6±1821.9[a]	12.3±2.1	0.1±0.1	0.3±0.3[a]
Q-G	Tomato	n.d.	n.d.	n.d.	n.d.	n.d.	0.4±0.7
	OF	n.d.	n.d.	n.d.	n.d.	n.d.	0.1±0.1
	ROOE	n.d.	n.d.	n.d.	n.d.	n.d.	0.5±0.8
Q-S	Tomato	n.d.	n.d.	n.d.	n.d.	n.d.	0.7±0.7[a]
	OF	n.d.	n.d.	n.d.	n.d.	n.d.	0.2±0.2[a]
	ROOE	n.d.	n.d.	n.d.	n.d.	n.d.	1.2±1.4

Results expressed as mean±SD. Values in a column with the same letters are significantly different($p < 0.05$). Oil free sauce(OF), Refined

Olive Oil Enriched(ROOE), maximum plasma concentration(C_{max}), time needed to reach the maximum plasma concentration(t_{max}), Concentration-versus-time curve from time 0 until the last detectable concentration(AUC_{last}), mean residence time(MRT), maximum cumulative amount excreted in urine(Qu_{∞}), Naringenin(N), Glucuronide(G), Ferulic acid(FA), Isoferulic acid(IFA), Sulfate(S), Quercetin(Q), not detected (n.d.).



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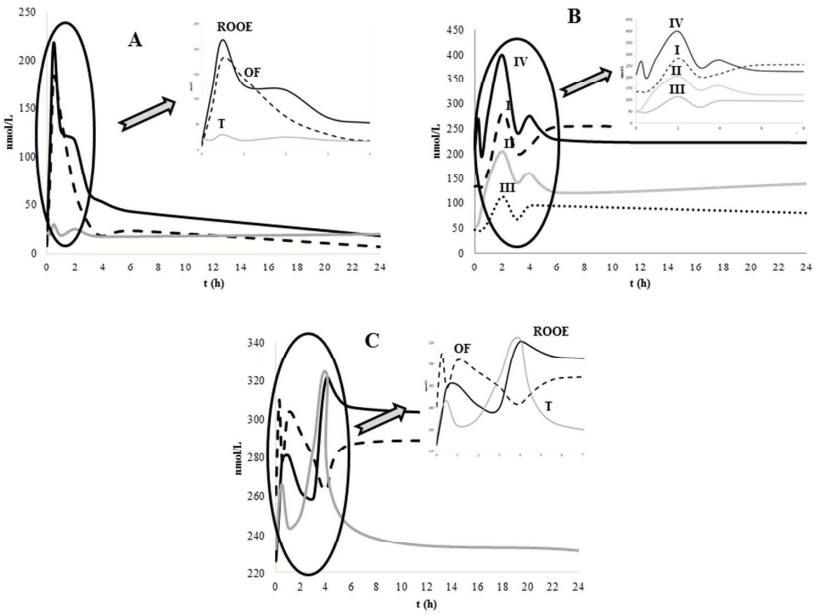

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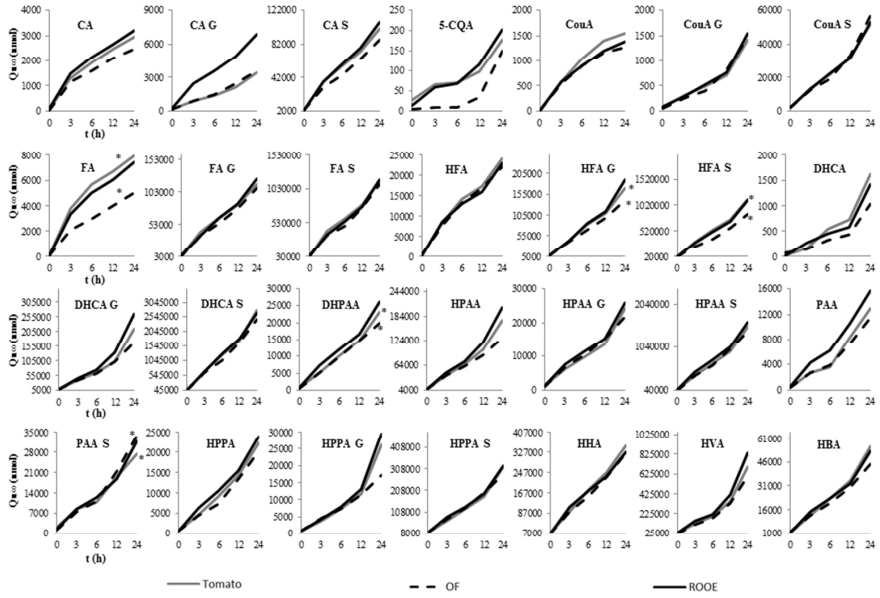

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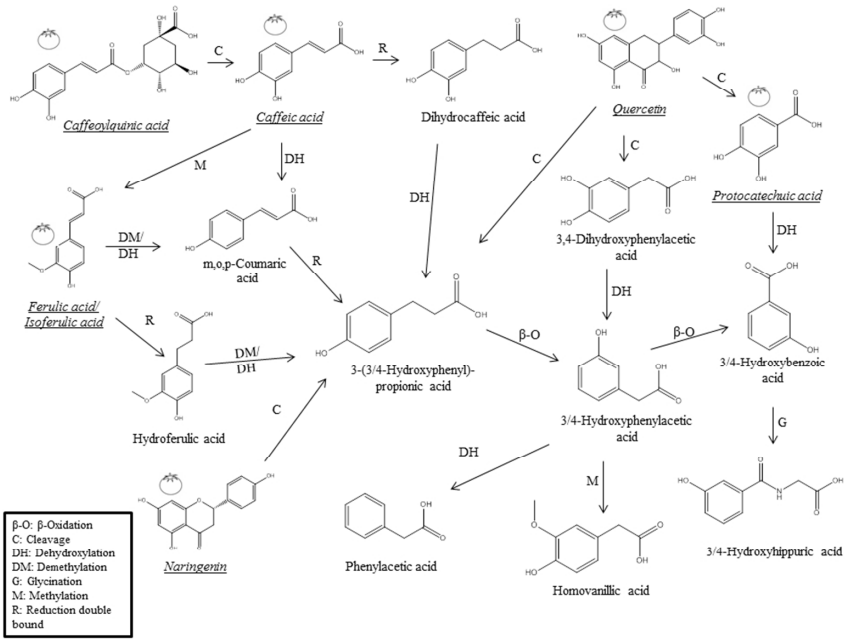

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DISCUSIÓN GENERAL

5. Discusión general

El tomate tiene propiedades beneficiosas contra el riesgo de padecer ciertos tipos de cáncer o enfermedades cardiovasculares gracias a la gran variedad de compuestos bioactivos que posee. Dentro de estos compuestos encontramos a la familia de los polifenoles. Se ha discutido mucho tanto sobre el efecto que tiene el procesado de tomate o sus derivados en la biodisponibilidad de los carotenoides como sobre el efecto de la adición de una matriz lipídica, pero no existen muchas evidencias científicas donde se estudie este efecto en los polifenoles. Por esta razón, la hipótesis de esta tesis doctoral se basó en si el procesado de tomate y la adición de una matriz lipídica durante el tratamiento del fruto favorecían la extractabilidad y, por lo tanto, la biodisponibilidad de los compuestos fenólicos contenidos en los productos. El objetivo de la tesis fue verificar si la biodisponibilidad de los compuestos fenólicos presentes en el tomate y sus derivados estaba influenciada por la tipología de la ingesta (tomate o salsa de tomate) y por la adición de una matriz lipídica añadida durante el procesado.

En primer lugar se validaron tres métodos analíticos, uno por HPLC-MS/MS (**publicación 1**) y dos más con UHPLC-MS/MS (**publicaciones 4 y 5**). En segundo lugar, se diseñaron tres ensayos clínicos de intervención para verificar si la biodisponibilidad de los compuestos fenólicos presentes en el tomate y sus derivados estaba influenciada por el tipo de producto consumido o por la adición de una matriz lipídica añadida durante el procesado. El primer estudio de intervención (**publicación 1**) estudia el efecto de la adición de una matriz lipídica añadida durante el procesado de salsas de tomate y el segundo y tercero (**publicaciones 3 y 6**) estudian en conjunto la influencia de la tipología de la ingesta y la adición de aceite.

- **Validación de métodos analíticos para muestras de alimentos y biológicas**

Previo al análisis de muestras, fue necesaria la validación de un buen método analítico para poder asegurar la correcta identificación de los compuestos y obtener la máxima sensibilidad para detectar los posibles metabolitos a concentraciones muy bajas. En el primer método de HPLC-MS/MS, se validaron 11 compuestos fenólicos en plasma y orina derivados del consumo de tomate y salsas de tomate permitiendo la identificación y cuantificación de cada uno de ellos en un tiempo total de 17 minutos. Las recuperaciones fueron superiores al 95% en ambas matrices, exceptuando el ácido 4-hidroxihipúrico con valores de 75% y 73% en orina y plasma, respectivamente, y para la quercetina con un 65% en orina. Los LODs y LOQs fueron similares o

mejoraron en el caso del ácido caféico, ácido 5-cafeoilquínico y la quercetina a comparación de otros valores reportados en las mismas matrices[80,81]. Tanto la exactitud como la precisión cumplieron los límites establecidos por la AOAC[82].

Posteriormente, se desarrollaron dos métodos y se validaron. Para ello, se adaptaron los métodos utilizados en HPLC-MS/MS[65,83] por nuestro grupo de investigación a UHPLC-MS/MS. Se validaron en muestras de alimentos, tales como, tomates cherry, salsa de tomate y zumo de tomate, y en muestras biológicas, plasma y orina. La validación en alimentos presentó recuperaciones superiores al 90% en todos los compuestos estudiados y los LODs y LOQs fueron inferiores a los encontrados por HPLC-MS/MS[65]. Resultados similares se observaron en la validación de las muestras biológicas. Se mejoró la sensibilidad y la eficacia de la mayoría de los compuestos estudiados y se disminuyó el tiempo de análisis de 17 a 3.5 min respecto al método anteriormente validado por HPLC-MS/MS[83]. Gracias a dichas mejoras, se pueden analizar más muestras en un menor tiempo, reduciendo de esta forma los costes relacionados con el análisis[84].

- **Estudios de intervención en humanos**

Se realizaron 2 ensayos de intervención en voluntarios sanos. El primer estudio (**publicación 2**) se llevó a cabo en 5 voluntarios varones a los que se les suministró 100 g de salsa de tomate sin aceite (OF), 100 g de salsa de tomate con aceite de oliva virgen extra (VOOE) y 100 g de salsa de tomate con aceite de oliva refinado (ROOE) para estudiar la biodisponibilidad de los compuestos fenólicos según la matriz lipídica añadida. En el segundo estudio se realizó un primer ensayo piloto, con 8 voluntarios sanos (**publicación 3**), y, a continuación, se elevó el número de voluntarios a 40 para obtener unos resultados más representativos de la población (**publicación 6**). Las intervenciones de estos dos estudios fueron 500 g de tomate fresco por 70 Kg de peso del voluntario y 250 g de ROOE u OF por cada 70 Kg de peso del voluntario.

En el análisis de los compuestos fenólicos de las salsas y tomates utilizados para los 3 estudios, se pudo observar que los fenoles encontrados pertenecían a la familia de las flavanonas, flavonoles y ácidos fenólicos, como ya se ha descrito en estudios anteriores[59,65,85–87]. En concreto, dentro de los ácidos fenólicos, destacaron los ácidos hidroxicinámicos y sus conjugados como el ácido 5-cafeoilquínico o el ácido homovanílico hexósido. La flavanona naringenina fue el compuesto mayoritario en las salsas junto con el flavonol, rutina. Cuando se compararon las concentraciones de los compuestos en tomate y en salsas se puede observar que para la naringenina y rutina,

la concentración encontrada es mayor en las salsas que en el tomate fresco. Este hecho, repetido en las intervenciones de los tres ensayos realizados, nos permite llegar a la conclusión que el tratamiento mecánico y térmico utilizado para la obtención de las salsas a partir del tomate fresco ayuda a la liberación de ciertos compuestos de la matriz del alimento[88]. En el caso de la naringenina existen estudios donde se demuestra que el compuesto está atrapado en la membrana de la matriz del fruto interactuando con poliésteres insolubles de la fibra del tomate. Los tratamientos mecánicos y térmicos permiten la rotura de estas interacciones incrementando la bioaccesibilidad de la naringenina[89]. Por lo que respecta a la adición de aceite en las salsas, no se observan grandes diferencias entre ROOE y OF, por lo que, esta matriz parece no favorecer la extracción de estos compuestos. Estudios similares en los que se estudiaba la interacción de aceite de oliva virgen extra en salsas de tomate demostraron cómo a mayor cantidad de aceite (10%) incrementaba la extractabilidad de los compuestos fenólicos contenidos en la salsa[62]. Una posible razón por la que este hecho no ocurriera en nuestras muestras sería que la cantidad de aceite añadida a la salsa (5%) no fuera lo suficientemente elevada para ayudar a los compuestos a extraerse mejor.

Tras el primer estudio de intervención, se detectaron dos metabolitos en plasma, naringenina glucurónido y ácido ferúlico glucurónido, mientras que en orina, a parte de estos dos compuestos, se cuantificó también la naringenina y los ácidos cafeico y ferúlico con sus respectivos glucurónidos. En el segundo estudio, se detectaron un mayor número de compuestos, cuatro en plasma y once en orina. En esta ocasión, a parte de los dos compuestos ya descritos en plasma en el estudio previo, se hallaron también la naringenina y el ácido cafeico glucurónido. En orina se sumaron a los 6 compuestos hallados con anterioridad, el ácido 5-cafeoilquínio, el ácido dihidrocafeico y el ácido dihidroferúlico, este último con sus respectivos metabolitos de fase II, glucurónido y sulfato. Este incremento probablemente debido a los cambios producidos en el diseño del estudio, ya que se aumentó la dosis de 100 g de salsas a 250 g, además de administrar la dosis según el peso del voluntario[90]. Por último, en el tercer estudio, el número de metabolitos detectados aumentó a 10 compuestos en plasma y 93 en orina. En esta ocasión, se pudieron detectar muchos más metabolitos posiblemente gracias a la mejora de los LODs y LOQs de los compuestos debido al uso de UHPLC-MS/MS. También en este estudio se realizó un *screening* de posibles metabolitos derivados de la microbiota y metabolitos de fase II no investigados en los estudios previos, por lo que, se ha conseguido determinar un amplio número de

compuestos, especialmente en las muestras de orina. Todos ellos fueron debidamente identificados por un espectrómetro de masas de alta resolución (UHPLC-LTQ-Orbitrap-MS).

Por lo que respecta a los parámetros farmacocinéticos de los tres estudios, se confirma lo observado en el análisis de las salsas, donde el procesado térmico y mecánico durante la preparación de éstas libera la flavanona naringenina de la matriz del tomate incrementando su bioaccesibilidad[19]. En los tres estudios, la concentración máxima (C_{max}) y área bajo la curva (AUC_{last}) es más elevada en las salsas que en los tomates frescos. En cambio, el tiempo máximo para llegar a la máxima concentración (t_{max}) son superiores tras el consumo de tomate crudo que en las salsas, demostrando con ello, que la absorción es más lenta en esta matriz. También se pudo observar el rápido metabolismo de la flavanona naringenina en el epitelio intestinal al aparecer su metabolito de fase II, naringenina glucurónido, a tiempos muy tempranos (t_{max} < 1 h) igual que unas elevadas C_{max} y AUC_{last} a comparación de su aglicona[91,92]. Si comparamos el metabolito entre intervenciones, también hay una clara diferencia entre salsas y tomate fresco, encontrándose valores superiores en las salsas como ya ocurría con su aglicona[19].

La adición de una matriz lipídica a las salsas también parece estar asociada a un aumento en la concentración de polifenoles, posiblemente debido a la afinidad del compuesto a los componentes lipofílicos del aceite de oliva o a que los ácidos grasos parecen favorecer la absorción de algunos polifenoles. Ban *et al.*[93] demostró la protección y la mejora de la bioaccesibilidad de algunos flavonoides (naringenina, quercetina y hesperidina) utilizando nanopartículas lipídicas; estas nanopartículas, compuestas por lípidos fisiológicos, eran resistentes a las duras condiciones en el sistema digestivo y mejoraron la bioaccesibilidad y la protección de los flavonoides hasta su absorción en los enterocitos. El aceite de oliva refinado utilizado para la elaboración de la salsa puede actuar como sistema de nanopartículas lipídicas, ayudando a la naringenina a ser absorbida o los ácidos grasos del aceite pueden favorecer su absorción. Estos resultados fueron corroborados al obtener valores mínimamente superiores de AUC_{last} en ROOE respecto a OF o tomate fresco, pero sin diferencias significativas, probablemente debido a la elevada variabilidad entre los voluntarios. Resultados similares se obtienen en los flavonoles, en particular, la quercetina. C_{max} y AUC_{last} fueron superiores en las salsas y ligeramente superiores en la salsa con aceite, hecho que demuestra que el proceso mecánico y térmico mejora la biodisponibilidad de

los flavonoles. La quercetina es un compuesto lipófilo, por lo tanto, la adición de una matriz lipídica a la salsa podría aumentar la biodisponibilidad del compuesto potenciando su presencia en plasma[94].

En el caso de los ácidos hidroxicinámicos, en particular, el ácido ferúlico y sus metabolitos, encontramos que el t_{max} fue ligeramente más alto que el obtenido por la naringenina o naringenina glucurónido, sobretodo en la salsa con aceite. Una posible razón para el retraso en su aparición en plasma puede ser debido al complejo metabolismo que experimenta el compuesto, ya que puede ser hidrolizado a ácido cafeico[95]. Otra posible razón es que el ácido ferúlico es un compuesto hidrófilo por lo que la presencia de una matriz lipídica puede no ayudar a su absorción resultando un incremento de t_{max}[96].

Se encontraron comportamientos bifásicos para la naringenina glucurónido, ácido ferúlico glucurónido y quercetina en las salsas con aceite. Este perfil bifásico también fue observado en otros estudios que atribuyen el segundo pico a un glucuronidación en el hígado con la participación de UDP-glucuronosiltransferasa. Después de ello, los compuestos pasan a la circulación enterohepática y vuelven a ser absorbidos, por lo que se produce el segundo pico presente en plasma[92,97,98]. Una segunda razón, en el caso de la quercetina, es su aparición tras la hidrólisis de la rutina. Shimoi *et al.*[94] demostró que la rutina se absorbe más lentamente que la quercetina. Este hecho puede hacer que la transformación de rutina a quercetina se produzca más lentamente, y la aparición de un segundo pico de quercetina en muestras de plasma pueda ser debido a esta conversión. Una vida media más larga de los compuestos fenólicos puede mejorar sus funciones beneficiosas en el organismo, ya que los metabolitos podrían ser fisiológicamente activos y además algunos tejidos a menudo poseen hidrolasas (por ejemplo, *β*-glucosidasas o sulfatasas) que pueden reconvertir estos metabolitos de nuevo en sus agliconas.

Una amplia variedad de compuestos fenólicos y metabolitos derivados de la microbiota pertenecientes a flavanonas, ácidos hidroxicinámicos y flavonoles se han cuantificado en muestras de orina. El metabolito glucuronidado de la naringenina presenta unos valores superiores de excreción máxima acumulada respecto a su aglicona, confirmando lo descrito en el plasma sobre el metabolismo de la naringenina[91]. De igual modo, se demostró la mejora en la biodisponibilidad del compuesto tras el procesado de tomate al obtener valores superiores de naringenina y naringenina glucurónido en las salsas en comparación con el fruto. Los flavonoles presentaron niveles muy bajos en comparación

con las flavanonas. Por lo que respecta a la excreción urinaria de los ácidos hidroxicinámicos, compuestos que no habían sido detectados en plasma, se cuantifican en orina como los ácidos 5-cafeoilquínico, ferúlico, cumárico y cafeico, así como sus metabolitos. Diversos estudios demuestran que la biodisponibilidad de estos ácidos es baja, pero además se forman una gran variedad de metabolitos de fase II o compuestos de la microbiota derivados de dichos ácidos hidroxicinámicos[99,100]. Este hecho es demostrado si se observan los valores superiores en la excreción máxima acumulada de los metabolitos respecto a sus agliconas. También se encontraron, en el último estudio, una diversidad de compuestos derivados de la microbiota a partir de la naringenina, ácidos hidroxicinámicos y flavonoles, como la rutina o quercetina. A partir de la naringenina, se pudo cuantificar el ácido 3-(4-hidroxifenilpropiónico), formándose a partir de éste, el ácido 4-hidroxihupúrico[101,102]. También se hallaron los ácidos 3,4-dihidroxifenilacético, 3-(3-hidroxifenilpropiónico), 3-hidroxifenilacético y fenilacético[95], derivados del metabolismo de la rutina y quercetina. Por último, a partir de los ácidos hidroxicinámicos, ácidos ferúlico, cafeico y 5-cafeoilquínico, se cuantificaron los ácidos dihidrocafeico, 3-(3-hidroxifenilpropiónico), hidroxihipúrico, hidroferúlico, 3-(4-hidroxifenilpropiónico) y homovanílico[95,102]. Metabolitos de fase II de estos compuestos derivados de la microbiota se hallaron a concentraciones elevadas. Estos compuestos podrían haber sido metabolizados en el hígado tras haber sido absorbidos por el sistema circulatorio[99]. No se observan diferencias significativas en ninguno de los compuestos cuantificados en orina, aunque aparentemente mostraran una tendencia de mayor excreción en las salsas con aceite. Este hecho se atribuye a la gran variabilidad interindividual que existe entre los voluntarios derivado de causas fisiológicas (peso, composición, hormonas o motilidad gástrica) y moleculares (diferencias en la actividad o la expresión de los transportadores o enzimas implicadas en la biotransformación)[103].

CONCLUSIONES

6. Conclusiones

De los resultados obtenidos en los diferentes trabajos realizados durante esta tesis doctoral derivan las siguientes conclusiones:

1. Para poder llevar a cabo los estudios de biodisponibilidad, se desarrollaron y validaron una metodología por cromatografía líquida de alta resolución acoplada a espectrometría de masas y dos metodologías por cromatografía líquida de ultra alta resolución acoplada a espectrometría de masas, que permitieron el análisis de compuestos fenólicos presentes en tomate, productos derivados y muestras biológicas.
2. La flavanona naringenina, los ácidos hidroxicinámicos como el ácido 5-cafeoilquínico o ácido homovanílico hexóxido, y el flavonol, rutina, se confirman como los compuestos fenólicos mayoritarios en tomates frescos y productos derivados de tomate.
3. El tratamiento mecánico y térmico favorecen la bioaccesibilidad de los compuestos fenólicos de los tomates y derivados.
4. La adición de una matriz lipídica al 5%, incrementa la concentración de los compuestos fenólicos pero de manera no significativa, por lo que parece no ser suficiente la concentración añadida para la buena extractabilidad de los compuestos fenólicos de la matriz del tomate y derivados.
5. El tratamiento térmico y mecánico y la adición de una matriz lipídica ayudan a incrementar la bioaccesibilidad de la naringenina y quercetina y de sus metabolitos.
6. La adición de aceite a las salsas de tomate retrasa la aparición en plasma de los ácidos hidroxicinámicos, en particular del ácido ferúlico y sus metabolitos.
7. La flavanona naringenina presenta un rápido metabolismo al aparecer su metabolito, naringenina glucurónido, a tiempos inferiores a una hora en plasma.
8. La matriz lipídica añadida a las salsas de tomate puede estimular la reabsorción por vía enterohepática de la naringenina glucurónido, el ácido ferúlico glucurónido y la quercetina, aumentando la vida media plasmática de los compuestos, pero no significativamente.

9. Los compuestos fenólicos del tomate presentan un gran metabolismo tanto de fase II como de la microbiota.
10. Existe una tendencia de mayor excreción urinaria de compuestos fenólicos tras la ingesta de salsas de tomate con aceite en comparación con salsa de tomate y tomate crudo, pero la gran variabilidad interindividual existente entre los voluntarios, no permite obtener diferencias significativas.

6. Conclusions

From the results obtained in the different studies performed during this thesis derived the following conclusions:

1. For the biavailability studies, one high resolution liquid chromatography coupled to mass spectrometry and two ultra-high resolution liquid chromatography coupled to mass spectrometry methodologies were developed and validated for the analysis of phenolic compounds present in tomato, its products and in biological samples.
2. Flavanone naringenin, hydroxycinnamic acids such as 5-caffeoylquinic acid and homovanillic acid hexoside or the flavonol, rutin, were confirmed as the major phenolic compounds in fresh tomatoes and tomato products.
3. The mechanical and thermal treatments help tomato phenolic compounds and derivatives to be more bioaccessible.
4. The proportion of the lipid matrix (5%) seems to be not enough for increasing the extractability of phenolic compounds and its derivatives from the matrix.
5. The thermal and mechanical treatments and the addition of a lipid matrix enhances the bioavailability of naringenin and quercetin and their metabolites.
6. The addition of oil to tomato sauces delays the appearance in plasma of hydroxycinnamic acids, including ferulic acid and its metabolites.
7. The flavanone naringenin has a fast metabolism as its metabolite, naringenin glucuronide, appears in plasma before an hour.
8. The lipid matrix added to tomato sauces can stimulate naringenin glucuronide, quercetin and ferulic acid glucuronide enterohepatic reabsorption, increasing plasma half-life of the compounds, but not significantly.
9. Tomato phenolic compounds present both substantial phase II and microbiota metabolisms.
10. There is a trend of increased urinary excretion in sauces with oil, but large interindividual variability exists among volunteers, yielding no significant differences.

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ANEXO

8. Anexo

8.1. Otras publicaciones en revistas

En este anexo se incluyen otras publicaciones que no forman parte propiamente del trabajo de tesis doctoral:

- **Publicación 7.** Perfil fenólico y capacidad antioxidante hidrofílica como marcadores quimiotaxonómicos de variedades de tomate.

  Phenolic Profile and Hydrophilic Antioxidant Capacity as Chemotaxonomic Markers of Tomato Varieties. Anna Vallverdú-Queralt, Alexander Medina-Remón, **Miriam Martínez-Huélamo**, Olga Jáuregui, Cristina Andres-Lacueva, and Rosa M. Lamuela-Raventós. *Journal of Agricultural and Food Chemistry*. 2011, 59:3994-4001.

- **Publicación 8.** Diferencias en el contenido de carotenoides en kétchups y gazpachos mediante el uso de HPLC/ESI(Li+)-MS/MS correlacionados con su capacidad antioxidante.

  Differences in the carotenoid content of ketchups and gazpachos through HPLC/ESI(Li+)-MS/MS correlated with their antioxidant capacity. Anna Vallverdú-Queralt, **Miriam Martínez-Huélamo**, Sara Arranz-Martínez, Esther Miralles, and Rosa M. Lamuela-Raventós. *Journal of the Science of Food and Agriculture.* 2012, 92:2043-2049.

- **Publicación 9.** Las condiciones analíticas son un paso crucial en la cuantificación de los polifenoles, los cuales son inestable en condiciones ácidas: Análisis de prenilflavanoides en muestras biológicas mediante cromatografía de líquidos acoplada a espectrometría de masas en triple cuadrupolo con ionización por electrospray.

  Analytical Condition Setting a Crucial Step in the Quantification of Unstable Polyphenols in Acidic Conditions: Analyzing Prenylflavanoids in Biological Samples by Liquid Chromatography−Electrospray Ionization Triple Quadruple Mass Spectrometry. Paola Quifer-Rada, **Miriam Martínez-Huélamo**, Olga Jáuregui, Gemma Chiva-Blanch, Ramón Estruch, and Rosa M. Lamuela-Raventós. *Analytical Chemistry*. 2013, 85(11):5547-5554.

- **Publicación 10.** Estudio exhaustivo del perfil fenólico de hierbas y especias culinarias ampliamente utilizadas: romero, tomillo, orégano, canela, comino y laurel.

  A comprehensive study on the phenolic profile of widely used culinary herbs and spices: Rosemary, thyme, oregano, cinnamon, cumin and bay. Anna Vallverdú-Queralt, Jorge Regueiro, **Miriam Martínez-Huélamo**, Jose Fernando Rinaldi Alvarenga, Leonel Neto Leal, and Rosa M. Lamuela-Raventós. *Food Chemistry*. 2014, 154:299-307.

- **Publicación 11.** Diferencias en el perfil de carotenoides de productos elaborados con tomate orgánico y convencional disponibles en el mercado.

  Differences in the carotenoid profile of commercially available organic and conventional tomato-based products. Anna Vallverdú-Queralt, **Miriam Martínez-Huélamo**, Isidre Casals-Ribes, and Rosa M. Lamuela-Raventós. *Journal of Berry Research*. 2014, 4:69-77.

- **Publicación 12.** La fracción no alcohólica de la cerveza aumenta las células estromales derivadas del factor 1 y el número de células progenitoras endoteliales circulantes en sujetos de alto riesgo cardiovascular: un ensayo clínico aleatorizado.

  The non-alcoholic fraction of beer increases stromal cell derived factor 1 and the number of circulating endothelial progenitor cells in high-cardiovascular risk subjects: a randomized clinical trial. Gemma Chiva-Blanch, Ximena Condines, Emma Magraner, Irene Roth, Palmira Valderas-Martínez, Sara Arranz, Rosa Casas, **Miriam Martínez-Huélamo**, Anna Vallverdú-Queralt, Paola Quifer-Rada, Rosa M. Lamuela-Raventos, and Ramon Estruch. *Atherosclerosis*. 2014, 233(2):518-24.

- **Publicación 13.** El isoxantohumol es un biomarcador específico y preciso del consumo de cerveza en orina.

  Urinary isoxanthohumol is a specific and accurate biomarker of beer consumption. Paola Quifer-Rada, **Miriam Martínez-Huélamo**, Gemma Chiva-Blanch, Olga Jáuregui, Ramon Estruch, and Rosa M. Lamuela-Raventós. *Journal of Nutrition*. 2014, 144(4):484-488.

- **Publicación 14.** Caracterización exhaustiva de los polifenoles de la cerveza por espectrometría de masas de alta resolución (LC-ESI-LTQ-Orbitrap-MS).
  A comprehensive characterization of beer polyphenols by high resolution mass spectrometry (LC-ESI-LTQ-Orbitrap-MS). Paola Quifer-Rada, Anna Vallverdú-Queralt, **Miriam Martínez-Huélamo**, Gemma Chiva-Blanch, Olga Jáuregui, Ramon Estruch, and Rosa M. Lamuela-Raventós. *Food Chemistry*. 2015, 169:336‑343.

- **Publicación 15.** Caracterización de los perfiles fenólicos y antioxidantes de hierbas y especias culinarias seleccionadas: comino, cúrcuma, eneldo, mejorana y nuez moscada.
  Characterization of the phenolic and antioxidant profiles of selected culinary herbs and spices: caraway, turmeric, dill, marjoram and nutmeg. Anna Vallverdú-Queralt, Jorge Regueiro, José Fernando Rinaldi Alvarenga, **Miriam Martínez-Huélamo**, Leonel Neto Leal, and Rosa M. Lamuela-Raventós. *Food Science And Technology*. 2015, 35(1):189-195.

- **Publicación 16.** Efectos del alcohol y los polifenoles de la cerveza sobre biomarcadores ateroscleróticos en hombres de alto riesgo cardiovascular: ensayo aleatorizado nutricional.
  Effects of alcohol and polyphenols from beer on atherosclerotic biomarkers in high cardiovascular risk men: A randomized feeding trial. Gemma Chiva-Blanch, Emma Magraner, Ximena Condines, Palmira Valderas-Martínez, Irene Roth, Sara Arranz, Rosa Casas, Margarita Navarro, Amparo Hervas, Antoni Sisó, **Miriam Martínez-Huélamo**, Anna Vallverdú-Queralt, Paola Quifer-Rada, Rosa M. Lamuela-Raventos, and Ramon Estruch. *Nutrition Metabolism and Cardiovascular Diseases*. 2015, 25(1):36-45.

- **Publicación 17.** Alta permeabilidad gastrointestinal y metabolismo local de la naringenina: influencia del tratamiento con antibiótico en la absorción y el metabolismo de la misma.
  High gastrointestinal permeability and local metabolism of naringenin: influence of antibiotic treatment on absorption and metabolism. Naiara Orrego-Lagarón, **Miriam Martínez-Huélamo**, Anna Vallverdú-Queralt, Rosa M. Lamuela-

Raventós, and Elvira Escribano-Ferrer. *British Journal of Nutrition*. 2015, 114:169-180.

- **Publicación 18.** Influencia del aceite de oliva en la absorción de carotenoides a partir de zumo de tomate y los efectos sobre la lipemia postprandial.
  Influence of olive oil on carotenoid absorption from tomato juice and effects on postprandial lipemia. Sara Arranz, **Miriam Martínez-Huélamo**, Anna Vallverdú-Queralt, Palmira Valderas-Martinez, Montse Illán, Emilio Sacanella, Elvira Escribano, Ramon Estruch, and Rosa M. Lamuela-Raventós. *Food Chemistry.* 2015, 168:203-210.

- **Publicación 19.** Un nuevo método para la cuantificación simultanea de antioxidantes: carotenos, xantofilas y vitamina A en plasma humano.
  A New Method to Simultaneously Quantify the Antioxidants: Carotenes, Xanthophylls, and Vitamin A in Human Plasma. Mariel Colmán-Martínez, **Miriam Martínez-Huélamo**, Esther Miralles, Ramon Estruch, and Rosa M. Lamuela-Raventós. *Oxidative Medicine and Cellular Longevity*. 2015, Article ID 246080 (aceptado).

8.2. Comunicaciones en congresos

8.2.1. Comunicación 1. Póster.

Título: A new LC-MS/MS method for the determination of tomato polyphenols in biological fluids.

Autores: **Miriam Martínez-Huélamo**, Sara Tulipani, Maria Rotchés-Ribalta, Cristina Andrés-Lacueva, and Rosa M. Lamuela-Raventós.

Congreso: 4th International Conference on Polyphenols and Health. Harrogate, Yorkshire, England, 2009.

A new LC-MS/MS method for the determination of tomato polyphenols in biological fluids

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INTRODUCTION

Tomato is a good source of antioxidants: vitamin C, carotenes (lycopene) and polyphenols. However, most of the bioavailability studies on tomato have focused on vitamin C and carotenes; few studies have evaluated the bioavailability of polyphenols from tomato. For the determination of polyphenols and their metabolites in urine and human plasma, it is necessary to ensure an efficient extraction in the sample treatment process. Optimization of this step is the key to obtaining a method of analysis that could be sufficiently sensitive to determine these substances in low concentration. One of the most widely used techniques for the preconcentration and clean-up of analytical samples is the solid-phase extraction (SPE).

Until now, only few studies have been focused on the tomato polyphenols in biological fluids, and the extraction procedures commonly used are complex and time-consuming. Here, we present the optimization of a new simple and rapid method for the determination of polyphenols and their metabolites in urine and human plasma after the administration of tomato sauces. The sample preparation included a SPE step with Oasis HLB 96-well plate to obtain cleaner extracts to be injected in the LC-MS/MS system.

The extraction method described is simple, rapid and friendly with the environment since it requires small volumes of solvent, more reproducible and cleaner extracts are obtained. For separation and quantification LC-MS/MS was confirmed to be a better powerful analytical tool, due to its high universality, sensitivity, and specific information than LC with UV detection or electrochemical, obtaining lower detection limits and adequate selectivity versus interfering substances of the matrix.

LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

HPLC conditions

- Luna C18, Phenomenex (5µm, 50 x 20 mm) column
- 600 µL/min of mobile phase
- Temperature column: 30 °C
- Injection volume: 20 µL

Time (min)	H_2O 0.1%HFo	MeCN 0.1%Hfo
5 (eq)	95	5
2	75	25
10	10	90
11	0	100
12	0	100

MS/MS conditions

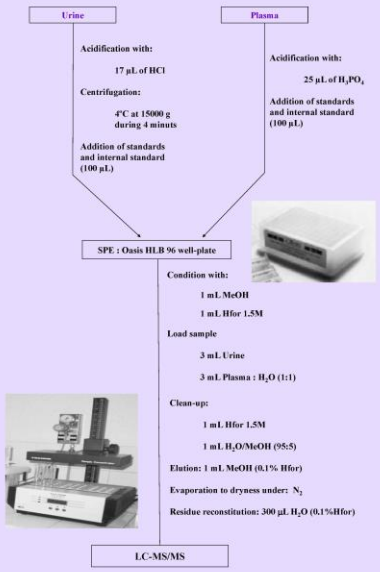
PE-SCIEX API 3000
Triple-Quadrupole
(Turbolonspray® source)

- Ionspray: -4000 V
- Auxiliar gas (N_2): 8000 mL.min^{-1}
- Temperature: 400°C
- Nebulizer gas (N_2): 10*
- Curtain gas (N_2): 12*
- CAD: 4*

* (arbitrary units)

	Caffeic acid	Chlorogenic acid	Dihydrocaffeic	Ethyl Gallate	Ferulic acid	Isoferulic	Kaempferol	Naringenin	Quercetin	Taxifolin
Parent ion (m/z)	179	353	181	197	193	193	285	271	301	303
Cone Voltage (V)	-40	-40	-40	-60	-50	-40	-70	-50	-50	-60
Focusing Pot. (V)	-170	-180	-170	-200	-220	-170	-265	-190	-210	-220
Entrante Pot. (V)	-11	-11	-11	-10	-11	-11	-11	-11	-11	-10
Fragment ions (m/z)	135 107 117 89 79 65	191 179 161 135 127 85	137 121 109 59	169 124	178 149 134 117	178 149 134	151 257	177 165 151 119 107 93	179 151	285 177 125
Transition quantification (Collision energy (V))	179→135 (-20)	353→191 (-20)	181→137 (-20)	197→169 (-25)	193→134 (-20)	193→178 (-20)	285→151 (-30)	271→151 (-30)	301→151 (-30)	303→285 (-20)
Transition identification (Collision energy (V))	179→107 (-30)	353→179 (-30)	181→121 (-30)	197→124 (-30)	193→178 (-20)	193→134 (-20)	285→257 (-30)	271→119 (-40)	301→179 (-30)	303→125 (-30)

SAMPLE PREPARATION

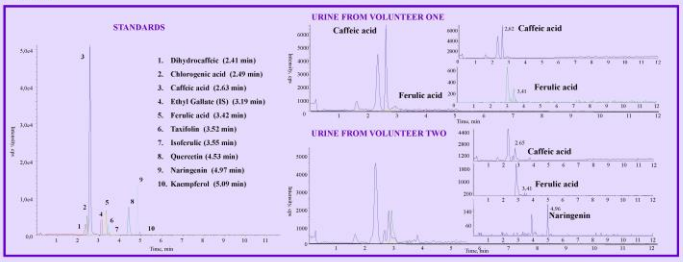


QUALITY PARAMETERS

PLASMA	Chlorogenic acid	Ferulic acid	Isoferulic	Kaempferol	Naringenin	Quercetin
Linearity ranges (ng/mL)	0,5-500	9-9000	9-9000	4-4000	1,5-1500	4-4000
Linear Fit (r)	0,9977	0,9966	0,9978	0,9957	0,9959	0,9941
Recovery (%)	76 ± 2	68 ± 2	75 ± 2	62 ± 2	74 ± 2	50 ± 1
LOD (ng/mL)	0,18	5,4	5,4	29	0,7	2,5
LOQ (ng/mL)	0,60	17,7	17,7	95	2,5	8,5
Intra-day 1 (%RSD)	7	4	8	1	6	2
Intra-day 2 (%RSD)	4	5	6	4	5	6
Intra-day 3 (%RSD)	7	6	7	6	4	2
Inter-day (%RSD)	7	6	8	7	8	7

URINE	Caffeic acid	Chlorogenic acid	Dihydrocaffeic	Ferulic acid	Isoferulic	Kaempferol	Naringenin	Quercetin
Linearity ranges (ng/mL)	1,5-1500	0,5-500	15-15000	9-9000	9-9000	4-4000	1,5-1500	4-4000
Linear Fit (r)	0,9913	0,9940	0,9964	0,9961	0,9951	0,9957	0,9977	0,9960
Recovery (%)	88 ± 4	99 ± 4	69 ± 2	84 ± 2	69 ± 2	103 ± 4	101 ± 2	109 ± 3
LOD (ng/mL)	0,8	0,18	8	5,4	5,4	29	0,7	2,5
LOQ (ng/mL)	3	0,60	30	17,7	17,7	95	2,5	8,5
Intra-day 1 (%RSD)	6	4	1	3	6	6	3	6
Intra-day 2 (%RSD)	3	7	6	5	5	7	5	6
Intra-day 3 (%RSD)	5	6	6	2	5	7	6	6
Inter-day (%RSD)	7	7	7	5	6	7	7	7

RESULTS



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CONCLUSIONS

- Easy and rapid method to determine polyphenols in biological fluids. Good quality parameters were obtained when the method was validated by LC-MS/MS.

8.2.2. Comunicación 2. Póster.

Título: Plasma pharmacokinetics and urinary excretion of phenolic metabolites from oil-free and oil-enriched tomato sauces.

Autores: Sara Tulipani, **Miriam Martínez-Huélamo**, Ramon Estruch, Elvira Escribano-Ferrer, Cristina Andrés-Lacueva, and Rosa M. Lamuela-Raventós.

Congreso: 4th International Conference on Polyphenols and Health. Harrogate, Yorkshire, England, 2009.

Plasma pharmacokinetics and urinary excretion of phenolic metabolites from oil-free and oil-enriched tomato sauces

Sara Tulipani[1,2], Miriam Martínez Huélamo[1], Ramon Estruch[3,4], Elvira Escribano Ferrer[5], Cristina Andrés-Lacueva[1,6], Rosa Maria Lamuela-Raventós[1,4*]

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INTRODUCTION

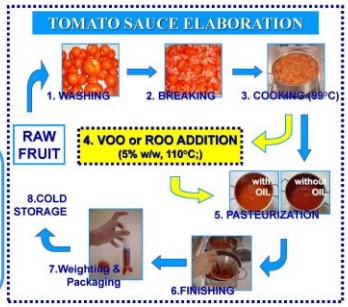
Tomato sauce is the most commonly consumed tomato product eaten right across the world, and is a major component of the so-called "Mediterranean diet". Several studies were recently focused on evaluating how the industrial processing leading to tomato sauce may affect compounds such as lycopene, β-carotene and vitamin C. Recent findings indicate that tomato processing may increase carotenoid bioavailability, particularly with the simultaneous intake of fats and triglycerides. Similar studies also suggest that the lipid matrix may favor the extractability and bioavailability of tomato carotenoids.

In contrast, very little is known about the extent of changes in the polyphenol/flavonoid content of tomato after the mechanical and thermic treatments leading to tomato sauce elaboration, such as about the impact of the tomato processing and tomato-olive oil combination on the phenolic bioavailability and bioefficacy in man.

AIMS

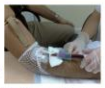
To verify whether the bioavailability of the phenolic compounds contained in tomato sauce is influenced by the lipid matrix eventually added, and by its typology:

Prospective, randomized, crossover study (n = 5, men; Age: 25-36 y; BMI = 25± 1.2) with three single dietary interventions (100 g): Oil-Free (O-F), Refined Olive Oil- (ROO-E) and Virgin Olive Oil (VOO-E) enriched tomato sauces



STUDY DESIGN

- PRE-STUDY: Days −3 to 0: Tomato-free diet; Day −1: Standardized Polyphenols-free diet
- IN-STUDY: Day 0 (after an overnight fast): O-F, ROO-E and VOO-E tomato sauces intake
  - → Urine collection @ 0h, 0-4h, 4-8h, 8-12h, 12-24h from the dietary intervention
  - → Blood pressure measurements
  - → Blood collection @ 0h, 15min, 30min, 1h, 2h, 3h, 4h, 5h, 6h, 8h, 12h, 24h from the intervention
  - → Routinary clinical-chemical laboratory tests
- Minimum WASHOUT period between interventions: 5 days



METHODOLOGY

✓ Raw tomatoes,tomato sauces, human urine and plasma → LC-MS/MS analysis by Triple Quadrupole mass spectrometer API 3000 (Applied Biosystems, PE Sciex, Concord, Ontario, Canada) with negative ion detection mode

✓ Human urine and plasma →Prior to LC-MS/MS analysis, acidification of the samples and solid-phase extraction (SPE) with Oasis® HLB 96-well plates.

Phenolic compounds were identified on the basis of their mass spectra in full scan mode, and MS-MS data from product ion scan (PIS) and multiple reaction monitoring (MRM) experiments were used to confirm their identity.

❖ Pharmacokinetic indexes *(WinNonlin Professional software 3.3, Pharsight Corporation, USA)*:
✓ C_{max}, T_{max}, AUC_{last}, MRT, C_{max}/AUC_{last}, Cumulative urinary excretion, Q_{ext}, Q_{ext}/Dose, CL_{ren}

❖ Statistical analysis *(SPSS software 12.0, Japan Inc., Tokyo, Japan)*:
✓ ANOVA analysis of variance → C_{max} , AUC_{last}, MRT, C_{max}/AUC_{last} (log-transformed data)
✓ Non-parametric Kruskal-Wallis one-way ANOVA on ranks (T_{max})
$P < 0.05$ was considered significant

RESULTS

Table 1
Identified phenolic compounds detected by LC-MS/MS in the raw tomato fruits and in O-F, VOO-E and ROO-E tomato sauces[1]

RT (min)[2]	compound	MRM (m/z)	Raw Fruit	O-F sauce	ROO-E sauce	VOO-E sauce
4.22	Caffeic acid	179 → 135	130.6 ± 21.2b	269.1 ± 7.8a	250.0 ± 3.9a	263.7 ± 29.8a
1.99	Caffeic acid-hexose *der 1*	341 → 179	235.3 ± 44.9b	437.1 ± 9.4a	280.3 ± 4.0b	286.8 ± 34.0b
3.50	Caffeic acid-hexose *der 2*	341 → 179	98.1 ± 15.0b	160.7 ± 1.3a	106.0 ± 4.2b	106.9 ± 12.2b
6.88	Caffeic acid-hexose *der 3*	341 → 179	5.5 ± 0.8	6.1 ± 0.1	7.4 ± 2.9	7.7 ± 0.7
3.96	Chlorogenic acid	353 → 191	234.4 ± 46.1c	814.3 ± 15.0a	552.2 ± 21.8b	651.6 ± 34.9b
1.17	Caffeoylquinic acid *der 2*	353 → 191	–b	2.58 ± 1.25a,b	4.65 ± 1.28a,b	5.90 ± 1.50a
1.90	Caffeoylquinic acid *der 3*	353 → 191	4.3 ± 0.6b	60.3 ± 1.1a	61.2 ± 2.1a	59.8 ± 6.4a
5.84	Caffeoylquinic acid *der 4*	353 → 191	12.4 ± 0.2b	26.5 ± 2.2a	17.3 ± 1.4b	22.2 ± 3.1a,b
12.93	Dicaffeoylquinic acid	515 → 353	36.0 ± 7.9	54.3 ± 5.1	61.1 ± 1.4	48.4 ± 8.5
8.05	Ferulic acid	193 → 134	113.7 ± 23.6a	23.5 ± 0.7b	44.0 ± 1.4b	43.5 ± 3.8b
3.42	Ferulic acid-hexose *der 1*	355 → 193	104.2 ± 15.1b	232.8 ± 9.1a	242.2 ± 45.6a	157.0 ± 2.9a,b
3.91	Ferulic acid-hexose *der 2*	355 → 193	34.2 ± 8.5c	127.3 ± 0.5a,b	103.3 ± 2.3b	128.5 ± 8.3a
1.50	Protocatechuic acid	153 → 109	–b	129.2 ± 6.7a	144.1 ± 5.6a	128.1 ± 14.4a
13.68	Naringenin	271 → 151	164.3 ± 12.1b	1347 ± 63.6a	1295.7 ± 211.9a	1304.7 ± 237.9a
11.76	Naringenin-7-glucoside	433 → 271	–b	16.0 ± 0.3a	17.7 ± 2.3a	17.5 ± 6.6a
13.71	Quercetin	301 → 151	84.5 ± 3.5	102.7 ± 3.2	110.7 ± 15.4	100.2 ± 13.1
10.09	Rutin	609 → 301	1.40 ± 0.19c	3.01 ± 0.04a	2.16 ± 0.04b	2.23 ± 0.30b
11.56	Kaempferol-3-rutinoside	593 → 285	317.6 ± 107.8	413.8 ± 124.4	362.6 ± 14.3	316.7 ± 12.8

[1] Values are means ± SEM. [2] RT: chromatographic retention (in minutes). [3] FW: fresh weight.

TOMATO SAUCES PHENOLIC COMPOSITION

The phenolic composition of all the three tomato sauces mainly differed from that of the raw intact tomatoes, particularly for the higher content of several hydroxycinnamates (caffeic, mono- and dicaffeoylquinic acids) and for the much higher content of naringenin and naringenin-7-glucoside. These data confirmed how the mechanical and thermic treatments during tomato sauce processing may induce biochemical changes in the starting product (Table 1).

However, the tomato-olive oil combination in ROO-E and VOO-E tomato sauces did not appear to relevantly affect the phenolic profile of the final product.

PHENOLIC METABOLITES in URINE

Six phenolic compounds, three aglycones and three glucuronides, were detected in urine after the single ingestion of all the three tomato sauces, reaching the maximum rate of excretion within 4 hours from the dietary intervention (Figure 1).

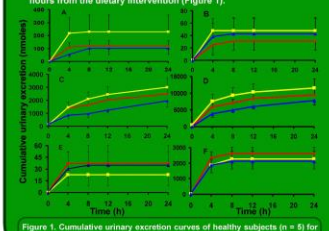


Figure 1. Cumulative urinary excretion curves of healthy subjects (n = 5) for caffeic acid (A), caffeic glucuronide (B), ferulic acid (C), ferulic glucuronide (D), naringenin (E) and naringenin glucuronide (F) after single ingestions of O-F, VOO-E and ROO-E tomato sauces. Values are means ± SEM.

An apparently higher urinary excretion of caffeic and ferulic acids (both in the native and glucuronide forms) was observed after ingestion of VOO-E tomato sauce. However, no statistically significant differences were observed in the CL_{ren}, the Q_{ext} (data not shown) of the excreted metabolites among all the three dietary interventions. The results were probably affected by the high interindividual response variability observed, thus emphasizing the need for further large-scale investigations.

Table 2
Q_{ext}[1]-to-ingested dose[2] ratio (Q_{ext}/Dose ratio) for naringenin and naringenin derivatives after single ingestion of O-F, VOO-E and ROO-E tomato sauces (n = 5)[3]

	Intervention	Q_{ext}/Dose ratio
Naringenin derivatives	O-F Salsa	0.0053 ± 0.0008
	VOO-E Salsa	0.0047 ± 0.0011
	ROO-E Salsa	0.0045 ± 0.0010

[1] The cumulative urinary excretion of the naringenin derivatives was calculated by summing the maximum urinary excretion of naringenin and the naringenin glucuronide metabolite (nmoles) confirmed by MRM and PIS experiments. [2] The ingested dose of naringenin

PHENOLIC METABOLITES in PLASMA

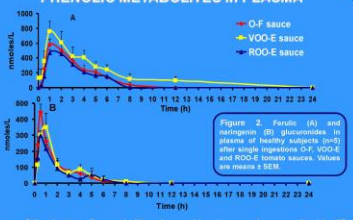


Figure 2. Ferulic (A) and naringenin (B) glucuronides in plasma of healthy subjects (n=5) after single ingestions O-F, VOO-E and ROO-E tomato sauces. Values are means ± SEM.

Only two of the six phenolic metabolites observed in urine samples were also identified in plasma after all the three interventions, and confirmed by PIS and MRM experiments (Figure 2A-B). The flavanone naringenin was rapidly excreted in an extremely lower extent in respect to its glucuronide metabolite, and was not observed in plasma after all the tomato sauce ingestions, suggesting a rapid metabolism of the compound.
Higher C_{max}, T_{max}, AUC_{last} and MRT were observed for the ferulic glucuronide in respect to the naringenin glucuronide in plasma, confirming a slower metabolism and urinary excretion of this metabolite (Figure 1-2).

Table 3
Pharmacokinetic indexes for naringenin and ferulic glucuronides after single ingestion (100 g) of O-F, VOO-E and ROO-E tomato sauces (n = 5) by healthy men[1]

		C_{max} nmol/L	T_{max} min	AUC_{last} nmol/L x min	MRT min	C_{max}/AUC_{last} ratio
Naringenin glucuronide	O-F Salsa	445.6 ± 48.8	30.0 ± 0.0	43559 ± 5630	98.6 ± 16.3	0.0105 ± 0.0011
	VOO-E Salsa	398.0 ± 86.8	45.0 ± 7.7	37649 ± 6794	91.7 ± 12.7	0.0106 ± 0.0013
	ROO-E Salsa	298.6 ± 30.1	30.0 ± 0.0	32009 ± 6705	106.0 ± 12.5	0.0113 ± 0.0025
Ferulic glucuronide	O-F Salsa	580 ± 89.4	72.0 ± 12.0	118949 ± 26786	157.6 ± 10.6	0.0056 ± 0.0008
	VOO-E Salsa	727.0 ± 164.7	75.0 ± 13.4	197702 ± 53384	218.2 ± 56.6	0.0056 ± 0.0019
	ROO-E Salsa	544.2 ± 101.3	84.0 ± 14.7	96531 ± 20455	149.4 ± 14.0	0.0061 ± 0.0008

[1] Values are means ± SEM

Interestingly, the plasma pharmacokinetic of naringenin glucuronide seemed to suggest a two-phase absorption only following the ingestion of oil-enriched tomato sauces (Figure 2B). These findings suggested the occurrence of re-absorption events by enterohepatic circulation, specifically stimulated by the tomato-oil combination. However, this hypothesis needs to be substantiated by further specific investigations of naringenin biotransformation by the liver in the presence/absence of the lipid matrix in the tomato sauce.
Particularly, the absence of a significant difference in the pharmacokinetic indexes calculated for the three interventions (Table 3) will stress the need for further large-scale consumption studies.

CONCLUSIONS

According to the present results, the tomato-olive oil combination during tomato sauce processing does not seem to significantly affect the extractability of phenolic compounds of intermediate hydrophobicity, such as the flavanone naringenin and the hydroxycinnamic acids, as well as their *in vivo* bioavailability and biotransformations. However, interesting differences in the plasma pharmacokinetic of naringenin glucuronide were observed after ingestion of the oil-enriched tomato sauces, suggesting the occurrence of re-absorption events by enterohepatic circulation stimulated by the lipid matrix in the sauces. By the way, the great interindividual response variability observed emphasized the need for further large-scale investigations, to confirm the differences among the oil-free and oil-enriched tomato sauces ingestions.

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8.2.3. Comunicación 3. Póster.

Título: Bioavailability and urinary excretion of phenolic metabolites from tomato by-products: the effect of the food matrix.

Autores: Sara Tulipani, **Miriam Martínez-Huélamo**, Ramon Estruch, Cristina Andrés-Lacueva, Montse Illán, Xavier Torrado, and Rosa M. Lamuela-Raventós.

Congreso: II Congreso de la Federación Española de Sociedades de Nutrición, Alimentación y Dietética. Barcelona, Spain, 2010.

Bioavailability and urinary excretion of phenolic metabolites from tomato by-products: the effect of the food matrix

S Tulipani[1,2], M Martínez Huélamo[1], R Estruch[3,4], C Andrés-Lacueva[1,5], M Illán[1], X Torrado[1], RM Lamuela-Raventós[1,4*]

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INTRODUCTION

- Tomato (*Lycopersicon esculentum* Mill., Solanaceae) is one of the key components of the so-called Mediterranean diet, and tomato sauce is the major tomato product eaten right across the world.
- The relevant content of phytochemical compounds such as polyphenols and carotenoids seems to play a crucial role in the health-promoting effects of tomato and by-products consumption.
- However, little is known on the extent of changes in the polyphenol/flavonoid content after the mechanical and thermal treatments leading to tomato sauce. Even less literature data are available on the effect of the addition of oil during sauce preparation, on the polyphenol bioaccessibility and bioavailability in humans.

AIMS

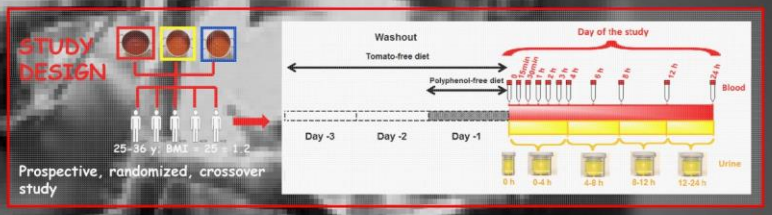
To assess the eventual differences in the absorption, plasma bioavailability and urinary excretion of phenolic metabolites, after a single-dose administration of

Oil-Free (O-F), Refined Olive Oil- (ROO-E) and Virgin Olive Oil- (VOO-E) enriched tomato sauces (100 g)

TOMATO SAUCE ELABORATION

- The phenolic composition of all the three tomato sauces mainly differed from that of the raw intact tomatoes, particularly for the higher content of several hydroxycinnamates (caffeic, mono- and dicaffeoylquinic acids) and for the much higher content of flavanones (naringenin).
- In contrast, the tomato-olive oil combination did not appear to relevantly affect the phenolic profile of the final product.

STUDY DESIGN



METHODOLOGY

- LC-MS/MS analysis of tomato/tomato sauce extracts, human plasma and urine by Triple Quadrupole mass spectrometer API 3000 (negative ion detection mode). Prior to analysis, the phenolic compounds in plasma and urine were extracted by solid-phase extraction (SPE). The identification of the compounds of interest was carried out on the basis of their mass spectra in full scan mode and confirmed by MS-MS experiments (PIS, NL). Multiple reaction monitoring (MRM) experiments were used for quantification.

RESULTS I: PHENOLICS in TOMATOES/SAUCES

Table 1

Identified phenolic compounds detected by LC-MS/MS in the raw tomato fruits and in O-F, VOO-E and ROO-E tomato sauces[1]

RT (min)[2]	compound	MRM (m/z)	Raw Fruit (μg/g FW[3])	O-F sauce	ROO-E sauce	VOO-E sauce
4.22	Caffeic acid	179 → 135	130.6 ± 21.2b	269.1 ± 7.8a	250.0 ± 3.9a	263.7 ± 29.8a
1.99	Caffeic acid-hexose der 1	341 → 179	235.3 ± 44.9b	437.1 ± 9.4a	280.3 ± 4.0b	286.8 ± 34.0b
3.50	Caffeic acid-hexose der 2	341 → 179	98.1 ± 15.0b	150.7 ± 1.3a	108.0 ± 4.2b	106.9 ± 12.2b
6.88	Caffeic acid-hexose der 3	341 → 179	5.5 ± 0.8	6.1 ± 0.1	7.4 ± 2.9	7.7 ± 0.7
3.96	Chlorogenic acid	353 → 191	234.4 ± 46.1c	814.3 ± 15.0a	552.2 ± 21.8b	651.6 ± 34.9b
1.17	Caffeoylquinic acid der 2	353 → 191	– b	2.58 ± 1.25ab	4.65 ± 1.28a,b	5.90 ± 1.50a
1.90	Caffeoylquinic acid der 3	353 → 191	4.3 ± 0.6b	60.3 ± 1.1a	61.2 ± 2.1a	59.8 ± 6.4a
5.84	Caffeoylquinic acid der 4	353 → 191	12.4 ± 0.2b	26.5 ± 2.2a	17.3 ± 1.4b	22.2 ± 3.1a,b
12.93	Dicaffeoylquinic acid	515 → 353	36.0 ± 7.9	54.3 ± 5.1	61.1 ± 1.4	48.4 ± 8.5
8.05	Ferulic acid	193 → 134	113.7 ± 23.6a	23.5 ± 0.7b	44.0 ± 1.4b	43.5 ± 3.8b
3.42	Ferulic acid-hexose der 1	355 → 193	104.2 ± 15.1b	232.8 ± 9.1a	242.2 ± 45.6a	157.0 ± 2.9a,b
3.91	Ferulic acid-hexose der 2	355 → 193	34.2 ± 8.5c	127.3 ± 0.5a,b	103.3 ± 2.3b	128.5 ± 8.3a
1.50	Protocatechuic acid	153 → 109	–b	129.2 ± 6.7a	144.1 ± 5.6a	126.1 ± 14.4a
13.68	Naringenin	271 → 151	164.3 ± 12.1b	1347 ± 63.6a	1295.7 ± 211.9a	1304.7 ± 237.9a
11.76	Naringenin-7-glucoside	433 → 271	–b	16.0 ± 0.3a	17.7 ± 2.3a	17.5 ± 6.6a
13.71	Quercetin	301 → 151	84.9 ± 3.5	102.7 ± 3.2	110.7 ± 15.4	100.2 ± 13.1
10.09	Rutin	609 → 301	1.40 ± 0.19c	3.01 ± 0.04a	2.19 ± 0.04b	2.23 ± 0.30b
11.56	Kaempferol-3-rutinoside	593 → 285	317.6 ± 107.8	413.8 ± 124.4	362.6 ± 14.3	316.7 ± 12.8

[1]Values are means ± SEM. [2]RT: chromatographic retention (in minutes). [3]FW: fresh weight.

RESULTS II: PHENOLIC METABOLITES in URINE

- Six phenolic compounds (three aglycones and three glucuronides) detected in urine after the single ingestion of all the 3 tomato sauces
- Maximum rate of excretion reached within 4 hours from the intervention
- No statistically significant differences observed in the CUE, CL_{ren}, Q_{inf} of the excreted metabolites among all the three dietary interventions

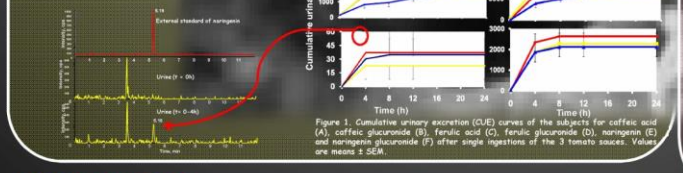


Figure 3. Cumulative urinary excretion (CUE) curves of the subjects for caffeic acid (A), caffeic glucuronide (B), ferulic acid (C), ferulic glucuronide (D), naringenin (E) and naringenin glucuronide (F) after single ingestions of the 3 tomato sauces. Values are means ± SEM.

RESULTS III: PHENOLIC METABOLITES in PLASMA

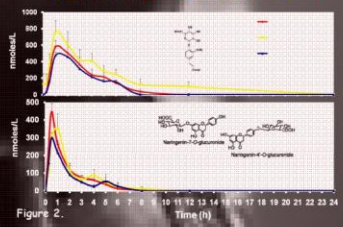


Figure 2.

- Ferulic (A) and naringenin (B) glucuronides were observed in both urine and plasma after all the three interventions (Figure 2).
- The flavanone naringenin was rapidly excreted in an extremely lower extent in respect to its glucuronide metabolite, suggesting a rapid metabolism of the compound.
- The higher C_{max}, T_{max}, AUC_{last} and MRT were observed for the ferulic glucuronide confirmed a slower metabolism and urinary excretion of this metabolite (Figure 1-2).

Table 3

Pharmacokinetic indexes for naringenin and ferulic glucuronides after single ingestion (100 g) of O-F, VOO-E and ROO-E tomato sauces (n = 6) by healthy men[1]

		C_{max} nmol/L	T_{max} min	AUC_{last} nmol/L x min	MRT min	C_{max}/AUC_{last} ratio
Naringenin glucuronide	O-F Salsa	445.6 ± 48.8	30.0 ± 0.0	43559 ± 5630	98.8 ± 16.3	0.0105 ± 0.0011
	VOO-E Salsa	398.0 ± 86.9	45.0 ± 7.7	37649 ± 6794	91.7 ± 12.7	0.0106 ± 0.0013
	ROO-E Salsa	296.6 ± 30.1	30.0 ± 0.0	32009 ± 5705	106.0 ± 12.5	0.0113 ± 0.0021
Ferulic glucuronide	O-F Salsa	580 ± 85.4	72.0 ± 12.0	118949 ± 26786	157.6 ± 10.6	0.0056 ± 0.0008
	VOO-E Salsa	727.0 ± 164.7	75.0 ± 13.4	197702 ± 53354	218.2 ± 56.6	0.0056 ± 0.0019
	ROO-E Salsa	544.2 ± 101.3	84.0 ± 14.7	99531 ± 20455	149.4 ± 14.0	0.0061 ± 0.0009

[1]Values are means ± SEM

- The plasma pharmacokinetic of naringenin glucuronide seemed to suggest a two-phase absorption only following the ingestion of oil-enriched tomato sauces (Figure 2B).
- No statistically significant differences observed in the pharmacokinetic indexes among the three interventions → need for larger-scale consumption studies.

CONCLUSIONS

- The mechanical and thermal treatments leading to tomato sauce elaboration seem to induce relevant changes in the phenolic composition of the starting product (Results I)
- The tomato-olive oil combination during tomato sauce processing does not seem to affect the extractability of the phenolic compounds in study, as well as their *in vivo* bioavailability and biotransformations (Results II - III).
- The plasma pharmacokinetic of naringenin glucuronide after ingestion of the oil-enriched tomato sauces suggest the occurrence of re-absorption events stimulated by the lipid matrix in the sauces (Results III).
- The great interindividual response variability observed emphasized the need for further large-scale investigations.

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- de Lourdes Mata-Bilbao M, *et al. Br J Nutr* 98:86-92, 2007

8.2.4. Comunicación 4. Póster.

Título: Phenolic profile and antioxidant capacity in tomato varieties from markers.

Autores: Anna Vallverdú-Queralt, Alexander Medina-Remón, **Miriam Martínez-Huélamo**, Olga Jáuregui-Pallarès, Cristina Andrés-Lacueva, and Rosa M. Lamuela-Raventos.

Congreso: 25th International Conference on Polyphenols. Montpellier, France, 2010.

Phenolic profile and antioxidant capacity in tomato varieties from markers.

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1. Introduction

Tomato (*Lycopersicon esculentum* Mill.) is the second most important vegetable crop worldwide. Epidemiological studies have pointed out that consumption of fruits and vegetables imparts health benefits, e.g. reduced risk of coronary heart disease and stroke, as well as certain types of cancer. Tomatoes are a key component in the so-called "Mediterranean diet" which is strongly associated with a reduced risk of chronic degenerative diseases.

Aim: Total polyphenols, individual polyphenols and antioxidant activity of seven commercial varieties of tomato produced in Spain were studied. The Folin-Ciocalteau (F-C) assay was used to analyse total polyphenol (TP) after solid phase extraction (SPE). Liquid chromatography coupled to mass spectrometry in tandem mode (LC/MS/MS) with negative ion detection was carried out to identify and quantify flavonols, flavanones, cinnamic acid derivatives and phenolic acids. Antioxidant capacity was analyzed by the ABTS assay.

2. Method

Sample preparation

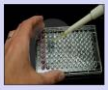
0.5 g tomato samples + 5 mL EtOH:H_2O (80:20) → Centrifugation: 4000 rpm, 20 min, 4 ºC → Evaporation: until 4 mL under nitrogen flow

supernatant + 5 mL EtOH:H_2O (80:20)

Sample cleaning-up: Oasis MAX® 96 well plate
- **1ª Condition**: 1 mL of methanol 98-100%.
- **2ª Condition**: 1 mL of sodium acetate 50 mM pH 7.
- **Load**: 1 mL samples + 1 mL Milli-Q water + 34 µL HCl.
- **Cleaning**: 1 mL sodium acetate 50 mM pH 7/ 5% methanol
- **Elution**: 1.8 mL methanol at 2 % formic acid.

Analysis of total polyphenols in tomatoes, in thermo microtiter 96 well plate.
- 20 µL of each methanolic fraction eluted after SPE.
- 188 µL of Milli-Q water.
- 12 µL of F-C reagent.
- 30 µL of sodium carbonate (200 g/L).

Incubation 60 min. in the darkroom.
After the reaction period: 50 µL of Milli-Q
Measure absorbance at 765 nm in the Spectrophotometers UV/VIS. **Results were expressed as mg of gallic acid equivalents (GAE)/100 g fresh material (FM).**



Analysis of total antioxidants in tomatoes
The antioxidant activity of the compounds is determined by the decolorization of the $ABTS^{\bullet+}$, through measuring the reduction of the radical cation as the percentage inhibition of absorbance at 734 nm in the Spectrophotometers UV/VIS. **Results of Trolox equivalent antioxidant capacity (TEAC) were expressed as (mmol trolox equivalent (TE)/100 g FM).**

calibration

Trolox mM	Trolox mL	ABTS⁺ mM	ABTS⁺ mL
0	5	0	245
5	5	250	245
8	5	400	245
10	5	500	245
15	5	750	245

sample

Dilution	Sample mL	PB mL
0	0	200
40	5	195
20	10	190
10	20	180

Trolox mM	Trolox (1mM) mL	Trolox mg	Final Volume mL
0	0	0	250
5	1,25	0,3125	
8	2	0,5	
10	2,5	0,625	
15	3,7	0,9375	

Calculation

$$INH\% = \frac{(\text{A solvent} - \text{A compound})}{(\text{A solvent}) \times 100}$$

$$TEAC = \frac{INH\% \text{ of sample}}{INH\% \text{ of trolox}}$$

Analysis of flavonols, flavanones, cinnamic acid derivatives and phenolic acids

Time (min)	H_2O 0.1%HFo	ACN 0.1%Hfo
0	95	5
10	82	18
13	0	100
14	0	100
15	95	5

Column: 50 x 2.0 mm i.d., 5 µm Luna C18
Injection volume: 20 µL
Flow: 4 mL / min
Column temperature : 30 ºC
Modes: Full scan, Product ion scan, neutral loss, multiple reaction monitoring

3. Results

Table 1. Total polyphenols and antioxidant activity expressed as mean ± SD of the seven varieties.

Varieties	Total polyphenols (mg GAE/100g FM)	Antioxidant activity (mmol TE/100 g FM)
malva	10.04 ± 0.30	1.79 ± 0.10
H-9661	13.32 ± 0.70	2.80 ± 0.20
H-9776	8.64 ± 0.20	1.29 ± 0.10
H-9997	9.01 ± 0.40	1.40 ± 0.10
albastro	8.60 ± 0.30	1.25 ± 0.09
guadiva	9.06 ± 0.20	1.63 ± 0.08
elegy	12.69 ± 0.40	1.98 ± 0.10

Albastro variety contained the lowest concentrations of phenolics, followed by H-9997 and Guadiva. The varieties with highest TP concentrations were H-9661, Elegy and Malva. A similar trend was observed for antioxidant activity.

Table 2: Quantification of cinnamic and phenolic acids (mean ± SD) expressed as µg/mg fresh weight of seven varieties described in the literature and investigated in this study.

Varieties	Ferulic acid (µg/kg FM)	Chlorogenic acid (µg/kg FM)	Caffeic acid (µg/kg FM)	Protocatechuic acid (µg/kg FM)
malva	N.Q.	0.36 ± 0.01	0.68 ± 0.01	0.20 ± 0.02
H-9661	0.35 ± 0.03	0.46 ± 0.01	1.02 ± 0.05	0.46 ± 0.02
H-9776	0.10 ± 0.05	0.40 ± 0.01	0.53 ± 0.01	N.Q.
H-9997	N.Q.	0.38 ± 0.03	0.53 ± 0.04	N.Q.
albastro	0.20 ± 0.01	0.39 ± 0.02	0.55 ± 0.02	N.Q
guadiva	N.Q.	0.38 ± 0.03	1.25 ± 0.01	N.Q.
elegy	0.21 ± 0.01	0.40 ± 0.02	0.63 ± 0.01	N.Q.

N.Q.: Not quantified

Table 3: Quantification of flavonols and flavanones (mean ± SD) expressed as µg/mg fresh weight of seven varieties described in the literature and investigated in this study.

Varieties	Quercetin (µg/kg FM)	Rutin (µg/kg FM)	Naringenin (µg/kg FM)	Kaempferol-3-*O*-Glucoside (µg/kg FM)
malva	0.73 ± 0.02	19.79 ± 0.77	3.03 ± 0.21	N.Q
H-9661	0.76 ± 0.04	20.44 ± 0.47	6.90 ± 0.19	N.Q
H-9776	0.42 ± 0.01	2.68 ± 0.05	0.75 ± 0.04	N.Q
H-9997	0.47 ± 0.04	4.65 ± 0.04	0.50 ± 0.01	0.27 ± 0.03
albastro	0.63 ± 0.04	0.79 ± 0.06	1.59 ± 0.25	N.Q
guadiva	0.69 ± 0.06	6.07 ± 0.51	1.71 ± 0.06	N.Q
elegy	0.69 ± 0.05	21.80 ± 1.68	0.70 ± 0.01	0.59 ± 0.01

N.Q.: Not quantified

The main polyphenol in all the varieties is rutin, which is present at levels ranging between 0.79 and 21.80 µg/g FM, followed by naringenin, which is found at levels between 0.50 and 6.90 µg/g FM. The variety with higher levels of individual polyphenols is H-9661 followed by Malva and Elegy, as it is reflected in the analysis of total phenolics and antioxidant activity, as well.

4. Conclusions

- It is the first time that SPE has been used to eliminate interfering non-phenolic reductants before the analysis of tomato varieties.
- The variables studied allowed the differentiation of H-9661, H-9997, H-9661, *malva, guadiva, albastro* and *elegy*. Significant differences ($P<0.05$) among varieties were obtained.
- H-9661 possessed the highest concentrations of total polyphenols and antioxidants, followed by *elegy* and *malva*.
- Rutin (quercetin-3-*O*-rutinoside) was the dominant flavonol amongst all the samples, followed by naringenin.

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25th International Conference on Polyphenols, Montpellier France

8.2.5. Comunicación 5. Póster.

Título: The effect of food matrix on the bioavailability of phenolic compounds from raw tomato and tomato sauces.

Autores: Sara Tulipani, **Miriam Martínez-Huélamo**, Ramon Estruch, Elvira Escribano, Montse Illán, Xavier Torrado, Cristina Andrés-Lacueva, and Rosa M. Lamuela-Raventós.

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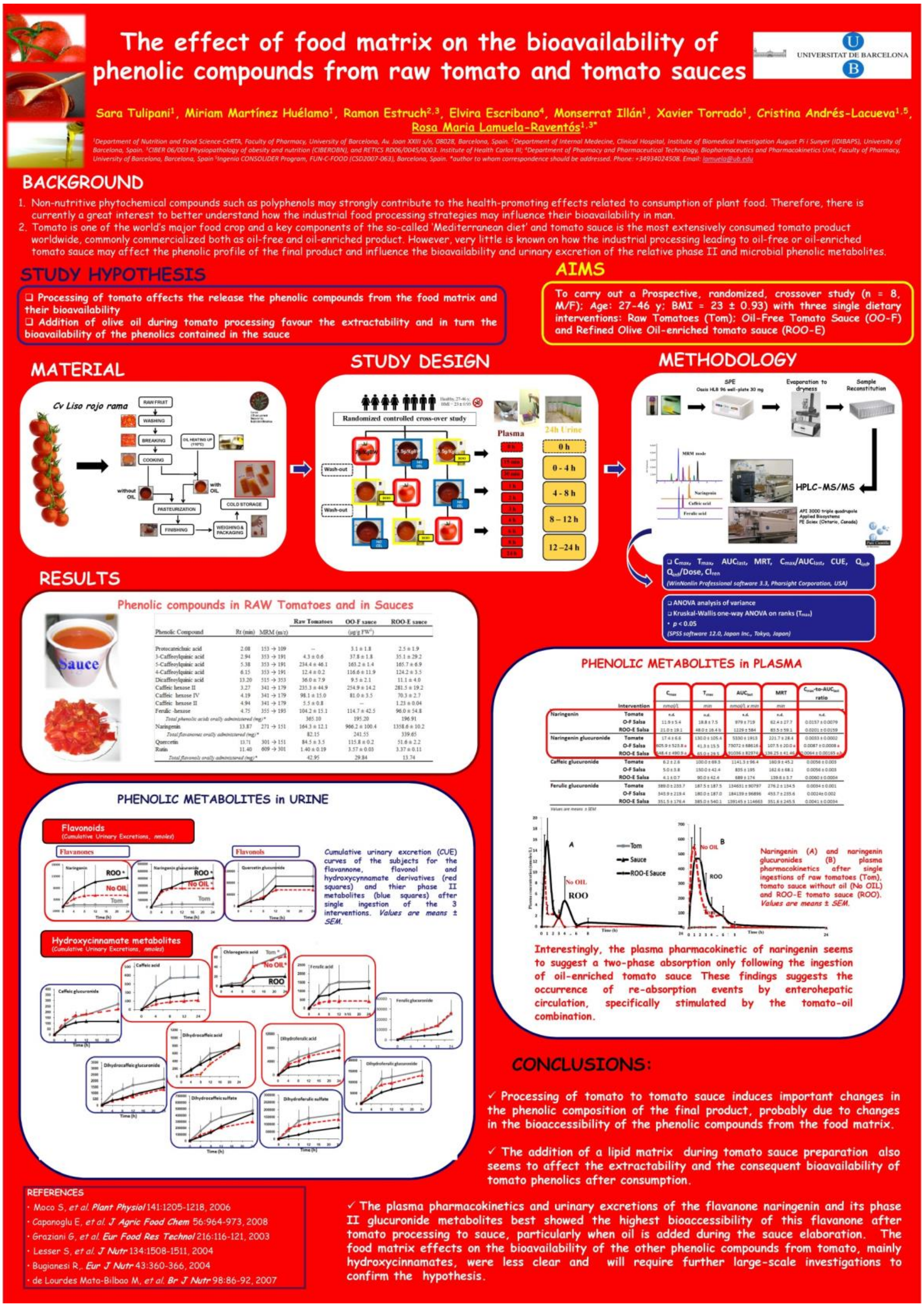
The effect of food matrix on the bioavailability of phenolic compounds from raw tomato and tomato sauces
UNIVERSITAT DE BARCELONA
Sara Tulipani[1], Miriam Martínez Huélamo[1], Ramon Estruch[2,3], Elvira Escribano[4], Monserrat Illán[1], Xavier Torrado[1], Cristina Andrés-Lacueva[1,5], Rosa Maria Lamuela-Raventós[1,3*]
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BACKGROUND
1. Non-nutritive phytochemical compounds such as polyphenols may strongly contribute to the health-promoting effects related to consumption of plant food. Therefore, there is currently a great interest to better understand how the industrial food processing strategies may influence their bioavailability in man.
2. Tomato is one of the world's major food crop and a key components of the so-called 'Mediterranean diet' and tomato sauce is the most extensively consumed tomato product worldwide, commonly commercialized both as oil-free and oil-enriched product. However, very little is known on how the industrial processing leading to oil-free or oil-enriched tomato sauce may affect the phenolic profile of the final product and influence the bioavailability and urinary excretion of the relative phase II and microbial phenolic metabolites.
STUDY HYPOTHESIS
❑ Processing of tomato affects the release the phenolic compounds from the food matrix and their bioavailability
❑ Addition of olive oil during tomato processing favour the extractability and in turn the bioavailability of the phenolics contained in the sauce
AIMS
To carry out a Prospective, randomized, crossover study (n = 8, M/F); Age: 27-46 y; BMI = 23 ± 0.93) with three single dietary interventions: Raw Tomatoes (Tom); Oil-Free Tomato Sauce (OO-F) and Refined Olive Oil-enriched tomato sauce (ROO-E)
MATERIAL
Cv Liso rojo rama
RAW FRUIT
WASHING
BREAKING
COOKING
OIL HEATING UP
without OIL
with OIL
PASTEURIZATION
COLD STORAGE
FINISHING
WEIGHING & PACKAGING
STUDY DESIGN
Randomized controlled cross-over study
Wash-out
Plasma
24h Urine
0 h
0 - 4 h
4 - 8 h
8 – 12 h
12 –24 h
METHODOLOGY
SPE
Oasis HLB 96 well-plate 30 mg
Evaporation to dryness
Sample Reconstitution
MRM mode
Naringenin
Caffeic acid
Ferulic acid
HPLC-MS/MS
API 3000 triple quadrupole
Applied Biosystems
PE Sciex (Ontario, Canada)
❑ Cmax, Tmax, AUClast, MRT, Cmax/AUClast, CUE, Qindi, Qindi/Dose, Clren
(WinNonlin Professional software 3.3, Pharsight Corporation, USA)
❑ ANOVA analysis of variance
❑ Kruskal-Wallis one-way ANOVA on ranks (Tmax)
• p < 0.05
(SPSS software 12.0, Japan Inc., Tokyo, Japan)
RESULTS
Phenolic compounds in RAW Tomatoes and in Sauces
Sauce
PHENOLIC METABOLITES in PLASMA
Tom
Sauce
ROO-E Sauce
No OIL
ROO
Naringenin (A) and naringenin glucuronides (B) plasma pharmacokinetics after single ingestions of raw tomatoes (Tom), tomato sauce without oil (No OIL) and ROO-E tomato sauce (ROO). Values are means ± SEM.
Interestingly, the plasma pharmacokinetic of naringenin seems to suggest a two-phase absorption only following the ingestion of oil-enriched tomato sauce These findings suggests the occurrence of re-absorption events by enterohepatic circulation, specifically stimulated by the tomato-oil combination.
PHENOLIC METABOLITES in URINE
Flavonoids
(Cumulative Urinary Excretions, nmoles)
Flavanones
Flavonols
Naringenin
Naringenin glucuronide
Quercetin glucuronide
Cumulative urinary excretion (CUE) curves of the subjects for the flavannone, flavonol and hydroxycynnamate derivatives (red squares) and thier phase II metabolites (blue squares) after single ingestion of the 3 interventions. Values are means ± SEM.
Hydroxycinnamate metabolites
(Cumulative Urinary Excretions, nmoles)
Caffeic glucuronide
Caffeic acid
Chlorogenic acid
Ferulic acid
Ferulic glucuronide
Dihydrocaffeic acid
Dihydroferulic acid
Dihydrocaffeic glucuronide
Dihydroferulic glucuronide
Dihydrocaffeic sulfate
Dihydroferulic sulfate
Time (h)
CONCLUSIONS:
✓ Processing of tomato to tomato sauce induces important changes in the phenolic composition of the final product, probably due to changes in the bioaccessibility of the phenolic compounds from the food matrix.
✓ The addition of a lipid matrix during tomato sauce preparation also seems to affect the extractability and the consequent bioavailability of tomato phenolics after consumption.
✓ The plasma pharmacokinetics and urinary excretions of the flavanone naringenin and its phase II glucuronide metabolites best showed the highest bioaccessibility of this flavanone after tomato processing to sauce, particularly when oil is added during the sauce elaboration. The food matrix effects on the bioavailability of the other phenolic compounds from tomato, mainly hydroxycinnamates, were less clear and will require further large-scale investigations to confirm the hypothesis.
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8.2.6. Comunicación 6. Póster.

Título: A new UPLC-MS/MS method for the determination of tomato polyphenols in biological fluids.

Autores: **Miriam Martínez-Huélamo**, Sara Tulipani, Olga Jáuregui, Isidre Casals, Cristina Andrés-Lacueva, and Rosa M. Lamuela-Raventós.

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A new UPLC-MS/MS method for the determination of tomato polyphenols in biological fluids

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INTRODUCTION

Tomato is one of the most popular and extensively consumed vegetable crop worldwide. The consumption of tomatoes and tomato products has been associated with decreased risk of chronic degenerative diseases. They are rich in antioxidant micronutrients and constitute almost the sole available source of lycopene. Other carotenes (such as b-carotene and lutein), vitamin C, and various phenolic compounds are also thought to be health-promoting factors with antioxidant properties. Tomato antioxidant content depends on the cultivar (genetic factors), maturity, and both agronomic and environmental conditions during cultivation.

We carried out a prospective randomized, cross-over study administering to 40 healthy subjects a single dose of raw tomatoes, tomato sauce without oil and tomato sauce with the addition of refined olive oil during processing. In order to quantify and identify the polyphenols and their metabolites in biological fluids like human plasma and urine we had validated a method that could be sufficiently sensitive to determine these substances in low concentration.

A crucial step in the sample treatment process is the extraction of polyphenols and their metabolites in biological fluids.

Our work describes the optimization of an effective extraction method for the analysis of polyphenols and their metabolites in urine and human plasma, applying for this purpose a solid phase extraction (SPE). The determination is carried out by Ultra Performance Liquid Chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). The method concluded satisfactory results in term of linearity, precision, recovery and limits of quantification.

SAMPLE PREPARATION

SPE: Oasis HLB 96 well-plate 30 mg

Acidification with 1 µL of H_3PO_4 / mL

Addition of standards and internal standard

Acidification with 2 µL of HFor/ mL

Centrifugation: 4ºC at 15000 g during 4 min

Addition of standards and internal standard

Condition with: MeOH and HFor 1.5M (2 mL)
Load sample: Urine or Plasma : H_2O (1:1) (2 mL)
Clean-up: HFor 1.5M and H_2O/MeOH (95:5) (2 mL)
Elution: MeOH (0.1% Hfor) (2 mL)

Evaporation and reconstitution with 200 µL H_2O (0.1%Hfor)

Filtration with 96-well filter plate

Analysis by: UPLC-MS/MS

ACQUITY UPLC™
PE-SCIEX API 3000

UPLC AND MS/MS CONDITIONS

UPLC conditions

- Acquity UPLC BEH C18 1.7 µm (2.1 x 50 mm) column
- 600 µL/min of mobile phase
- Temperature column: 30ºC
- Injection volume: 10 µL

Time (min)	H_2O (0,025%Hfor)	MeCN (0,025%Hfor)
0.00	95	5
2.00	75	25
2.50	10	90
2.65	0	100
2.80	0	100
2.90	95	5
3.50	95	5

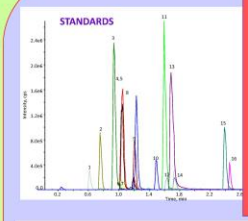
MS/MS conditions

- Ionspray: -3500 V
- Auxiliar gas (N_2): 5000 mL.min^{-1}
- Temperature: 400ºC
- Nebulizer gas (N_2): 10*
- Curtain gas (N_2): 12*
- CAD: 8*

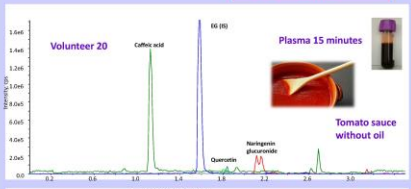
* (arbitrary units)

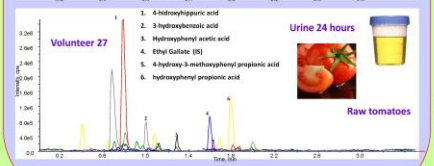
	Cone Voltage (V)	Focusing Pot. (V)	Entrance Pot. (V)	Transition quantification	Collision energy (V)
3-hydroxyphenyl acetic acid	-40	-170	-10	151 → 107	-10
3,3-hydroxyphenyl propionic acid	-40	-170	-10	165 → 121	-20
3,4-Dihydroxyphenyl acetic acid	-40	-170	-10	167 → 123	-10
4-hidroxyhippuric acid	-40	-170	-10	194 → 100	-20
4-hydroxy-3-methoxyphenyl acetic acid	-40	-170	-10	181→ 137	-20
Caffeic acid	-40	-170	-10	179 → 135	-20
Chlorogenic acid	-50	-180	-10	353 → 191	-20
Dihydrocaffeic acid	-50	-170	-10	181 → 137	-20
Ethyl Gallate	-60	-200	-10	197 → 169	-25
Ferulic acid	-40	-170	-10	193 → 134	-20
Hippuric acid	-40	-170	-10	178 → 134	-20
Isoferulic	-50	-220	-10	193 → 178	-20
Naringenina	-50	-190	-10	271 → 151	-30
Phenyl acetic acid	-50	-170	-10	135 → 91	-30
Quercetin	-60	-210	-10	301 → 151	-30
Quercetin-3-O-glucuronide	-60	-210	-10	477 → 301	-30

RESULTS



1. 4-hidroxyhippuric acid (0.68 min)
2. 3,4-Dihydroxyphenyl acetic acid (0.82 min)
3. Chlorogenic acid (1.00 min)
4. Hippuric acid (1.10 min)
5. 4-hydroxy-3-methoxyphenyl acetic acid (1.10 min)
6. Dihydrocaffeic acid (1.11 min)
7. Phenyl acetic acid (1.31 min)
8. Caffeic acid (1.18 min)
9. 3-hydroxyphenyl acetic acid (1.27 min)
10. Ethyl Gallate (IS) (1.56 min)
11. 3,3-hydroxyphenyl propionic acid (1.67 min)
12. Ferulic acid (1.7 min)
13. Quercetin-3-O-glucuronide (1.81 min)
14. Isoferulic (1.81 min)
15. Quercetin (2.46 min)
16. Naringenina (2.53 min)





VALIDATION PARAMETERS

	Linearity ranges (ng/mL)	Linear Fit (r)	Recovery (%)	LOD (ng/mL)	LOQ (ng/mL)	Intra-day 1 (%RSD)	Intra-day 2 (%RSD)	Intra-day 3 (%RSD)	Inter-day (%RSD)
3-hydroxyphenyl acetic acid	2-1152	0,9989	98 2	2	20	2	1	3	7
3,3-hydroxyphenyl propionic acid	10-5760	0,9968	94 3	2	22	2	4	5	7
3,4-Dihydroxyphenyl acetic acid	6-3168	0,9987	69 2	2	18	9	8	8	9
4-hidroxyhippuric acid	4-2016	0,9993	61 4	1	20	7	7	10	10
4-hydroxy-3-methoxyphenyl acetic acid	25-14400	0,9953	92 4	8	74	3	12	11	14
Caffeic acid	1-576	0,9976	91 2	1	7	2	3	1	3
Chlorogenic acid	5-2880	0,9991	92 2	1	6	8	8	5	11
Dihydrocaffeic acid	19-10656	0,9962	90 4	5	70	6	5	12	12
Ferulic acid	4-2016	0,9951	100 3	2	15	9	7	9	13
Hippuric acid	2-864	0,9952	99 4	2	24	3	4	6	6
Isoferulic	10-5760	0,9964	97 4	3	37	3	8	7	9
Naringenina	1-576	0,9996	96 2	0	4	4	9	6	11
Phenyl acetic acid	25-14400	0,9969	99 4	44	374	2	2	1	7
Quercetin	25-14400	0,9988	100 2	4	33	3	2	7	13
Quercetin-3-O-glucuronide	20-11520	0,9965	95 2	2	12	6	8	5	8

	Linearity ranges (ng/mL)	Linear Fit (r)	Recovery (%)	LOD (ng/mL)	LOQ (ng/mL)	Intra-day 1 (%RSD)	Intra-day 2 (%RSD)	Intra-day 3 (%RSD)	Inter-day (%RSD)
3-hydroxyphenyl acetic acid	2-1152	0,9998	99 ± 2	1	10	1	4	6	6
3,3-hydroxyphenyl propionic acid	10-5760	0,9952	98 ± 4	2	26	8	6	4	8
3,4-Dihydroxyphenyl acetic acid	6-3168	0,9968	78 ± 3	1	13	9	12	10	15
4-hidroxyhippuric acid	4-2016	0,9998	68 ± 3	1	11	8	13	4	14
4-hydroxy-3-methoxyphenyl acetic acid	25-14400	0,9980	97 ± 4	5	117	6	7	2	11
Caffeic acid	1-576	0,9969	100 ± 2	1	5	4	5	3	9
Chlorogenic acid	5-2880	0,9957	99 ± 4	1	8	4	6	6	6
Dihydrocaffeic acid	19-10656	0,9980	97 ± 5	6	65	6	7	5	8
Ferulic acid	4-2016	0,9987	100 ± 4	2	20	5	5	8	10
Hippuric acid	2-864	0,9994	97 ± 2	4	50	8	7	6	11
Isoferulic	10-5760	0,9963	100 ± 3	2	39	3	5	5	6
Naringenina	1-576	0,9998	99 ± 2	1	3	9	8	12	12
Phenyl acetic acid	25-14400	0,9960	95 ± 3	63	563	10	3	7	12
Quercetin-3-O-glucuronide	20-11520	0,9985	99 ± 2	1	13	7	6	8	10

CONCLUSIONS

A new very rapid method to quantify polyphenols in biological fluids has been developed. Good calibration parameters were obtained when the method was validated by UPLC-MS/MS.

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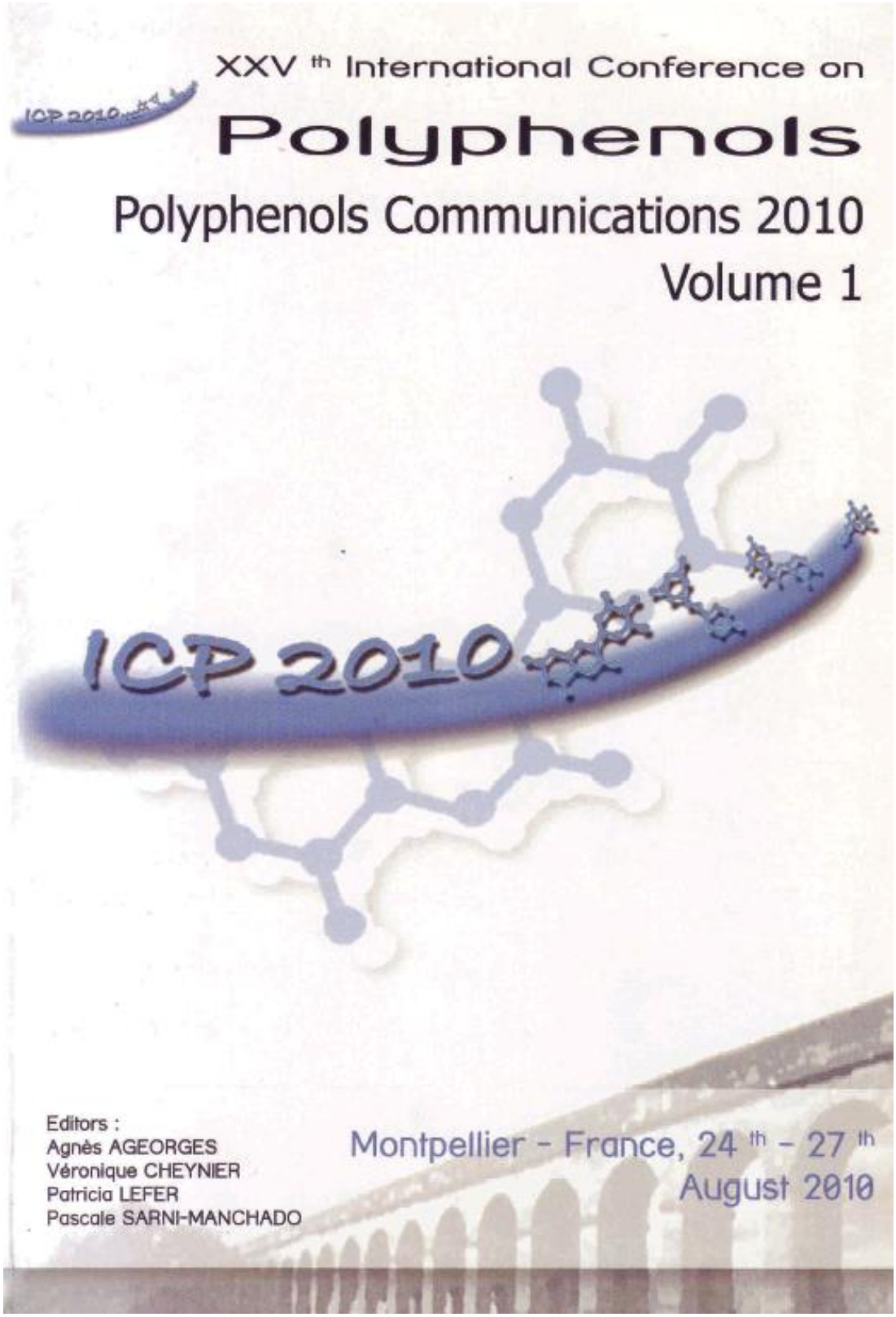
8.2.7. Comunicación 7. Comunicación Oral.

Título: Bioavailability and Urinary Excretion of Phenolic Metabolites from Raw Tomato and Tomato Sauces.

Autores: Sara Tulipani, **Miriam Martínez-Huélamo**, Ramon Estruch, Elvira Escribano, Montse Illán, Xavier Torrado, Cristina Andrés-Lacueva, and Rosa M. Lamuela-Raventós.

Congreso: 25th International Conference on Polyphenols. Montpellier, France, 2010.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Bioavailability and urinary excretion of phenolic metabolites from raw tomato and tomato sauces

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Abstract. The effects of the industrial processing leading to tomato sauce on the accessibility and further bioavailability of tomato phenolics is a topic of interest, given the large daily consumption of both tomato and tomato sauce in the Mediterranean region, and the typical combination with oil during cooking or industrial processing of tomato sauce. During the years 2008-2010, prospective randomized controlled cross-over studies were carrid out administering to healthy subjects single doses of raw tomatoes, tomato sauce without oil and tomato sauce elaborated with the addition of virgin or refined olive oil. The phenolic characterization of the dietary interventions, such as the identification and quantification of the expected phenolic metabolites present in plasma and urine samples was carried out, to evaluate the potential matrix effects on the plasma pharmacokinetics and urinary excretion of these compounds.

Introduction. Tomato (*Lycopersicon esculentum* Mill., Solanaceae) is one of the world's major food crop and a key component of the so-called Mediterranean diet. It is consumed both as fresh vegetable and processed products, most of all tomato sauce. In addition, the combination of tomatoes and tomato sauce with olive oil in food preparation is a typical Mediterranean habit. The regular consumption of tomato and tomato sauce has been long associated with lower risk of several types of cancer and coronary heart disease, and increasing evidence suggest that their relevant content of vitamin C and other phytochemical compounds such as polyphenols and carotenoids may play a crucial role in the health-promoting effects observed.

As already hypothesized for tomato carotenoids, the domestic or industrial-scale processing leading to tomato sauce production may produce changes in the extractability of phenolics due to the disruption of the plant cell wall and, thus, result in an easier release of bound polyphenolic and flavonoid compounds [1]. As well, the typical combination of oil during tomato sauce processing may influence the bioavalability of the phenolics in tomato, by modifying their bioaccessibility from the food matrix [2, 3]. However, very few studies using relatively small subjects numbers have investigated the absorption and excretion of phenolic compounds from raw tomatoes and tomato sauces, and even less is known on the impact of tomato-olive oil combination during processing on the phenolic bioavailability and bioefficacy in man. The aim of the present study was to assess the eventual differences in the absorption, plasma bioavailability and urinary excretion of phenolic metabolites, after single-dose administrations of raw tomatoes, oil-free and oil-enriched tomato sauces.

Materials and Methods. During tres consecutive years 2008-2010, we carried out three prospective randomized controlled cross-over study administering to healthy subjects single-doses of raw tomatoes and tomato sauces with or without the addition of olive oil during processing. Subjects were asked to follow a tomato-free diet during the 3 days preceding the dietary intervention, and a polyphenol-free diet during the 24 h immediately preceding the test. The day of each test, the overnight fasted subjects consumed their interventions in a randomized order, after a baseline heparinized blood sample was collected. Blood was then collected at several timepoints during the 24 h after the ingestion of the tomatoes and tomato sauces, and plasma samples immediately stored at -80ºC until analysis. Also urine

was collected before consumption of the interventions (baseline, 0 h) and during the 24 h-urine after ingestion in separate fractions, and the amount of urine in each fraction was measured.
The phenolic characterization of the raw tomatoes and tomato sauces, as well as the detection, identification and quantification of the corresponding phenolic metabolites in human plasma and urine, coming from phase II transformations and microbiota metabolism, were carried out by newly optimized HPLC and UPLC-MS/MS analysis by Triple Quadrupole mass spectrometer API 3000, with negative ion detection mode. To ensure an efficient extraction of phenolics from plasma and urine samples, the biological fluids were subjected to solid-phase extraction (SPE) with Oasis® HLB 96-well plates, before LC-MS analysis. The identification of the compounds of interest was carried out on the basis of their mass spectra in full scan mode, then confirmed by MS-MS experiments (product ion scan, precursor ion scan, neutral loss). Multiple reaction monitoring (MRM) experiments were used for quantification purposes.

Results and Discussion. The LC-MS characterization of the phenolic compounds in the three dietary interventions firstly confirmed the flavanone naringenin (4', 5, 7-trihydroxyflavanone) and the hydroxycinnamate chlorogenic acid (5-caffeoylquinic acid) among the most abundant phenolics in tomato [4], already extensively studied for their multiple potential biological and antioxidant. Secondly, the data showed how heat and mechanical treatments during tomato sauce processing may induce biochemical changes in the food matrix, increasing the bioaccessibility of several phenolics from the processed products. The flavanone naringenin was the most affected compound, its content being significantly higher in the sauces than in raw tomatoes. Also the plasma kinetics and urinary excretion of the flavanone naringenin and the phase II naringenin glucuronide metabolites inequivocally suggested the highest bioaccessibility of this flavanone after tomato processing to sauce, confirming the strong effect of the food matrix in polyphenols bioavailability.

References

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8.2.8. Comunicación 8. Póster

Título: Varietal differences among the phenolic profiles of tomatoes and differences between two tomato juice recipes.

Autores: **Miriam Martínez-Huélamo**, Palmira Valderas-Martínez, Olga Jáuregui, Montse Illán, and Rosa M. Lamuela-Raventós

Congreso: Recent Advances in Food Analysis, RAFA. Prague, Czech Republic, 2011.

Varietal differences among the phenolic profiles of tomatoes and differences between two tomato juice recipes

Miriam Martínez-Huélamo[1,2], Palmira Valderas-Martínez[2,3], Olga Jáuregui[4], Montse Illán[1], Rosa M. Lamuela-Raventós[1,2*]

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INTRODUCTION

The health-promoting properties of tomato and tomato products seem to reside in the high amount of phytochemical constituents such as carotenoids, vitamin C, vitamin E and the wide variety of phenolic components ranging from flavonoids to phenolic acids. Despite increasing knowledge of the effect of industrial food processing on carotenoid and vitamin bioavailability, there is a lack of information regarding the extent of changes in the polyphenol/flavonoid content of tomato after mechanical and/or thermal processing treatments, and there is even less available data in the literature on the effects of food matrix on bioavailability. Furthermore, very little is known about the impact of tomato and tomato-olive oil combination on the phenolic bioavailability and bioefficacy in humans.

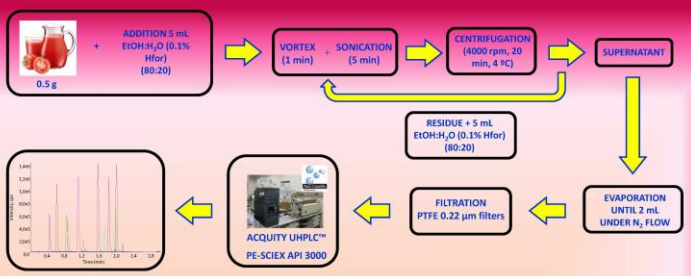
Previous work on phenolic characterization of tomato fruits has been performed using different techniques including nuclear magnetic resonance, gas chromatography and capillary electrophoresis, but the most common is high-performance liquid chromatography (HPLC) coupled to a photodiode detector (UV/Vis) or mass spectrometry (MS). Among the methods used to determine phenolic compounds, liquid chromatography coupled to mass spectrometry with electrospray ionization (ESI) is one of the most powerful tools for the analysis of non-volatile and thermally labile classes of compounds. High performance liquid chromatography has been improved by the introduction of ultra performance liquid chromatography (UPLC), which increases the signal-to-noise ratio (S/N), enhances peak resolution, and reduces both analysis time and costs.

AIMS

Analyse the phenolic profile of different Spanish tomatoes in order to get the juice with the highest phenolic content.

Study of two tomato juices with and without refined olive oil in order to verify how the oil combination affects the phenolic bioavailability.

SAMPLE PREPARATION



TOMATO JUICE

RESULTS

PHENOLIC COMPOUNDS IN "RAMA", PEAR AND "LISO" TOMATOES

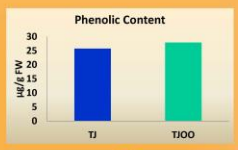
Compound	RT (min)[a]	MRM (m/z)	"RAMA" µg/g FW[b] skin	"RAMA" µg/g FW[b] pulp	PEAR µg/g FW[b] skin	PEAR µg/g FW[b] pulp	"LISO" µg/g FW[b] skin	"LISO" µg/g FW[b] pulp
Protocatechuic	0,7	153 → 109	n.d	n.d	n.d	0,009 ± 0,001	n.d	0,001 ± 0,000
Caffeic hexose I	0,74	341 → 179	0,285 ± 0,006	0,028 ± 0,004	n.d	0,706 ± 0,087	n.d	0,414 ± 0,042
Coumaric hexose I	0,77	325 → 163	0,044 ± 0,007	0,086 ± 0,001	0,039 ± 0,001	0,100 ± 0,013	0,037 ± 0,002	0,057 ± 0,006
Ferulic hexose	0,9	355 → 193	12,61 ± 1,81	8,473 ± 0,629	5,111 ± 0,690	10,29 ± 1,29	8,778 ± 0,998	6,719 ± 5,828
Caffeic hexose II	0,97	341 → 179	0,282 ± 0,019	0,228 ± 0,009	0,387 ± 0,013	0,253 ± 0,034	0,249 ± 0,036	0,158 ± 0,017
3-Caffeoylquinic acid	1,02	353 → 191	0,065 ± 0,009	0,049 ± 0,006	1,923 ± 0,222	0,165 ± 0,022	1,137 ± 0,087	0,054 ± 0,003
Coumaric hexose II	1,07	325 → 163	2,338 ± 0,284	0,022 ± 0,003	2,039 ± 0,285	0,028 ± 0,004	2,368 ± 0,308	0,017 ± 0,001
5-Caffeoylquinic acid	1,09	353 → 191	n.d	0,036 ± 0,003	n.d	0,064 ± 0,007	n.d	0,032 ± 0,004
Caffeic acid	1,17	179 → 135	0,121 ± 0,015	0,178 ± 0,023	0,144 ± 0,006	0,445 ± 0,063	0,130 ± 0,005	0,081 ± 0,012
4-Caffeoylquinic acid	1,26	353 → 191	2,412 ± 0,289	0,123 ± 0,017	3,990 ± 0,589	0,226 ± 0,026	3,485 ± 0,524	0,075 ± 0,065
o-Coumaric acid	1,53	163 → 119	n.d	0,024 ± 0,001	n.d	0,033 ± 0,029	n.d	0,015 ± 0,001
Rutin	1,7	609 → 301	33,77 ± 4,29	n.d	43,53 ± 5,49	n.d	38,80 ± 5,55	n.d
Ferulic acid	1,7	193 → 134	n.d	0,148 ± 0,016	n.d	0,750 ± 0,090	n.d	0,277 ± 0,022
Dicaffeoylquinic acid	1,97	515 → 353	n.d.	0,005 ± 0,003	0,090 ± 0,004	0,006 ± 0,006	0,096 ± 0,084	0,002 ± 0,003
Naringenin-O-hexoside	2,08	433 → 271	1,010 ± 0,068	0,010 ± 0,001	0,259 ± 0,225	n.d	0,311 ± 0,270	n.d
Naringenin	2,52	271 → 151	20,15 ± 17,50	n.d.	51,32 ± 6,00	n.d.	43,57 ± 3,22	n.d.

[a]RT: Chromatographic retention. [b]FW: Fresh weight

HIGHER PHENOLIC CONTENT

PEAR TOMATO

PHENOLIC COMPOUNDS IN TOMATO JUICE (JC) AND TOMATO JUICE WITH REFINED OLIVE OIL (TJOO)



HIGHER PHENOLIC CONTENT

Compound	RT (min)[a]	MRM (m/z)	TJ[b] µg/g FW[d]	TJOO[c] µg/g FW[d]
Caffeic hexose I	0,74	341 → 179	1,848 ± 0,032	2,676 ± 0,267
Caffeic hexose II	0,97	341 → 179	1,043 ± 0,014	1,271 ± 0,229
3-Caffeoylquinic acid	1,02	353 → 191	4,905 ± 0,191	7,509 ± 0,835
Coumaric hexose II	1,07	325 → 163	0,144 ± 0,012	0,175 ± 0,154
5-Caffeoylquinic acid	1,09	353 → 191	2,751 ± 0,282	4,688 ± 0,403
Caffeic acid	1,17	179 → 135	1,960 ± 0,160	2,191 ± 0,360
4-Caffeoylquinic acid	1,26	353 → 191	0,680 ± 0,040	0,795 ± 0,089
o-Coumaric acid	1,53	163 → 119	0,238 ± 0,027	0,179 ± 0,156
Rutin	1,7	609 → 301	5,975 ± 0,102	6,407 ± 0,972
Ferulic acid	1,7	193 → 134	1,392 ± 0,095	1,423 ± 0,201
Dicaffeoylquinic acid	1,97	515 → 353	0,790 ± 0,075	0,368 ± 0,038
Naringenin-O-hexoside	2,08	433 → 271	0,054 ± 0,008	0,051 ± 0,005
Naringenin	2,52	271 → 151	3,928 ± 3,413	0,141 ± 0,051

[a]RT: Chromatographic retention [b]TJ: Tomato juice [c]TJOO: Tomato juice with olive oil [d]FW: Fresh weight

CONCLUSIONS

* Three tomato varieties ("rama", pear and "liso") were studied. The results showed a higher phenolic content in pear tomato.

* Moreover, it was noticed that the process for preparing tomato juice and the addition of a lipid matrix during juice preparation change the phenolic composition of the product, probably due to changes in the bioaccessibility of phenolics from the food matrix.

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8.2.9. Comunicación 9. Póster

Título: Bioavailability of phenoliccompounds of raw tomato and tomato sauces.

Autores: **Miriam Martínez-Huélamo**, Sara Tulipani, Palmira Valderas-Martínez, Giuseppe Di Lecce, Anna Vallverdú-Queralt, Ramón Estruch, Xavier Torrado, Elvira Escribano-Ferrer, and Rosa M. Lamuela-Raventós.

Congreso: 5th International Conference on Polyphenols and Health. Sitges, Spain, 2011.

Bioavailability of phenolic compounds of raw tomato and tomato sauces

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INTRODUCTION

Tomato is widely consumed either fresh or after processing into various cooked products. The health-promoting properties of tomato and tomato based products seem to reside in the high amount of bioactive molecules such as carotenoids, vitamin C, and phenolic compounds. Food matrix seems to be an important factor modulating absorption and further biological activity of dietary polyphenols. In contrast, very little is known about the extent of changes in the individual polyphenols of tomato after the mechanical and thermal treatments leading to tomato sauce preparation, such as the impact of the tomato processing and tomato-olive oil combination on the phenolic bioavailability and bioefficacy in human.

AIMS

Verify whether the oral absorption and metabolism of the phenolic compounds contained in tomato and tomato sauces is influenced either by the processing or the addition of a lipid matrix during sauce processing.

Prospective randomized, cross-over study was conducted in 40 healthy volunteers aged 28 11 years (BMI = 23.30 3.86kg/m²). The study included three interventions: raw tomatoes (RT), oil-free tomato sauce (OF) and refined olive oil-enriched tomato sauce (ROOE).

STUDY DESIGN

PRE-STUDY :

Day -3: Tomato free diet
Day -1: Polyphenol free diet

STUDY DAY :

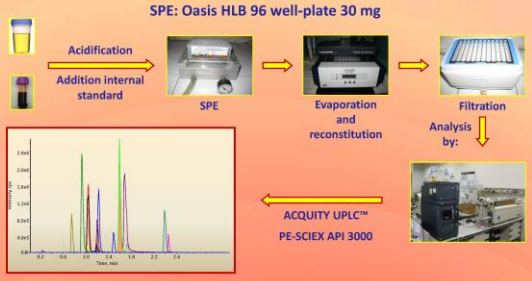
RT ROOE OF

WASHOUT: 1 Month between interventions

TOMATO SAUCE PREPARATION

SAMPLE PREPARATION

SPE: Oasis HLB 96 well-plate 30 mg



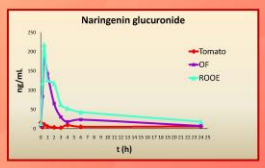
RESULTS

PHENOLIC COMPOUNDS IN RAW TOMATOES, OF AND ROOE SAUCES

Compound	RT (min)[a]	MRM (m/z)	RAW TOMATO ng/g FW[b]	OF ng/g FW[b]	ROOE ng/g FW[b]
Coumaric hexose 1	0,95	325 → 163	35,2 ± 0,6	29,7 ± 2,5	20,2 ± 2,1
Protocatechuic	0,98	153 → 109	23,9 ± 3,0	137,4 ± 9,3	77,4 ± 8,5
Caffeic hexose 1	1,00	341 → 179	1641,0 ± 108,8	1545,5 ± 175,5	1088,4 ± 55,0
Coumaric hexose 2	1,02	325 → 163	235,3 ± 4,8	51,7 ± 4,1	53,6 ± 6,7
3-Caffeoylquinic acid	1,07	353 → 191	135,7 ± 1,0	189,5 ± 12,2	298,2 ± 14,8
Ferulic hexose	1,14	355 → 193	1437,2 ± 54,2	822,3 ± 12,1	832,6 ± 8,0
Caffeic hexose 2	1,22	341 → 179	647,8 ± 20,9	722,5 ± 49,8	675,8 ± 19,8
Homovanillic hexose 1	1,23	343 → 181	4525,1 ± 361,6	6985,3 ± 445,0	8312,9 ± 524,1
Homovanillic hexose 2	1,26	343 → 181	636,6 ± 54,5	738,7 ± 43,0	923,6 ± 55,3
5-Caffeoylquinic acid	1,26	353 → 191	385,5 ± 10,6	899,1 ± 39,9	704,6 ± 78,9
Coumaric hexose 3	1,31	325 → 163	201,4 ± 1,8	374,2 ± 6,6	380,3 ± 8,6
Caffeic acid	1,45	179 → 135	379,5 ± 18,1	498,6 ± 18,7	527,7 ± 16,9
4-Caffeoylquinic acid	1,49	353 → 191	832,5 ± 7,1	533,8 ± 38,6	542,7 ± 12,4
3-Hydroxybenzoic acid	1,55	137 → 93	40,8 ± 3,6	13,3 ± 1,0	1,6 ± 0,2
Rutin	1,77	609 → 301	1889,4 ± 9,1	3849,9 ± 74,7	3628,5 ± 63,9
Naringenin chalcone	1,80	271 → 151	185,7 ± 2,6	207,5 ± 14,0	223,5 ± 1,1
Ferulic acid	1,85	193 → 134	48,2 ± 4,9	n.d.	n.d.
Dicaffeoylquinic acid	1,91	515 → 353	57,8 ± 0,3	48,0 ± 0,3	50,0 ± 0,5
Apigenin-7-glucoside	1,94	431 → 269	77,0 ± 2,4	97,9 ± 2,3	88,7 ± 11,0
Naringenin-O-hexoside	1,94	433 → 271	62,5 ± 4,1	60,0 ± 9,0	71,0 ± 6,6
Tricaffeoylquinic acid	2,06	677 → 515	116,3 ± 3,8	65,3 ± 5,7	63,1 ± 1,7
p-Coumaric acid	2,08	163 → 119	21,0 ± 1,8	26,8 ± 3,0	12,4 ± 1,6
Naringenin	2,26	271 → 151	3499,9 ± 379,2	3349,3 ± 38,7	3746,8 ± 102,2

[a]RT: Chromatographic retention (in minutes). [b]FW: Fresh weight

PHENOLIC METABOLITES IN PLASMA

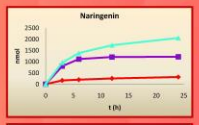


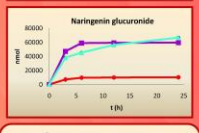
	Intervention	C_{max} ng/mL	T_{max} h	AUC_{last} ng/mL x h	MRT h	C_{max}-to-AUC_{last} ratio
Naringenin	Raw tomato	24,6 ± 19,9	3,9 ± 7,5	247,2 ± 210,0	10,7 ± 5,0	0,3 ± 0,4
	OF sauce	21,7 ± 17,3	2,6 ± 6,2	153,4 ± 174,6	8,0 ± 6,	0,6 ± 0,6
	ROOE sauce	23,1 ± 14,6	1,9 ± 4,7	180,8 ± 172,7	7,6 ± 5,6	0,4 ± 0,4
Naringenin glucuronide	Raw tomato	53,3 ± 64,1	2,1 ± 1,8	109,1 ± 105,5	3,2 ± 0,6	0,4 ± 0,2
	OF sauce	130,7 ± 80,8	0,8 ± 0,4	173,1 ± 139,0	1,2 ± 0,4	1,1 ± 0,6
	ROOE sauce	253,0 ± 236,7	0,8 ± 0,4	373,4 ± 433,2	2,3 ± 3,3	0,8 ± 0,4
Ferulic glucuronide 1	Raw tomato	84,8 ± 76,2	2,7 ± 2,0	817,8 ± 894,3	8,0 ± 4,4	0,2 ± 0,1
	OF sauce	103,0 ± 108,8	5,3 ± 7,0	1166,8 ± 1328,3	9,7 ± 4,7	0,2 ± 0,1
	ROOE sauce	114,2 ± 102,1	5,0 ± 5,7	1026,4 ± 960,9	7,6 ± 4,0	0,2 ± 0,1
Ferulic glucuronide 2	Raw tomato	144,7 ± 113,9	1,32 ± 0,8	249,4 ± 272,1	1,9 ± 1,1	0,8 ± 0,4
	OF sauce	139,8 ± 110,8	1,8 ± 1,5	299,4 ± 253,4	2,6 ± 2,1	0,6 ± 0,2
	ROOE sauce	188,4 ± 172,8	3,7 ± 5,1	475,8 ± 587,4	4,6 ± 5,4	0,6 ± 0,3
Ferulic glucuronide 3	Raw tomato	123,4 ± 145,8	1,1 ± 0,7	224,7 ± 213,3	4,1 ± 4,3	0,7 ± 0,3
	OF sauce	168,2 ± 263,5	1,5 ± 1,1	213,1 ± 222,1	3,1 ± 2,7	0,9 ± 0,8
	ROOE sauce	94,4 ± 76,6	2,0 ± 1,2	233,8 ± 288,5	3,8 ± 3,8	0,9 ± 0,5
Ferulic glucuronide 4	Raw tomato	160,6 ± 157,7	2,8 ± 5,3	1387,9 ± 1604,2	10,1 ± 5,4	0,3 ± 0,2
	OF sauce	144,7 ± 123,9	3,3 ± 6,7	1293,8 ± 1399,4	10,6 ± 6,9	0,2 ± 0,2
	ROOE sauce	n.d.	n.d.	n.d.	n.d.	n.d.
Ferulic sulfate 1	Raw tomato	n.d.	n.d.	n.d.	n.d.	n.d.
	OF sauce	92,1 ± 58,6	2,3 ± 1,5	531,1 ± 611,4	10,0 ± 9,2	0,5 ± 0,4
	ROOE sauce	84,0 ± 71,8	7,3 ± 11,3	402,9 ± 701,9	6,2 ± 8,0	1,3 ± 0,9
Ferulic sulfate 2	Raw tomato	156,3 ± 212,2	1,4 ± 1,3	181,1 ± 277,8	1,64 ± 0,9	1,2 ± 0,6
	OF sauce	186,6 ± 139,6	1,5 ± 1,3	678,7 ± 678,9	10,7 ± 7,5	0,5 ± 0,4
	ROOE sauce	163,6 ± 177,9	1,6 ± 0,5	358,4 ± 152,0	8,7 ± 7,8	0,5 ± 0,4
Isoferulic	Raw tomato	154,2 ± 75,4	3,0 ± 6,8	550,2 ± 527,5	4,6 ± 6,1	0,5 ± 0,3
	OF sauce	180,3 ± 126,7	4,3 ± 8,3	994,6 ± 1326,2	7,6 ± 7,3	0,6 ± 0,5
	ROOE sauce	232,4 ± 176,8	4,6 ± 8,2	1607,5 ± 2156,7	8,5 ± 7,4	0,4 ± 0,6
Quercetin	Raw tomato	150,9 ± 84,2	4,9 ± 7,8	1931,7 ± 1219,1	11,7 ± 3,2	0,1 ± 0,1
	OF sauce	151,6 ± 67,3	4,1 ± 6,9	2097,8 ± 1323,8	11,2 ± 2,9	0,1 ± 0,1
	ROOE sauce	181,9 ± 118,1	4,2 ± 6,9	2743,7 ± 1800,9	12,4 ± 2,2	0,1 ± 0,0

The plasma pharmacokinetic of Naringenin glucuronide seems to suggest a two-phase absorption only following the ingestion of oil-enriched tomato sauce. These findings suggested the occurrence of re-absorption events by enterohepatic circulation, specifically stimulated by the tomato-oil combination.

PHENOLIC METABOLITES IN URINE

Compound	Tomato nmol	OF nmol	ROOE nmol	Compound	Tomato nmol	OF nmol	ROOE nmol
Caffeic acid	2939	2489	3199	4-Hydroxybenzoic acid	262	295	354
Caffeic glucuronide	3434	3562	6917	Dihydrocaffeic acid	1620	1045	1428
Caffeic sulfate	100087	87310	107850	Dihydrocaffeic glucuronide	215534	168940	264582
Coumaric glucuronide	1418	1479	1538	Dihydrocaffeic sulfate	2782485	2455967	2672168
Coumaric sulfate	51863	56918	52902	Dihydroxyphenylacetic acid	23335	20041	26215
Chlorogenic acid	178	151	202	Hydroxyhippuric acid	351606	323138	328110
Ferulic acid	7972	5009	7453	Homovanillic acid	695849	601121	838278
Ferulic glucuronide	113748	108259	122858	Hydroxyphenylacetic glucuronide	24175	21915	26004
Ferulic sulfate	1142697	1089336	1162266	Hydroxyphenylacetic sulfate	1519276	1418998	1628891
Isoferulic	84319	68377	86159	Hydroxyphenylpropionic glucuronide	26243	17308	29390
m-Coumaric	420	311	306	Hydroxyphenylpropionic sulfate	315251	284042	321625
Naringenin	316	1218	2055	Hydroferulic acid	24192	21963	23039
Naringenin glucuronide	10231	59587	66731	Hydroferulic glucuronide	171014	141417	189281
o-Coumaric	448	567	519	Hydroferulic sulfate	1128186	855576	1115637
p-Coumaric	683	397	554	Hydroxyphenylacetic acid	175164	130743	204840
Quercetin	392	292	332	Hydroxyphenylpropionic acid	22308	20287	24005
Quercetin glucuronide	17387	9470	11738	Phenylacetic glucuronide	25	30	34
Quercetin sulfate	350	1919	423	Phenylacetic sulfate	27545	33928	31948
3-Hydroxybenzoic acid	55363	44508	52657	Phenylacetic acid	12892	11496	15687





Cumulative urinary excretion curves for Naringenin and Naringenin glucuronide after single ingestions of RAW TOMATO, O-F and ROOE sauces

CONCLUSIONS

The plasma kinetics and urinary excretion of the flavanone Naringenin and Naringenin glucuronide after the interventions confirmed the highest bioaccessibility of this flavanone after processing tomato to sauce.

The plasma pharmacokinetic of Naringenin glucuronide suggests a two-phase absorption only following the ingestion of oil-enriched tomato sauce. These findings suggest the occurrence of re-absorption events by enterohepatic circulation.

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8.2.10. Comunicación 10. Póster

Título: Determination of prenylflavonoids in different spanish beers using liquid chromatography-mass spectrometry.

Autores: Paola Quifer-Rada, Ramón Estruch, and Rosa M. Lamuela-Raventós.

Congreso: 5th International Conference on Polyphenols and Health. Sitges, Spain, 2011.

DETERMINATION OF PRENYLFLAVANOIDS IN DIFFERENT SPANISH BEERS USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY.

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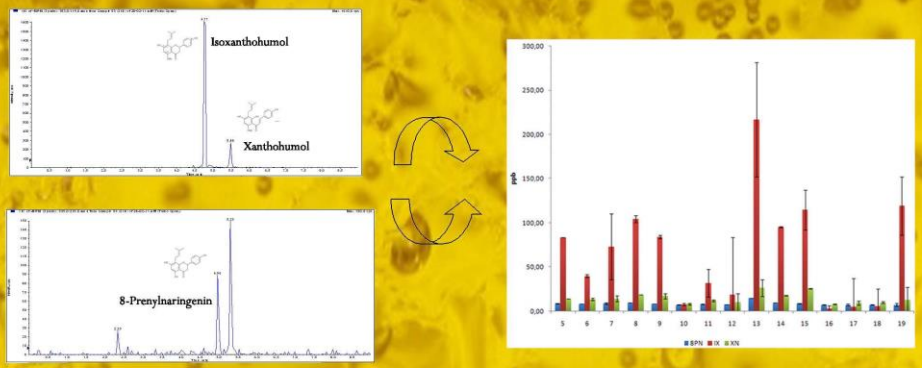
1. INTRODUCTION:

Beer is one of the most commonly consumed fermented and undestilled alcoholic beverages in many countries. About 20-30% of beer polyphenols come from hops and the remaining 70-80% are malt derived (1). The most abundant phenols in hop are the prenylflavanoids group which includes xanthohumol and its derivates (isoxanthohumol and 8-prenylnaringenin) and humulons, which all come exclusively from hops (2). Xanthohumol (XN) represents 95% of hop prenylflavanoids fraction and is a prenylchalcona that isomerizes to isoxanthohumol during the brewing process (3). In the intestinal tract, isoxanthohumol (IX) is converted to 8-prenylnaringenin (8-PN) through an O-desmetilization catalyzed by gut bacteria (4,5).

The aim of this work was to analyzed hop poyphenol levels in 19 Spanish beers from the market by liquid chromatography coupled to a mass spectrometry.

2. MATERIALS AND METHOD:

3. RESULTS:



4. CONCLUSIONS:

All Spanish beers showed levels of prenylflavanoids. IX compound was the most abundant; XN was found in the range of 7.67-26.27 µg/L, 8-PN in the range of 7.25-14.54 µg/L and IX in the range of 2.74-216.79 µg/L. However, the content of prenylflavanoids did not depend on the type of beer and it may be due to the sort or the amount of hop used during brewing process.

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6. ACKNOWLEDGEMENTS

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8.2.11. Comunicación 11. Comunicación Oral

Título: Oil addition increase anti-inflammatory effects of tomato.

Autores: **Miriam Martínez-Huélamo**, Sara Tulipani, Palmira Valderas-Martínez, Giuseppe Di Lecce, Anna Vallverdú-Queralt, Ramón Estruch, Xavier Torrado, Elvira Escribano-Ferrer, and Rosa M. Lamuela-Raventós.

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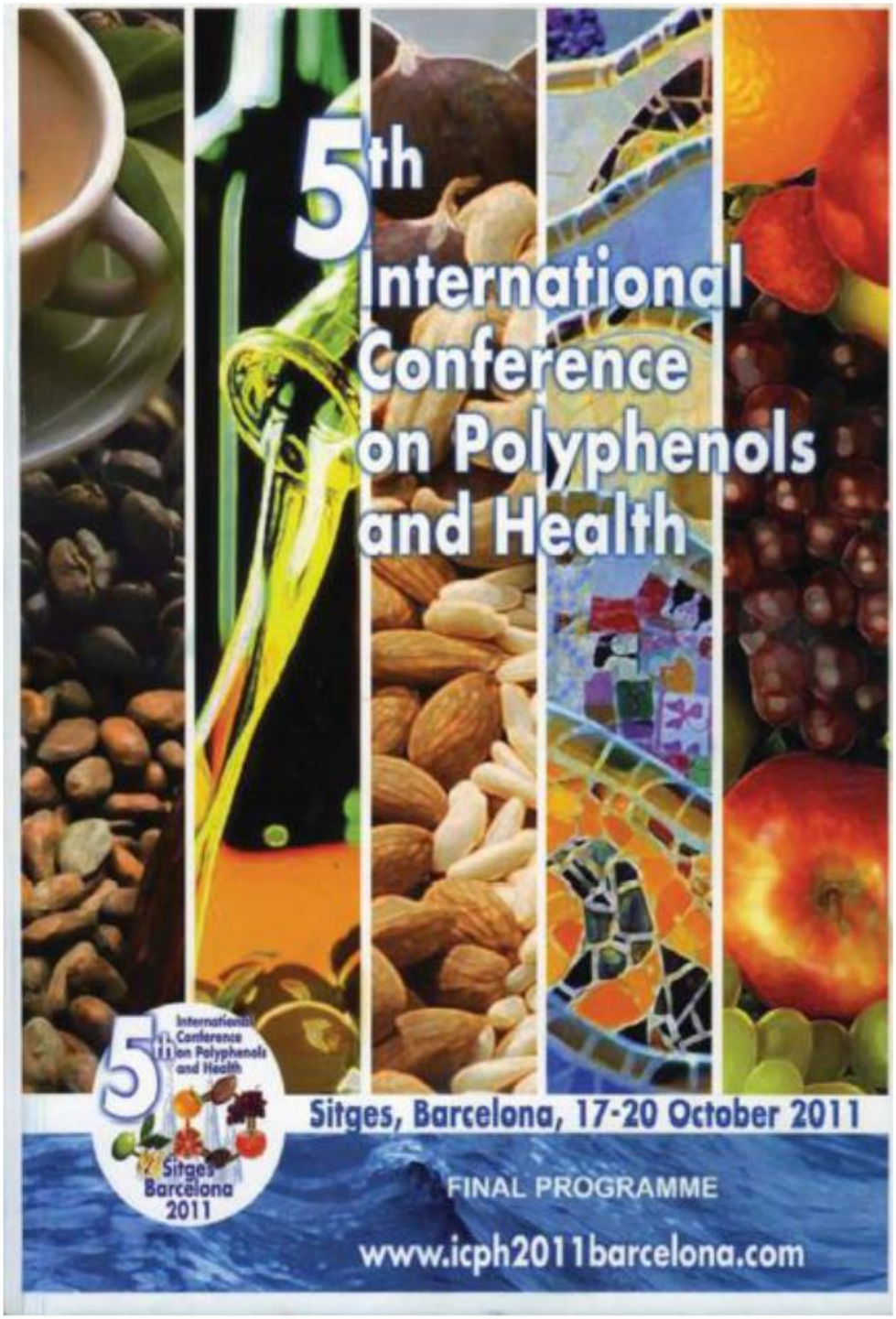
5th International Conference on Polyphenols and Health
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O52 - Co-sponsored Lecture by IOOC

NUTRIGENOMIC EFFECTS OF MEDITERRANEAN DIET AND OLIVE OIL CONSUMPTION ON CARDIOVASCULAR RISK

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Introduction Adherence to traditional Mediterranean diet, in which olive oil is the main source of fat, has been associated with reductions in cardiovascular morbidity and mortality. The objective of the present study was to assess the in vivo gene expression changes, protective for cardiovascular disease, associated with the consumption of traditional Mediterranean diet, virgin olive oil, and its phenolic compounds, in healthy volunteers.

Materials and methods: A randomized, parallel, controlled, double-blind trial (ISRCTN53283428) with three dietary interventions was performed. Ninety Volunteers (20-50 years) were recruited. Whole blood and urine samples were collected at 0 hours (baseline) and after 3 months of intervention. Volunteers were grouped into three groups: a) Group A: Traditional Mediterranean diet with virgin olive oil high in phenolic content; b) Group B: Traditional Mediterranean diet with olive oil with very low phenolic content; Group C: habitual diet. Changes in the expression of several cardiovascular disease-related genes were assessed. Plasma glucose, lipid profile, oxidative damage, tyrosol/ hydroxytyrosol, and in flammation markers were also measured.

Results: Traditional Mediterranean diet consumption, decreased: 1) plasma oxidative and inflammatory status and; 2) the expression of genes related with both inflammation (interferon-gamma; IFNgamma, Rho-GTPase activating protein15; ARHGAP15, interleukin-7 receptor; IL7R) and oxidative stress (adrenergic-beta-2 receptor; ADRB2, and polymerase (DNA directed)-kappa; POLK) in mononuclear cells. All effects, with the exception of the decrease in POLK expression, were particularly observed when virgin olive oil was present in the TMD dietary pattern.

Conclusions: Olive oil phenolic compounds, within the frame of the traditional Mediterranean diet, enhance the health protective nutrigenomic effect. Our results support the idea that the benefits associated with a Mediterranean diet, virgin olive oil, and olive oil phenolic compounds consumption on cardiovascular risk could be mediated through changes in the expression of cardiovascular risk-related genes.

O53 - Co-sponsored Lecture by IOOC

OIL ADDITION INCREASE ANTI-INFLAMMATORY EFFECTS OF TOMATO

Miriam Martínez-Huélamo[1,2], Sara Tulipani[1,3], Palmira Valderas-Martínez[2,4], Giuseppe Di Lecce[1], Anna Vallverdú-Queralt[1,2], Ramón Estruch[2,4], Xavier Torrado[1], Elvira Escribano Ferrer[5], Rosa M. Lamuela-Raventós[1,2]

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Introduction: Food matrix seems to be an important factor modulating absorption and further biological activity of dietary polyphenols. In order to verify whether the oral absorption and metabolism of the phenolic compounds contained in tomato and tomato sauces is influenced by the processing and the addition of a lipid matrix during sauce processing, we performed a prospective randomized, cross-over study with 40 healthy volunteers and three interventions: raw tomatoes, oil-free tomato sauce and refined olive oil-enriched tomato sauce.

Methods and materials: The phenolic content of the raw tomatoes and tomato sauces was characterized by UPLC-MS/MS analysis by Triple Quadrupole mass spectrometer, with negative ion detection mode. The corresponding phenolic metabolites were identified in plasma and urine by UPLC-MS/MS after solid-phase extraction of the biological samples with Oasis HLB 96-well plates. The identification of the compounds was carried out on the basis of their mass spectra in full scan mode and confirmed by MS/MS experiments (PIS, NL). Multiple

reaction monitoring experiments were used for quantification purposes. Peripheral blood mononuclear cells were separated by Fycoll-Hypaque gradient. Adhesion molecule expression on T-lymphocytes and monocytes surface was determined by using a double-direct immunofluorescence test. Determinations were performed using a FACSCalibur flow cytometer. Bio-Plex cytokines assays were performed in soluble inflammatory biomarkers, using Luminex-100 equipment.

Results and conclusions: The processing of tomato to sauce and the addition of a lipid matrix during sauce preparation changes the phenolic composition of the product, probably due to changes in the bioaccessibility of phenolics from the food matrix. The use of oil in tomato sauce increases the anti-inflammatory effect since it reduces the expression of certain adhesion molecules associated with atherosclerosis than after raw tomatoes, oil-free tomato sauce. The plasma kinetics and urinary excretion of the flavanone naringenin and naringenin glucuronides after the interventions confirmed the highest bioaccessibility of this flavanone after tomato processing to sauce. The plasma pharmacokinetic of naringenin suggests a two-phase absorption only following the ingestion of oil-enriched tomato sauce. These findings suggest the occurrence of re-absorption events by enterohepatic circulation.

Polyphenols as preventive ingredients: in vitro experiment, animal models and human intervention studies

O54 - Invited Speaker

POLYPHENOLS IN THE PREVENTION OF CHRONIC DISEASES AND RELATED COMPLICATIONS: IN VITRO STUDIES WITH THE RIGHT MOLECULES

Daniele Del Rio, Luca Calani, Margherita Dall'Asta

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On the basis of prospective, cross-sectional and intervention studies linking polyphenols to human health, several experimental papers in the literature have tried to evaluate the molecular mechanisms involved in their bioactivity. Polyphenols are reported to in vitro inhibit cancer cell proliferation, reduce vascularisation, protect neurons, stimulate vasodilation and improve insulin secretion, but are often studied as aglycones or as sugar conjugates and at non-physiological concentration. However, it is now well established that polyphenols undergo substantial metabolism after being ingested by humans in dietary relevant amount and that concentrations of plasma metabolites after a normal dietary intake rarely exceed nmol/L. This lecture intends to highlight that uncritical judgments made on the basis of the published literature about polyphenol bioactivity, may sometimes have been misled and misleading and to conclude that it is phenolic metabolites, formed in the small intestine and hepatic cells, and low molecular weight metabolic products of the colonic microbiota to travel around the human body in the circulatory system or reach body tissues to elicit bioactive effects. Therefore, this lecture will bring to the audience attention the most recent advancements in the field of polyphenol physiological activities, with particular attention to their antiinflammatory, estrogenic and antiglycative effects investigated with the most innovative and conservative experimental techniques.

O55 - Invited Speaker

PREVENTION OF AGING-RELATED ENDOTHELIAL DYSFUNCTION BY POLYPHENOLS: EVIDENCE FROM ANIMAL STUDIES

Cyril Auger, Noureddine Idris-Khodja, Valérie B. Schini-Kerth

Cardiovascular Pharmacology and Pathophysiology group, UMR CNRS 7213-Laboratory of Biophotonics and Pharmacology, Faculty of Pharmacy, University of Strasbourg Illkirch-Graffenstaden-France.

Several cardiovascular diseases, such as hypertension, diabetes, and aging are associated with an endothelial dysfunction. Since in vitro and ex-vivo studies have demonstrated that polyphenols can induce the formation of vasoprotective factors such as NO and EDHF-mediated responses, they may also exert beneficial effects on the endothelial function in vivo. Indeed, experimental studies indicate that chronic intake of polyphenol-rich products can prevent or improve the endothelial dysfunction in various animal models of cardiovascular diseases such as hypertension, and vascular aging. This presentation will also discuss the possible mechanistic aspects underlying the beneficial effects of polyphenols on the endothelial function.

8.2.12. Comunicación 12. Póster

Título: How oil combination affects the phenolic content in tomato juice?

Autores: **Miriam Martínez-Huélamo,** Palmira Valderas-Martínez, Olga Jáuregui, Xavier Torrado, Ramón Estruch, Rosa M. Lamuela-Raventós.

Congreso: IX Congreso Internacional de Barcelona sobre la Dieta Mediterránea. Barcelona, Spain, 2012.

HOW OIL COMBINATION AFFECTS THE PHENOLIC CONTENT IN TOMATO JUICE?

Miriam Martínez-Huélamo[1,2], Palmira Valderas-Martínez[2,3], Olga Jáuregui[4], Xavier Torrado[1], Ramón Estruch[2,3], Rosa M. Lamuela-Raventós[1,2*]

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INTRODUCTION

The health-promoting properties of tomato and tomato products seem to reside in the high amount of phytochemical constituents such as carotenoids, vitamin C, vitamin E and the wide variety of phenolic components ranging from flavonoids to phenolic acids. Despite increasing knowledge of the effect of industrial food processing on carotenoid and vitamin bioavailability, there is a lack of information regarding the extent of changes in the polyphenol/flavonoid content of tomato after mechanical and/or thermal processing treatments, and there is even less available data in the literature on the effects of food matrix on bioavailability. Furthermore, very little is known about the impact of tomato and tomato-olive oil combination on the phenolic bioavailability and bioefficacy in humans.

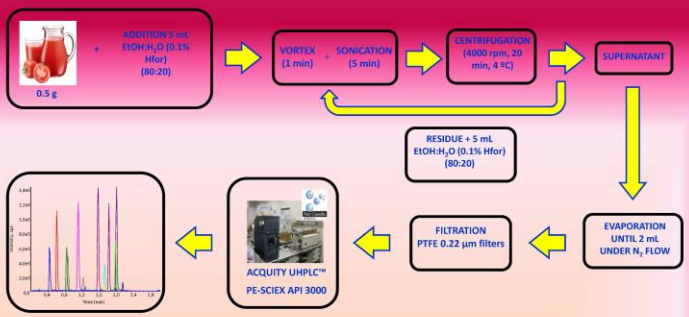
Previous work on phenolic characterization of tomato fruits has been performed using different techniques including nuclear magnetic resonance, gas chromatography and capillary electrophoresis, but the most common is high-performance liquid chromatography (HPLC) coupled to a photodiode detector (UV/Vis) or mass spectrometry (MS). Among the methods used to determine phenolic compounds, liquid chromatography coupled to mass spectrometry with electrospray ionization (ESI) is one of the most powerful tools for the analysis of non-volatile and thermally labile classes of compounds. High performance liquid chromatography has been improved by the introduction of ultra performance liquid chromatography (UPLC), which increases the signal-to-noise ratio (S/N), enhances peak resolution, and reduces both analysis time and costs.

AIMS

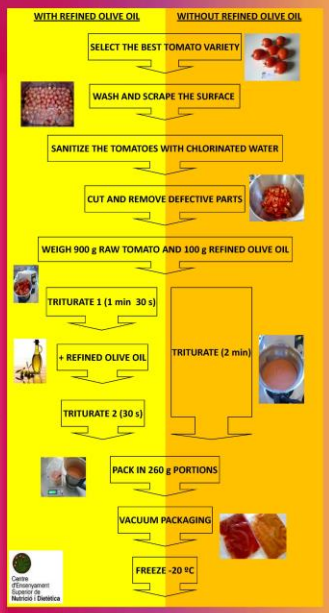
Quantify the phenolic profile of different Spanish tomato varieties in order to get the juice with the highest phenolic content.

Study of two tomato juices with and without refined olive oil in order to verify how the oil combination affects the phenolic bioavailability.

SAMPLE PREPARATION



TOMATO JUICE

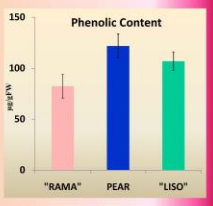


RESULTS

PHENOLIC COMPOUNDS IN "RAMA", PEAR AND "LISO" TOMATOES

Compound	"RAMA" µg/g FW[b]		PEAR µg/g FW[b]		"LISO" µg/g FW[b]	
	skin	pulp	skin	pulp	skin	pulp
Protocatechuic	n.d	n.d	n.d	0,009 ± 0,001	n.d	0,001 ± 0,000
Caffeic hexose I	0,285 ± 0,006	0,028 ± 0,004	n.d	0,706 ± 0,087	n.d	0,414 ± 0,042
Coumaric hexose I	0,044 ± 0,007	0,086 ± 0,001	0,039 ± 0,001	0,100 ± 0,013	0,037 ± 0,002	0,057 ± 0,006
Ferulic hexose	12,61 ± 1,81	8,473 ± 0,629	5,111 ± 0,690	10,29 ± 1,29	8,778 ± 0,998	6,719 ± 5,828
Caffeic hexose II	0,282 ± 0,019	0,228 ± 0,009	0,387 ± 0,013	0,253 ± 0,034	0,249 ± 0,036	0,158 ± 0,017
3-Caffeoylquinic acid	0,065 ± 0,009	0,049 ± 0,006	1,923 ± 0,222	0,165 ± 0,022	1,137 ± 0,087	0,054 ± 0,003
Coumaric hexose II	2,338 ± 0,284	0,022 ± 0,003	2,039 ± 0,285	0,028 ± 0,004	2,368 ± 0,308	0,017 ± 0,001
5-Caffeoylquinic acid	n.d	0,036 ± 0,003	n.d	0,064 ± 0,007	n.d	0,032 ± 0,004
Caffeic acid	0,121 ± 0,015	0,178 ± 0,023	0,144 ± 0,006	0,445 ± 0,063	0,130 ± 0,005	0,081 ± 0,012
4-Caffeoylquinic acid	2,412 ± 0,289	0,123 ± 0,017	3,990 ± 0,589	0,226 ± 0,026	3,485 ± 0,524	0,075 ± 0,065
o-Coumaric acid	n.d	0,024 ± 0,001	n.d	0,033 ± 0,029	n.d	0,015 ± 0,001
Rutin	33,77 ± 4,29	n.d	43,53 ± 5,49	n.d	38,80 ± 5,55	n.d
Ferulic acid	n.d	0,148 ± 0,016	n.d	0,750 ± 0,090	n.d	0,277 ± 0,022
Dicaffeoylquinic acid	n.d.	0,005 ± 0,003	0,090 ± 0,004	0,006 ± 0,006	0,096 ± 0,084	0,002 ± 0,003
Naringenin-*O*-hexoside	1,010 ± 0,068	0,010 ± 0,001	0,259 ± 0,225	n.d	0,311 ± 0,270	n.d
Naringenin	20,15 ± 17,50	n.d.	51,32 ± 6,00	n.d.	43,57 ± 3,22	n.d.

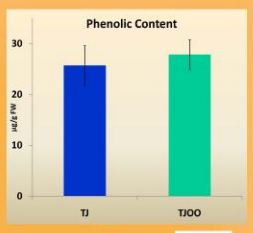
[a]RT: Chromatographic retention. [b]FW:Fresh weight



HIGHER PHENOLIC CONTENT

PEAR TOMATO

PHENOLIC COMPOUNDS IN TOMATO JUICE (JC) AND TOMATO JUICE WITH REFINED OLIVE OIL (TJOO)



HIGHER PHENOLIC CONTENT

Compound	RT (min)[a]	MRM (m/z)	TJ[b] µg/g FW[d]	TJOO[c] µg/g FW[d]
Caffeic hexose I	0,74	341 → 179	1,848 ± 0,032	2,676 ± 0,267
Caffeic hexose II	0,97	341 → 179	1,043 ± 0,014	1,271 ± 0,229
3-Caffeoylquinic acid	1,02	353 → 191	4,905 ± 0,191	7,509 ± 0,835
Coumaric hexose II	1,07	325 → 163	0,144 ± 0,012	0,175 ± 0,154
5-Caffeoylquinic acid	1,09	353 → 191	2,751 ± 0,282	4,688 ± 0,403
Caffeic acid	1,17	179 → 135	1,960 ± 0,160	2,191 ± 0,360
4-Caffeoylquinic acid	1,26	353 → 191	0,680 ± 0,040	0,795 ± 0,089
o-Coumaric acid	1,53	163 → 119	0,238 ± 0,027	0,179 ± 0,156
Rutin	1,7	609 → 301	5,975 ± 0,102	6,407 ± 0,972
Ferulic acid	1,7	193 → 134	1,392 ± 0,095	1,423 ± 0,201
Dicaffeoylquinic acid	1,97	515 → 353	0,790 ± 0,075	0,368 ± 0,038
Naringenin-*O*-hexoside	2,08	433 → 271	0,054 ± 0,008	0,051 ± 0,005
Naringenin	2,52	271 → 151	3,928 ± 3,413	0,141 ± 0,051

[a]RT: Chromatographic retention [b]TJ: Tomato juice [c]TJOO: Tomato juice with olive oil [d]FW: Fresh weight

CONCLUSIONS

* Three tomato varieties ("rama", pear and "liso") were studied. The results showed a higher phenolic content in the pear tomato.

* Moreover, it was noticed that the process for preparing tomato juice and the addition of a lipid matrix during juice preparation change the phenolic composition of the product, probably due to changes in the bioaccessibility of phenolics from the food matrix.

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8.2.13. Comunicación 13. Póster

Título: ¿Es necesario estandarizar las recomendaciones de consumo de frutas y verduras que establecen las guías alimentarias?

Autores: Palmira Valderas-Martínez, Sara Arranz, Gemma Chiva-Blanch, **Miriam Martínez-Huélamo**, Rosa Casas, Mireia Urpí-Sardà, Rosa M. Lamuela-Raventós, and Ramón Estruch.

Congreso: IX Congreso Internacional de Barcelona sobre la Dieta Mediterránea. Barcelona, Spain, 2012.

IX Congreso Internacional de Barcelona sobre la DIETA MEDITERRÁNEA 27 y 28 de marzo de 2012

¿ES NECESARIO ESTANDARIZAR LAS RECOMENDACIONES DE CONSUMO DE FRUTAS Y VERDURAS QUE ESTABLECEN LAS GUÍAS ALIMENTARIAS?

Valderas-Martínez Palmira [1,2,3], Arranz Sara [1,2,3], Chiva-Blanch Ghiva [1,2,3], Miriam Martínez-Huélamo [2,3], Rosa Casas [1,3], Urpi-Sarda Mireia [1,3], Rosa Maria Lamuela-Raventós [2,3], Ramón Estruch [1,3]

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INTRODUCCIÓN

Las frutas y las verduras (F&V) son componentes esenciales de una dieta saludable y existen evidencias científicas de que un consumo regular puede contribuir a la prevención de enfermedades cardiovasculares (ECV) y algunos cánceres. De acuerdo con el *World Health Report 2002*, se estima que el bajo consumo de F&V causa alrededor del 14% de las muertes por cáncer gastrointestinal y el 20% de las muertes por ECV; 1.7 millones de vidas podrían salvarse cada año si se incrementara suficientemente este consumo.

OBJETIVO

El objetivo de este trabajo es evaluar las recomendaciones sobre el consumo de F&V en guías dietéticas de diferentes países.

ANTECEDENTES

Mientras que las últimas guías dietéticas recomiendan un consumo de entre 5-13 porciones de F&V/día un informe reciente de la FAO/OMS recomienda una ingesta mínima de 400g/día para la prevención de enfermedades crónicas como las ECV, el cáncer, la diabetes o la obesidad.

CONCLUSIONES

Las recomendaciones deben basarse en resultados obtenidos en ensayos clínicos y el uso de biomarcadores nutricionales puede añadir varias ventajas sobre la percepción subjetiva de los datos.
La disponibilidad y preferencia de alimentos y los antecedentes culturales relacionados con los hábitos dietéticos dificultan el establecimiento de pautas comunes. No obstante, es necesario estandarizar las medidas de las porciones y la estimación de la ingesta de F&V para proporcionar mensajes de validez universal encaminados a promocionar la salud en todo el mundo.

AGRADECIMIENTOS

Palmira Valderas-Martinez agradece al programa de becas APIF-UB. Gemma Chiva-Blanch agradece al programa de becas Manuel de Oya. Mireia Urpi-Sarda y Sara Arranz agradecen al programa de becas Sara Borrell (CD09/00134 y CD10/00151, respectivamente) del MICINN. Miriam Martínez agradece al programa de becas FPI del MICINN.

8.2.14. Comunicación 14. Póster

Título: Prenylflavanoid as new biomarker of beer consumption.

Autores: Paola Quifer-Rada, **Miriam Martínez-Huélamo**, Ramon Estruch, and Rosa M. Lamuela-Raventós.

Congreso: IX Congreso Internacional de Barcelona sobre la Dieta Mediterránea. Barcelona, Spain, 2012.

PRENYLFLAVANOIDS AS NEW BIOMARKER OF BEER CONSUMPTION

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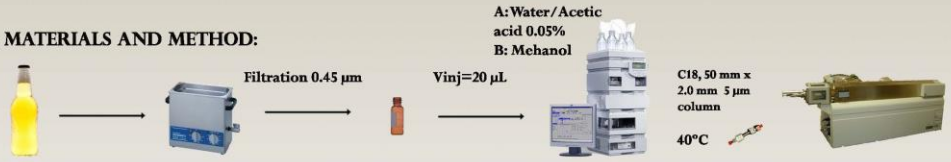
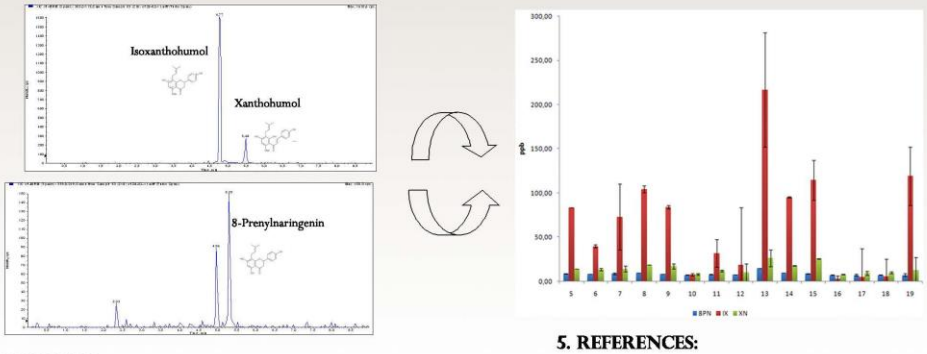
1. INTRODUCTION:

Beer is one of the most commonly consumed fermented and undestilled alcoholic beverages in many countries. However it's consumption in the Mediterranean area is lower than Northern European countries due to its consumption follows the Mediterranean diet pattern.
The PREDIMED study showed that moderate beer consumers had a lower incidence of diabetes type 2 and hypertension compared to abstemious although no differences in obesity or overweight was observed.
Several studies have pointed out that moderate alcohol consumption reduces overall mortality and incidence of coronary heart disease. Although beer and spirits may have protective effects on the cardiovascular system, some authors consider that wine intake may have additional benefits. These discrepancies are probably due to the difficulties to accurately assess dietary habits (including alcoholic beverage consumption) in epidemiological studies. Biomarkers have several advantages over self-reported data, since they are more precise and provide better objective measures than data obtained from food frequency questionnaires (FFQ). For this fact, a biomarker for beer intake is needed in order to assess the benefits and risks of beer consumption and evaluate its potential role within the Mediterranean Diet.
About 20-30% of beer polyphenols come from hops and the remaining 70-80% are malt derived. The most abundant phenols in hop are the prenylflavanoids group which includes xanthohumol and its derivates (isoxanthohumol and 8-prenylnaringenin). Xanthohumol (XN) represents 95% of hop prenylflavanoids fraction and is a prenylchalcona that isomerizes to isoxanthohumol during the brewing process. In the intestinal tract, isoxanthohumol (IX) is converted to 8-prenylnaringenin (8-PN) through an O- demethylation catalyzed by gut bacteria.
The aim of this work was to analyzed hop polyphenol levels in 19 Spanish beers from the market by liquid chromatography coupled to mass spectrometry

2. MATERIALS AND METHOD:



3. RESULTS:



4. CONCLUSIONS:

All Spanish beers showed levels of prenylflavanoids. IX compound was the most abundant; XN was found in the range of 7.67-26.27 µg/L, 8-PN in the range of 7.25-14.54 µg/L and IX in the range of 2.74-216.79 µg/L. However, the content of prenylflavanoids did not depend on the type of beer; it may be due to the sort or the amount of hop used during brewing process. This results show that IX could be a potential biomarker for beer consumption due to its specificity and the amount found in beer.

6. ACKNOWLEDGEMENTS

this research was supported by Centro de Información Cerveza y Salud through the Manuel de Oya grant.

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8.2.15. Comunicación 15. Póster

Título: Absorción gástrica del ácido salicílico por perfusión gástrica de un solo paso en ratón.

Autores: Elvira Escribano, Naiara Orrego, **Miriam Martinez-Huélamo** and Rosa M. Lamuela-Raventos.

Congreso: XI Congreso de la Sociedad Española de Farmacia Industrial y Galénica. Alicante, Spain, 2013.

ABSORCIÓN GÁSTRICA DEL ÁCIDO SALICÍLICO POR PERFUSIÓN GÁSTRICA DE UN SOLO PASO EN RATÓN

E. Escribano[1], N. Orrego[1], M. Martinez-Huélamo[2] and Rosa M. Lamuela-Raventos[2]

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1. Introducción y objetivo

La vía oral es la vía de administración más conveniente y comúnmente utilizada para tratamientos crónicos y/o administraciones frecuentes (1). A su vez, es la mejor aceptada por parte de los pacientes y la de menor coste sanitario. Tras su administración oral, el principio activo debe disolverse en los fluidos del tracto gastrointestinal y atravesar las membranas biológicas accediendo así a la circulación sistémica.

A pesar de que de los principios activos son absorbidos mayoritariamente en el intestino delgado, algunos fármacos se absorben también a nivel gástrico (ranitidina (2), aspirina y secobarbital (3)) . También está descrita la absorción gástrica de algunos polifenoles, como los ácidos ferúlico (4) y clorogénico (5). En estos casos, la permeabilidad en el estómago juega un papel importante biodisponibilidad oral de los mismos.

El objetivo del trabajo ha sido poner a punto una técnica de perfusión gástrica de un solo paso en ratón , y estudiar la absorción gástrica del ácido salicílico.

2. Metodología

Ensayos de perfusión gástrica

La metodología se expone en la Figura 1. Las condiciones de ensayo fueron:

- **Solución de perfusión:** ácido salicílico 11.5mM (6) en pH 2 (297mOsm) con 0.1mg/mL de rojo de fenol (7)***(C_{in})***
- **Animales:** ratones macho CD1 (30-35g).
- **Anestesia:** i.p. mezcla ketamina/xilacina (100 mg/kg y 10 mg/kg, respectivamente
- **Flujo:** 0.2mL/min ***($Ø_{in}$)***
- **Muestras:** cada 10min, durante 5min y hasta 1h.

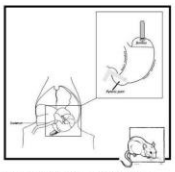

Fig. 1. Técnica quirúrgica gástrica

Estudios de estabilidad

Se estudió la estabilidad de la solución de perfusión de ácido salicílico a pH2 y 37ºC, a los tiempos: inicial, 1 y 2 h. El nº de replicados por tiempo: n=5

Técnica analítica

Las concentraciones de ácido salicílico y de rojo de fenol en las muestras biológicas fueron determinadas por HPLC a 237 nm ***(C_{out})*** y por espectrofotometría en medio alcalino a 558 nm, respectivamente.

Análisis de datos

A partir de las C_{out} corregidas en estado de equilibrio estacionario, se estimó para cada ratón, el porcentaje de ácido salicílico absorbido y el coeficiente de permeabilidad (Peff) de acuerdo con la siguiente ecuación (8):

$$Peff = \frac{-\phi_{in} \cdot Ln(C_{out.corr} / C_{in})}{2 \cdot \pi \cdot r \cdot L} \quad \text{(Ecuación 1)}, \text{ donde}$$

C_{in} and $C_{out.corr}$ son las concentraciones de salida del estómago corregidas por el método gravimétrico (9)y/o por las concentraciones del rojo de fenol, y ***L*** la longitud del estómago.

El radio ***r*** del estómago fue estimado a partir de la ecuación 2, y asumiendo un volumen cilíndrico de 0.4mL (10):

$$r = \sqrt{\frac{0.4}{\pi \cdot L}} \quad \text{(Ecuación 2)}$$

El flujo neto de agua (NWF) se determinó mediante la ecuación 3, de acuerdo con Issa (8) y Lindahl (11) :

$$NWF = \frac{(1 - C_{out} / C_{in}) \cdot \phi_{in}}{L} \quad \text{(Ecuación 3)}, \text{ donde}$$

C_{out} y C_{in} son los flujos (o concentraciones de rojo de fenol) a la entrada y salida del estómago.

3. Resultados y discusión

- **Estabilidad:** - La solución de ácido salicílico (pH 2, 37ºC) resultó estable durante 2h (ANOVA, p= 0.448), con lo que el descenso de las concentraciones en el lumen gástrico es atribuible al proceso de absorción.
- **Flujo neto de agua:**
  - Método gravimétrico: 0.798 ± 0.106 $mL \cdot h^{-1} \cdot cm^{-1}$.
  - Método de rojo de fenol: 0.425 ± 0.220 $mL \cdot h^{-1} \cdot cm^{-1}$.
- Las relaciones $C_{out.corr} / C_{in}$ *vs.* tiempo (Figura 2) corregidas por el método gravimétrico resultaron inferiores a las determinadas por el rojo de fenol. Ésto se tradujo en un mayor valor estimado para el coeficiente de permeabilidad (y porcentaje de fármaco absorbido) a partir del método gravimétrico (Tabla1).

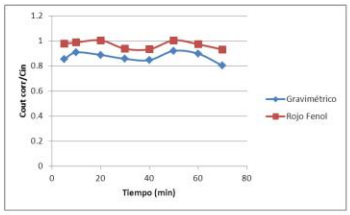

Fig. 2. *Cout.corr/Cin vs. tiempo*

Tabla 1. *Valores del Peff y porcentaje de fármaco absorbido por los métodos gravimétrico (1) y rojo de fenol (2).*

Ratón	Peff _1 (cm/s)	Peff _2 (cm/s)	% 1	% 2
1	0.00071006	0.00061442	41.36	36.97
2	0.00090182	0.00069697	53.98	45.21
3	0.00149319	0.00015621	28.35	11.07
4	0.00028644	0.00011509	21.31	9.18
5	0.00076694	0.00056772	44.34	35.19
6	0.00014749	0.00005321	10.81	4.26
Media	**0.000718**	**0.000367**	**33.36**	**23.65**
SD	**0.000479**	**0.000289**	**16.05**	**17.43**

4. Conclusiones

- El estado de equilibrio estacionario fue alcanzado entre los 5-10 min de iniciada la perfusión.
- La determinación del flujo neto de agua por el método del rojo de fenol subestimó dicho proceso, a la vez que aumentó la variabilidad interindividual (%cv 51.91% vs. 13.27).
- El Peff gástrico del ácido salicílico en ratón resultó ser de $7.18 \cdot 10^{-4} \pm 4.79 \cdot 10^{-4}$ cm/s. Otros autores (12) hallaron un Peff intestinal para el piroxicam (alta permeabilidad) en ratón del mismo orden ($10.21 \cdot 10^{-4} \pm 4.11 \cdot 10^{-4}$ cm/s).
- El porcentaje promedio de ácido salicílico absorbido mediante la técnica de perfusión gástrica ha resultado ser un 33.36±16.05%, similar al hallado por otros autores (6).
- Dado que a mayor tiempo de permanencia del fármaco en el lumen mayor probabilidad de incrementar su absorción, resulta conveniente repetir el ensayo a un menor flujo de perfusión.

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8.2.16. Comunicación 16. Póster

Título: Differences in the carotenoid profile between organic and conventional tomato-based products.

Autores: Anna Vallverdú-Queralt, **Miriam Martínez-Huélamo**, Isidre Casals, and Rosa M. Lamuela-Raventós.

Congreso: Sustainable diet and Food Security. Lille, France, 2013.

Differences in the carotenoid profile between organic and conventional tomato-based products

Anna Vallverdú-Queralt[1,2], Miriam Martínez-Huélamo[1,2], Isidre Casals[3] and Rosa M. Lamuela-Raventós[1,2*]

[1]Nutrition and Food Science Department, XaRTA, INSA. School o Pharmacy, University of Barcelona, Av. Joan XXIII s/n Barcelona, Spain.[2]CIBER 06/003 Physiopathology of Obesity and Nutrition, (CIBEROBN)n.[3]Scientific and Technical Services, University of Barcelona, Barcelona, Spain. *Nutrition and Food Science Department, University of Barcelona. Telephone +34-934034843. Fax +34-93-4035931; http://www.polyphenolresearch.com, e-mail: lamuela@ub.edu

Introduction

Fundamental differences between organic and conventional production systems may affect the nutritive composition of plants, including secondary plant metabolites. Conventional and organic systems differ in the amount of nutrients applied as fertilizers and in organic matter applied to the soil as crop residues, winter legume cover crops or composted manure. The availability of inorganic nitrogen in particular has the potential to influence the synthesis of secondary plant metabolites, proteins and soluble solids. Carotenoids are important antioxidant compounds in the human diet. Due to their constrained polyene structure, carotenoids can exist in *cis* and *trans* isomeric forms. In ketchups and tomato juices, all *trans* isomeric forms are predominant. *cis*-Isomers are believed to have a higher antioxidant capacity and to be more bioavailable than *trans*-isomers.

The aim of this study is to compare the carotenoid and lipophilic antioxidant capacity of organic and conventional tomato-based products.

Material and Methods

Extraction of carotenoid compounds

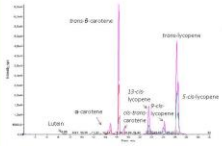
0.5 g tomato –based products EtOH: Hexane (4:3, v/v)

Centrifugation: 4000 rpm, 20 min, 4 ºC

supernatant + 5 mL EtOH:Hexane (4:3, v/v)

Evaporation and recosntitution with 1 mL MBTE

Analysis of carotenoids compounds



Time (min)	H_2O	MeOH	MTBE
0	4	70	26
10	4	20	76
20	4	6	90
21	4	6	90
23	4	70	26
33	4	70	26

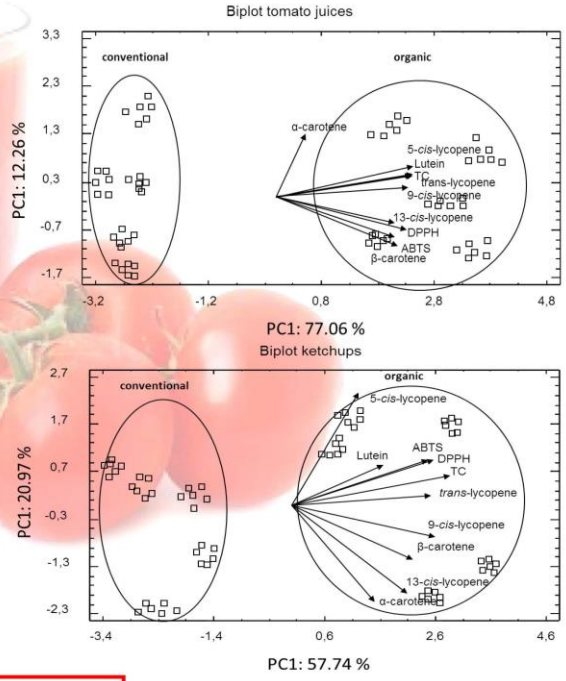
Antioxidant capacity (AC)

-5 µL of each extract
-245 µL of ABTS** or DPPH radicals.
Incubation for 60 min in the darkroom
Measurement of absorbance at 734 nm (ABTS**) and 515 (DPPH).

Results: percentage of antioxidant capacity related to that of the untreated samples.

Results

Comparisons of the analyses of ketchups and tomato juices from conventional and organic production systems showed statistically higher levels ($P<0.05$) of carotenoid compounds and lipophilic antioxidant capacity in organic counterparts.



Conclusions

The results obtained in this study of carotenoid profiling led to the distinction of features that differentiate between organic and conventional tomato-based products. Statistical analysis was performed to identify metabolites that may serve as markers for organic and conventional ketchups and tomato juices. Organic counterparts tend to provide food with a higher content of carotenoids. However, more studies are required to study carotenoids from a standpoint of bioavailability.

Acknowledgments

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Sustainable Diet and Food Security, May 28-29, Lille, France

8.2.17. Comunicación 17. Póster

Título: Carotenoid profile of organic and conventional ketchups and tomato juices.

Autores: Jose Fernando Rinaldi di Alvarenga, Anna Vallverdú-Queralt, **Miriam Martínez-Huélamo**, and Rosa M. Lamuela-Raventós.

Congreso: Simposio Latino Americano de Ciencia de Alimentos. Sao Paulo, Brazil, 2013.

T437

CAROTENOID PROFILE OF ORGANIC AND CONVENTIONAL KETCHUPS AND TOMATO JUICES

Alvarenga, J.F.R., Vallverdú-Queralt, A.[1,2], Martínez-Huélamo, M.[1,2] and Lamuela-Raventós, R. M.[1,2*]

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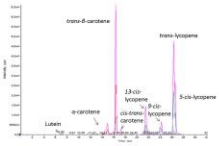

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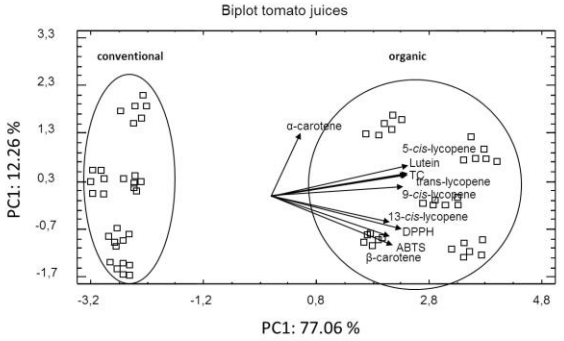

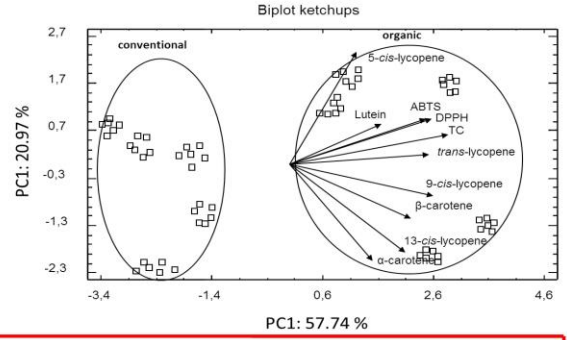

Conclusions

The results obtained in this study of carotenoid profiling led to the distinction of features that differentiate between organic and conventional tomato-based products. Statistical analysis was performed to identify metabolites that may serve as markers for organic and conventional ketchups and tomato juices. Organic counterparts tend to provide food with a higher content of carotenoids. However, more studies are required to study carotenoids from a standpoint of bioavailability.

Acknowledgments

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8.2.18. Comunicación 18. Póster

Título: Analysis of Prenylflavanoids in biological samples requires neutral pH. Differences in urinary excretion of Isoxanthohumol according to Gender.

Autores: Paola Quifer-Rada, **Miriam Martinez-Huelamo**, Olga Jáuregui, Gemma Chiva-Blanch, Ramon Estruch, and Rosa M. Lamuela-Raventós.

Congreso: Diet and Optimum Health Conference. Corvallis, Oregon. EEUU. 2013.

Analysis of Prenylflavanoids in biological samples requires neutral pH. Differences in urinary excretion of Isoxanthohumol according to Gender.

Paola Quifer-Rada[1,2], Miriam Martinez-Huelamo [1,2], Olga Jauregui [3], Gemma Chiva-Blanch [2,4], Ramon Estruch [2,4], Rosa M Lamuela-Raventos [1,2].

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[4]Department of Internal Medicine, Hospital Clinic, Institute of Biomedical Investigation August Pi I Sunyer (IDIBAPS), University of Barcelona, Spain.

Introduction

The interest in studying the prenylflavanoids isoxanthohumol, xanthohumol and 8-prenylnaringenin in biological samples has increased in recent years due to their biological activity as strong phytoestrogens and potent cancer chemopreventive agents, and their role in osteoporosis prevention (1,2,3). Hop and beer prenylflavanoids have been the subject of various studies and have been quantified by LC-MS, but these methods failed to account for the instability of these compounds in acidic medium(4). In order to develop a new sensitive and specific method based on SPE-LC-ESI-MS/MS that maintains prenylflavanoid stability throughout the analytical process, the effect of the pH during SPE and the chromatographic run was investigated by applying different pH (from 1.0 to 7.0) during the analytical process. In addition, the use of an antioxidant, such as ascorbic acid, to prevent analyte degradation was evaluated.
The urinary excretion of prenylflavanoids was also studied in a dose-response, randomized, cross-over clinical trial.

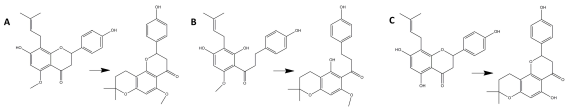
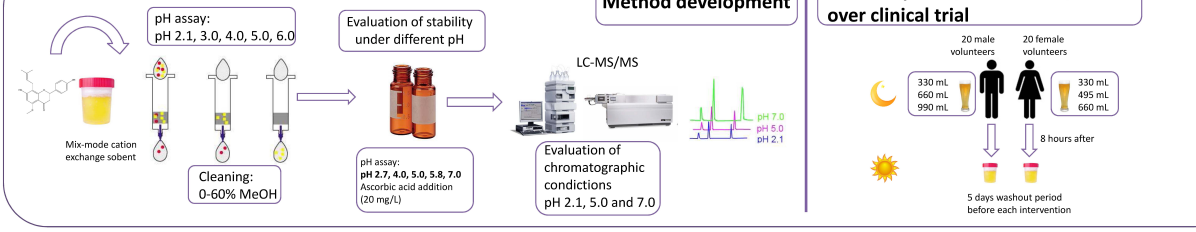


Figure 1. Prenyl chain cyclation in acidic medium of IX (A), XN (B) and 8PN (C).

Materials and methods



Results

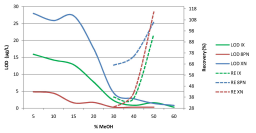


Figure 2. LOD and recovery obtained cleaning with different % MeOH during SPE using MCX sorbent.

Extraction pH	LOD (µg·L⁻¹) IX	XN	8PN	Recovery (%) IX	XN	8PN
2.1	0.03	0.02	0.02	99	234	123
3	0.01	0.02	0.02	111	139	126
4	0.01	0.02	0.02	104	114	105
5	0.01	0.03	0.02	95	113	109
6	0.01	0.02	0.03	75	82	98

Table 1. LOD and recovery obtained using different pH during SPE using MCX sorbent.

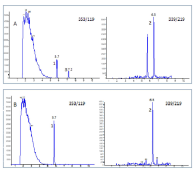


Figure 3. Multiple Reaction Monitoring (MRM) chromatograms.
A: Spiked urine with standards (1 µg L⁻¹).
B: male volunteer urine 8 hours after the consumption of 330 mL of beer.
1: IX, 2: 8PN, 3: XN.

Table 2. Coefficient correlation, accuracy, precision and stability obtained in different media.

Medium	r^2 IX	8PN	XN	Accuracy (%) n=10 IX	8PN	XN	Precision (%CV) n=10 IX	8PN	XN	Stability (% of degradation) 12 hours IX	8PN	XN	24 hours IX	8PN	XN
pH 2.7	0.82	0.84	0.60	1.6	4.4	>15	10.6	13	>15	-*	-*	-*	-*	-*	-*
pH 4	0.98	0.99	0.94	5	5	>15	8.8	8.3	>15	-*	-*	-*	-*	-*	-*
MilliQ water	0.94	0.97	0.97	11	3.5	>15	12.4	5.2	>15	-*	-*	-*	-*	-*	-*
pH 5	0.97	0.97	0.97	14	1	>15	7.1	3.8	>15	7.9	37	91	-*	-*	-*
pH 5 + AA	1.00	1.00	0.97	7.8	6.8	>15	4.2	3.8	2.5	0	13.5	12.2	4.5	33.3	32.2
pH 7 + AA	1.00	0.98	1.00	11	12	15	2.2	13.2	3.5	0	0	7.8	0	3.72	15.7

LOD: Limit of Detection; LOQ: Limit of Quantification; AA: ascorbic acid
-*: Stability not determined since results in accuracy and precision obtained were not acceptable.

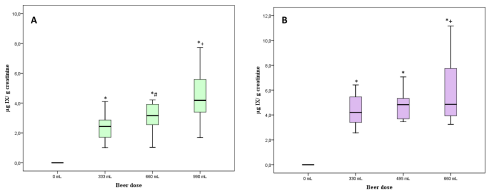


Figure 4. Urinary excretion of Isoxanthohumol in male (A) and female (B) volunteers 8 hours after the intake of different volumes of beer.
*Significant differences between washout period and beer intervention ($P<0.05$), # Significant differences between the first and the second beer intervention ($P<0.001$), + Significant differences between the second and the third beer intervention ($P<0.05$) (Wilcoxon test).

Conclusions

-A neutral pH is necessary for the analysis of prenylflavanoids, in order to maintain the stability of compounds for at least 24 hours (5).
-The addition of ascorbic acid to the media improved calibration curves coefficients of correlation, accuracy, and precision parameters (5).
-Cleaning with 50% MeOH during SPE mixed-mode cation exchange sorbent provided the lowest matrix effect and best recoveries. Consequently, the limit of detection of the new method was 10-times lower than of other published methods (5).
-Women seem to absorb IX greater (two times more) after a regular beer dose of 330 mL. However, it seems that IX absorption in women may be saturated promptly since no differences in IX excretion is observed after the intake of higher doses of beer. In contrast, men excreted IX linearly with the dose size of beer.

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Diet and Optimum Health May 15-18, 2013 Corvallis, Oregon

http://polyphenolresearch.com

8.2.19. Comunicación 19. Póster

Título: Olive oil increases postprandial absorption of carotenoids from tomato juice in humans.

Autores: Sara Arranz, **Miriam Martínez-Huélamo**, Anna Vallverdú-Queralt, Palmira Valderas-Martínez, Montse Illán Ramón Estruch, and Rosa M. Lamuela-Raventós.

Congreso: World Forum for Nutrition Research Conference. Reus, Spain, 2013.

WORLD FORUM for NUTRITION RESEARCH CONFERENCE 2013

OLIVE OIL INCREASES POSTPRANDIAL ABSORPTION OF CAROTENOIDS FROM TOMATO JUICE IN HUMANS

Sara Arranz [a,b], Miriam Martínez-Huélamo [b,c], Anna Vallverdu-Queralt [b,c], Palmira Valderas-Martinez [a,b], Montse Illán [c], Ramón Estruch [a,b] and Rosa Mª Lamuela-Raventos [b,c,*]

[a] Department of Internal Medicine, Institut d'Investigacions Biomèdiques August Pi Sunyer (IDIBAPS), Hospital Clinic, Faculty of Medicine, University of Barcelona, Barcelona, Spain.
[b] CIBER CB06/03 Fisiopatología de la Obesidad y la Nutrición, (CIBERobn), Institute of Health Carlos III, Madrid, Spain.
[c] Nutrition and Food Science Department, XaRTA, INSA. Pharmacy School, University of Barcelona, Barcelona, Spain. * lamuela@ub.edu

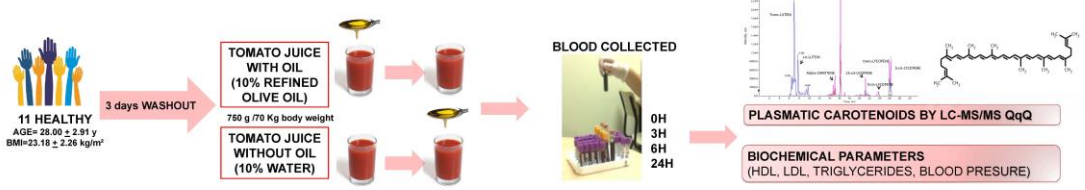
BACKGROUND AND OBJECTIVES

Tomato-rich diets may produce a potential vascular health benefit that has been related to the high plasma carotenoid level achieved after its intake. The matrix in which carotenoids are found in foods appear to be an important determinant of their bioavailability.

The aim of the present study was to evaluate the effect of oil addition on tomato juice not treated with heat on the bioavailability of plasma carotenoids and the postprandial lipid response.

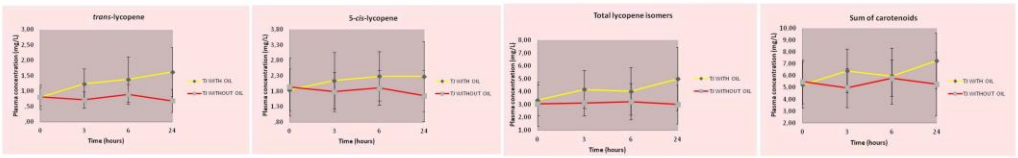
STUDY DESIGN

Open, controlled and cross-over feeding clinical trial



RESULTS

The presence of refined olive oil in tomato juice **enhances the bioavailability of carotenoids** in plasma. All lycopene isomers increased significantly when subjects consume tomato juice with oil reaching the maximum concentration at 24 h.



Low density lipoprotein (LDL) cholesterol **decreased** significantly (p=0.036) 6 h after the consumption of **tomato juice with oil**, which was significantly **correlated with an increase of trans-lycopene** (r2=0.56; p=0.049).

Total cholesterol reduction after tomato juice with oil consumption correlates with an increase of 5-cis-lycopene (r2=0.67; p=0.023).

CONCLUSION

The addition of oil in tomato juice increases postprandial absorption of carotenoids, mainly 5-cis and trans-lycopene which seem to affect lipid metabolism on healthy humas.

ACKNOWLEDGEMENTS

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8.2.20. Comunicación 20. Póster

Título: Influence of olive oil in carotenoids absorption from tomato juice and effects on postprandial lipemia.

Autores: Sara Arranz, **Miriam Martínez-Huélamo**, Anna Vallverdú-Queralt, Palmira Valderas-Martínez , Montse Illán, Emilio Sacanella, Elvira Escribano, Ramon Estruch, and Rosa M. Lamuela-Raventós.

Congreso: X Congreso Internacional de Dieta Mediterránea. Barcelona, Spain, 2014.

INFLUENCE OF OLIVE OIL IN CAROTENOIDS ABSORPTION FROM TOMATO JUICE AND EFFECTS ON POSTPRANDIAL LIPEMIA

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BACKGROUND AND OBJECTIVES

The potential benefits of tomato-rich diets on the cardiovascular system have been related to the concentrations of plasmatic carotenoids. In addition, the bioavailability of carotenoids from foods depends on their chemical structure, processing, and the food matrix.

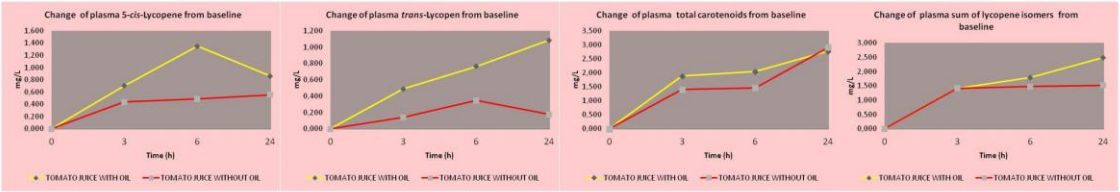
The aim of the present study was to evaluate the effect of oil addition on tomato juice not treated with heat on the bioavailability of plasma carotenoids and the postprandial lipid response.

STUDY DESIGN

Open, controlled and cross-over feeding clinical trial

RESULTS

The consumption of tomato juice with oil provides a significant increase in lycopene exposition over time mainly due to trans-lycopene and 5-cis-lycopene isomers which clearly demonstrated major contribution to the total carotenoid concentrations in plasma. Kinetic parameters provided evidence on how the oil matrix tends also to increase the absorption rate of some carotenoids, mainly lycopene isomers.



Figures: Plasma concentration change from baseline of different lycopene isomers and total carotenoids through both interventions.

Low density lipoprotein (LDL) cholesterol was significantly lower after consumption of tomato juice with oil, being associated with a higher absorption of lycopene isomers, mainly trans-lycopene (r2=0.56; p=0.049). The reduction in total cholesterol after the intake of tomato juice with oil was associated with an increase of 5-cis-lycopene isomer in plasma (r2=0.67; p=0.023).

CONCLUSION

The addition of oil in tomato juice increases postprandial absorption of carotenoids, mainly 5-cis and trans-lycopene which seem to affect lipid metabolism on healthy humans.

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CICYT (AGL2010-22319-C03) from the Spanish Ministry of Science and Innovation (MICINN). MAPFRE Foundation. The CIBERobn CB06/03 is an initiative of the Instituto de Salud Carlos III, Spain. Arranz, S. thanks the postdoctoral Sara Borrell program (CD10/00151) from the Instituto de Salud Carlos III, Spain. Vallverdu-Queralt, A. and Martínez-Huelamo, M received support from MICINN. Valderas-Martínez, P. thanks the APIF predoctoral fellowship from the University of Barcelona.

8.2.21. Comunicación 21. Póster

Título: Identification of the phenolic metabolites in a functional food made with grape extract and, in human urine, by a novel high resolution LTQ-Orbitrap-MS approach.
Autores: Gemma Sasot, **Miriam Martínez-Huélamo**, Anna Vallverdú-Queralt, Mireia Roig, Jorge Regueiro, Ramón Estruch, Gemma Chiva-Blanch, Mercè Mercader Martí, and Rosa M. Lamuela-Raventós.
Congreso: X Congreso Internacional de Dieta Mediterránea. Barcelona, Spain, 2014.

Identification of the phenolic metabolites in a functional food made with grape extract and, in human urine, by a novel high resolution LTQ-Orbitrap-MS approach

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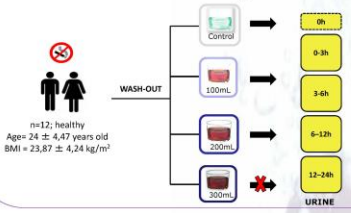
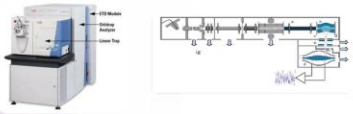
Introduction

Grape pomaces are known to be a rich source of polyphenols with biological activity, which may be used as functional ingredients for the development of new interesting products. Numerous studies have reported that bioactive compounds may act through multiple mechanisms, like antioxidant, anticarcinogen or anti-inflammatory (1).
However, an exhaustive identification of the polyphenols present in this functional compound, grape extract (GE) and, in biological samples obtained after the consumption of this novel food is required. In last years, there has been an increased interest in metabolites formed by the intestinal microbiota to understand the benefits derived from consumption (2). The application of high resolution mass spectrometry (HR-MS) allows the identification of phenolics using accurate mass measurements.

Material and Methods

In order to verify the oral absorption and metabolism of the polyphenols content, we performed a prospective randomized, cross-over study in 12 healthy volunteers with three interventions: 500 mL of a functional beverage enriched with 100 mL of grape extract, 500 mL of the same beverage with 200 mL of GE and 500 mL of a control beverage without GE





LTQ-Orbitrap-MS enabled the identification of phenolics in grape extract and in human urine collected

Results

Compound	R_t (min)	Accurate mass [M-H]⁻	MS/MS ions (%Intensities)	mDa	Molecular formula
Pyrogallol	1,08	125,02406	97 (100)	0,4	$C_6H_6O_3$
	1,38	125,02415	97 (100)	0,2	$C_6H_6O_3$
2-O-Methylpyrogallol	4,99	139,03987	124 (100)	0,2	$C_7H_8O_3$
Gallic acid	1,53	169,01396	125 (100)	0,5	$C_7H_6O_5$
3-O-Methylgallic acid / 4-O-Methylgallic acid	1,81	183,02962	139 (100), 95 (20)	0,3	$C_8H_8O_5$
	3,35	183,02951	168 (100), 139 (70), 124 (20)	0,4	$C_8H_8O_5$
	3,64	183,02934	168 (100)	0,6	$C_8H_8O_5$
3-Hydroxyhippuric acid / 4-Hydroxyhippuric acid	2,47	194,04587	100 (100)	0,3	$C_9H_9NO_4$
	3,11	194,04587	150 (100)	0,3	$C_9H_9NO_4$
3,4-O-Dimethylgallic acid / Gallic acid ethyl ester	1,68	197,0451	137 (100), 182 (10)	0,4	$C_9H_9O_5$
	2,62	197,04133	182 (100), 153 (30)	0,3	$C_9H_9O_5$
	5,91	197,04125	182 (100), 153 (40)	0,3	$C_9H_9O_5$
5-(3,4-Dihydroxyphenyl)-γ-valerolactone	6,96	207,06627	163 (100), 122 (20)	0,4	$C_{11}H_{11}O_4$
	9,09	207,08627	181(100), 179 (40), 163 (40), 143(40)	0,4	$C_{11}H_{11}O_4$
4-O-Methylgallic acid-3-O-sulfate	2,67	262,98642	183 (100)	0,3	$C_8H_8O_8S$
	3,17	262,98633	219 (40), 183 (100)	0,4	$C_8H_8O_8S$
Dihydroferulic acid 4-sulfate	5,42	273,02222	195 (100)	0,9	$C_{10}H_{11}O_7S$
	5,58	273,02264	195 (100), 177 (90)	0,4	$C_{10}H_{11}O_7S$
	7,33	273,02239	195 (100)	0,9	$C_{10}H_{11}O_7S$
	12,25	273,02246	195 (100)	0,6	$C_{10}H_{11}O_7S$
Catechin	5,56	289,0712	245 (100), 205 (60)	0,6	$C_{15}H_{13}O_6$
Epicatequin	7,08	289,07086	245 (100), 207 (70)	0,9	$C_{15}H_{13}O_6$
Quercetin	11,46	301,03537	265 (100), 243 (45)	0,0	$C_{15}H_9O_7$
Dihydroquercetin	11,53	303,5319	223 (100)	2,2	$C_{15}H_{11}O_7$
trans-Resveratrol-3-O-sulfate	12,97	307,02817	227 (100)	0,3	$C_{14}H_{11}O_6S$
trans-Resveratrol-4'-O-sulfate	14,43	307,02817	227 (100)	0,6	$C_{14}H_{11}O_6S$
cis-Resveratrol-3-O-sulfate	17,23	307,02817	227 (100)	0,9	$C_{14}H_{11}O_6S$
cis-Resveratrol-4-O-sulfate	18,68	307,02817	227 (100)		$C_{14}H_{11}O_6S$
Protocatechuic-O-glucoside	1,84	315,07347	153 (100)	0,7	$C_{13}H_{16}O_9$
Dihydrocaffeic acid 3-O-glucuronide	5,53	357,08270	181 (100), 113 (45)	0,9	$C_{15}H_{17}O_{10}$
Catechin sulfate	5,56	369,02795	289 (100), 245 (30)	0,6	$C_{15}H_{13}O_9S$
Epicatechin sulfate	7,08	369,02798	290 (100), 245 (30)	0,6	$C_{15}H_{13}O_9S$
Dihydroferulic acid 4-O-glucuronide	4,73	371,0983	195 (60), 175 (90), 113 (100)	0,3	$C_{16}H_{19}O_{10}$
	9,89	371,09778	195 (40), 175 (90), 113 (100)	0,6	$C_{16}H_{19}O_{10}$
	10,35	371,09772	195 (25), 175 (90), 113 (100)	0,6	$C_{16}H_{19}O_{10}$
Methyl-catechin sulfate	6,56	383,04391	303 (100), 217 (80), 137 (30)	0,3	$C_{16}H_{15}O_9S$
Methyl-epicatechin sulfate	7,12	383,03364	303 (100), 245 (30), 217 (80), 137 (30)	0,6	$C_{16}H_{15}O_9S$
	8,19	383,04416	303 (100), 217 (60), 137 (30)	0,3	$C_{16}H_{15}O_9S$
	8,76	383,04337	303 (100), 217 (70), 137 (30)	0,8	$C_{16}H_{15}O_9S$
Epigallocatechin 3-O-glucuronide / Epigallocatechin 3'-O-glucuronide / Epigallocatechin 7-O-glucuronide	11,7	481,09674	305 (100)	1,7	$C_{21}H_{21}O_{13}$
Catechin-glucuronide	4,37	465,10297	289 (100)	0,9	$C_{21}H_{21}O_{12}$
Epicatechin-glucuronide	5,84	465,10355	289 (100), 245 (30)	0,3	$C_{21}H_{21}O_{12}$
Methyl-catechin-glucuronide	5,85	479,11871	303 (100)	0,8	$C_{22}H_{23}O_{12}$
3'-O-Methyl-epicatechin-7-O-glucuronide	5,73	479,1189	303 (100)	0,6	$C_{22}H_{23}O_{12}$
4'-O-Methyl-epicatechin-7-O-glucuronide	6,59	479,11902	303 (100)	0,5	$C_{22}H_{23}O_{12}$
	11,76	479,11633	461(20), 303 (100)	3,2	$C_{22}H_{23}O_{12}$
4'-O-Methyl-epigallocatechin-3'-O-glucuronide / 4'-O-Methyl-epigallocatechin-7-O-glucuronide	8,98	495,11439	319 (100)		$C_{22}H_{23}O_{13}$
Catechin-glucuronide sulfate	2,52	545,06662	465 (45), 369 (100)	0,2	$C_{21}H_{21}O_{15}S$
Epicatechin glucuronide sulfate	5,10	545,06073	465 (45), 369 (100)	0,1	$C_{21}H_{21}O_{15}S$
trans-Resveratrol-3-O-glucuronide	12,49	403,10344	227 (20), 175 (100)	0,2	$C_{20}H_{19}O_9$
trans-Resveratrol-4'-O-glucuronide	15,45	403,10344	227 (100)	0,8	$C_{20}H_{19}O_9$
cis-Resveratrol-3-O-glucuronide	17,62	403,10344	227 (90), 175 (100)	1,4	$C_{20}H_{19}O_9$
cis-Resveratrol-4'-O-glucuronide	18,79	403,10344	341 (100), 227 (30)	1,6	$C_{20}H_{19}O_9$
Dihydroresveratrol-3-O-sulfate	12,87	309,04382	229 (100), 123 (15)	0,8	$C_{14}H_{13}O_6S$
Dihydroresveratrol-4-O-sulfate	13,55	309,04382	229 (100), 125 (15)	1	$C_{14}H_{13}O_6S$
Dihydroresveratrol glucuronide-sulfate	8,88	485,07590	309 (100), 233(20), 175 (35)	0,4	$C_{20}H_{21}O_{12}S$
3-Hydroxybenzoic acid	2,99	137,02441	93(100)	0,33	$C_7H_6O_3$
4-Hydroxybenzoic acid	4,66	137,02441	93(100)	0,43	$C_7H_6O_3$
2-Hydroxyphenylacetic acid	4,8	151,04006	107 (100)	0,4	$C_8H_8O_3$
3-Hydroxyphenylacetic acid	7,05	151,04006	107 (100)	0,3	$C_8H_8O_3$
2-Hydroxy-2-phenylacetic acid	7,1	151,04006	107 (100)	0,4	$C_8H_8O_3$
4-Hydroxyphenylacetic acid	10,3	151,04006	107 (100)	0,5	$C_8H_8O_3$
2-Hydroxy-2-phenylacetic acid	10,3	151,04006	107 (70), 93 (100)	0,3	$C_8H_8O_3$
3,4-Dihydroxybenzoic acid (Protocatechuic acid)	2,8	153,01932	109 (100)	0,5	$C_7H_6O_4$
3,4-Dihydroxyphenylacetic acid (Homoprotocatechuic acid)	2,43	167,03457	123 (100)	0,4	$C_8H_8O_4$
Hippuric acid	4,92	178,05096	134 (100)	0,01	$C_9H_9NO_3$
Caffeic acid	3,38	179,03497	135 (100)	2,2	$C_9H_8O_4$
3,4-Dihydroxyphenylpropionic acid (Dihydrocaffeic acid)	3,5	181,05062	163 (100), 135 (40)	0,6	$C_9H_{10}O_4$
Homovanillic acid	3,52	181,05062	137 (100)	0,5	$C_9H_{10}O_4$
4-Hydroxy-5-(3,4-dihydroxyphenyl)valeric acid	13,14	225,07883	207 (30), 181 (40), 137 (100), 83 (20)	0,5	$C_{11}H_{14}O_5$
Vanillic acid 4-sulfate	3,36	246,99179	203 (80), 167 (100), 123 (100)	0,05	$C_8H_8O_7S$
Caffeic acid 3-sulfate	4,56	258,99179	179 (100)	0,9	$C_9H_8O_7S$
Caffeic acid 4-sulfate	6,03	258,99179	179 (100)	0,8	$C_9H_8O_7S$
Homovanillic acid 4-sulfate	5,95	261,00744	181 (100)	0,4	$C_9H_{10}O_7S$
Isoferulic acid 3-sulfate	6,32	273,00677	229 (20), 193 (100)	0,7	$C_{10}H_{10}O_7S$
Ferulic acid 4-sulfate	6,36	273,00744	193 (100)	0,9	$C_{10}H_{10}O_7S$
Epigallocatechin	6,42	305,06668	225 (100)	2,2	$C_{15}H_{14}O_7$
p-Coumaric acid	6,64	163,03969	119 (100)	0,4	$C_9H_8O_3$
	8,96	163,3958	119 (100)	0,5	$C_9H_8O_3$
3-(3-Hydroxyphenyl)propionic acid / 3-(4-Hydroxyphenyl)propionic acid	6,78	165,05571	147 (20), 121 (100)	0,4	$C_9H_{10}O_3$
3,5-Dimethoxy-4-hydroxybenzoic acid (Syringic acid)	1,69	197,04553	137 (100)	0,3	$C_9H_{10}O_5$

Table 1. List of compounds identified in urine samples

Compound	R_t (min)	Accurate mass [M-H]⁻	MS/MS ions (%Intensities)	mDa	Molecular formula
Procyanidin trimer type B 1	1,50	865.1985	695 (100), 739 (70), 577 (65), 407 (40), 287 (20)	4	$C_{45}H_{38}O_{18}$
Gallic acid	1,57	169.0142	125 (100)	1,3	$C_7H_6O_5$
Galloylglucose	1,6	331.0665	169 (100), 125 (10)	1,1	$C_{13}H_{16}O_{10}$
Protocatechuic acid-O-hexoside	1,86	315.0721	153 (100), 109 (20)	1,2	$C_{13}H_{16}O_9$
Gentisic acid	2,70	153.0188	123 (100), 109 (10)	0,4	$C_7H_6O_4$
Protocatechuic acid	2,79	153.0193	153 (40), 109 (90)	0,9	$C_7H_6O_4$
Methyl gallate	3,09	183.0293	139 (100), 95 (40)	1,3	$C_8H_8O_5$
Caffeic acid	3,22	311.0408	149 (100)	2	$C_{15}H_{12}O_8$
Procyanidin dimer type B 1	3,40	577.1346	425 (100), 451 (60), 407 (50), 559(30), 289 (30)	2,9	$C_{30}H_{26}O_{12}$
Caffeic acid-C-hexoside 1	3,54	341.0877	281 (90), 251 (100), 221 (40), 179 (60), 135 (10)	2,1	$C_{15}H_{18}O_9$
Procyanidin dimer type B 2	4,09	577.1346	425 (100), 451 (60), 407 (50), 559(30), 289 (30)	2,9	$C_{30}H_{26}O_{12}$
Procyanidin trimer type B monogalloylate 1	4,38	1.017.2034	729 (100), 891 (80), 865 (60), 999 (50), 407 (30)	6,4	$C_{52}H_{42}O_{22}$
Caffeic acid-C-hexoside 2	4,39	341.0877	281 (90), 251 (100), 221 (40), 179 (60), 135 (10)	2	$C_{15}H_{18}O_9$
Coumaric-O-hexoside	4,36	325,0928	163 (100), 119 (12)	1,2	$C_{15}H_{18}O_8$
Catechin	4,43	289.0718	245 (100)	1,7	$C_{15}H_{14}O_6$
p-hydroxybenzoic acid	4,49	137,0244	93 (100)	0,7	$C_7H_6O_3$
Procyanidin trimer type B 2	4,75	865,1985	695 (100), 739 (70), 577 (65), 407 (40), 287 (20)	4	$C_{45}H_{38}O_{18}$
Procyanidin trimer type B 3	5,30	865,1985	695 (100), 739 (70), 577 (65), 407 (40), 287 (20)	4	$C_{45}H_{38}O_{18}$
Procyanidin trimer type B monogalloylate 2	5,60	1.017.2034	729 (100), 891 (80), 865 (60), 999 (50), 407 (30)	6,4	$C_{52}H_{42}O_{22}$
Caffeic acid	5,72	179.0349	135 (100)	1,1	$C_9H_8O_4$
Procyanidin trimer type B monogalloylate 3	6,04	1.017.2034	729 (100), 891 (80), 865 (60), 999 (50), 407 (30)	6,4	$C_{52}H_{42}O_{22}$
Epicatechin	6,47	289.0718	245 (100)	1,9	$C_{15}H_{14}O_6$
Eriodictyol-O-hexoside	6,64	449.1089	287 (100), 259 (40), 269 (38)	2,6	$C_{21}H_{22}O_{11}$
Procyanidin dimer type B monogalloylate 1	6,85	729.1461	711 (20), 577 (80), 559 (60), 407 (100), 289 (40)	3,8	$C_{37}H_{30}O_{15}$
Procyanidin trimer type B 4	7,29	865,1985	695 (100), 739 (70), 577 (65), 407 (40), 287 (20)	4	$C_{45}H_{38}O_{18}$
Procyanidin dimer type B monogalloylate 2	7,83	729,1461	711 (20), 577 (80), 559 (60), 407 (100), 289 (40)	3,8	$C_{37}H_{30}O_{15}$
Coumaric acid	8,24	163,0400	119 (100)	0,9	$C_9H_8O_3$
Myricetin-O-hexoside	8,49	479,0826	316 (100), 317 (50)	3,7	$C_{21}H_{20}O_{13}$
Kaempferol-3-O-rutinoside	8,79	593,1511	285 (100)	3,6	$C_{27}H_{30}O_{15}$
Ferulic acid	9,17	193,0506	178 (70), 149 (100), 134 (50)	1,2	$C_{10}H_{10}O_4$
Picnid acid 1	9,50	389,1241	227 (100), 193 (80), 341 (70)	2	$C_{17}H_{26}O_9$
Procyanidin dimer type B digalloylated	9,53	881,1520	729 (100), 711 (30) , 559 (20), 441 (10), 407 (20), 289 (5)	2,1	$C_{44}H_{34}O_{20}$
Epicatechin-O-gallate	9,8	441,0822	289 (100), 331 (20), 271 (15), 169 (30)	1,8	$C_{22}H_{18}O_{10}$
Kaempferol-3-O-glucoside	10,20	447,0932	285 (100)	1,4	$C_{21}H_{20}O_{11}$
Quercetin-O-hexoside	10,20	463,0881	301 (100), 293 (40), 271 (15), 169 (30)	2,2	$C_{21}H_{20}O_{12}$
Procyanidin trimer type B monogalloylate 4	10,26	1.017.2034	729 (100), 891 (80), 865 (60), 999 (50), 407 (30)	6,4	$C_{52}H_{42}O_{22}$
Quercetin-O-rhamanoside	11,67	447,0932	301 (100), 284 (20), 285 (20)	1,9	$C_{21}H_{20}O_{11}$
Picnid acid 2	12,23	389,1241	227 (100)	2,2	$C_{17}H_{26}O_9$
Quercetin	16,30	301,0353	179 (100), 151 (90), 107 (30)	3,5	$C_{15}H_{10}O_7$
Resveratrol	16,58	227,0713	185 (100), 143 (30)	2,5	$C_{14}H_{12}O_3$
Naringenin	17,88	271,0611	151 (100), 177 (20), 119 (10)	1,7	$C_{15}H_{12}O_5$

Table 2 List of compounds identified in grape extract

Conclusions

Forty one polyphenols were identified in extract mainly procyanidins, phenolic acids and flavonols.
In urine samples, more than sixty metabolites of phenolic compounds were detected including microbiota metabolites, glucuronides and sulfate derivatives.
In conclusion, the LTQ-Orbitrap-MS enhances the identification of a large variety of polyphenols from functional ingredients and their metabolites originated in the human body.

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8.2.22. Comunicación 22. Póster

Título: Phenolic profiling of Spanish beers by high resolution mass spectrometry (LC-ESI-LTQ-Orbitrap-MS).

Autores: Paola Quifer-Rada, Anna Vallverdu-Queralt, **Miriam Martinez-Huelamo**, Olga Jáuregui, Gemma Chiva-Blanch, Ramon Estruch, and Rosa M Lamuela-Raventós.

Congreso: X Congreso Internacional de Dieta Mediterránea. Barcelona, Spain, 2014.

PHENOLIC PROFILING OF SPANISH BEERS BY HIGH RESOLUTION MASS SPECTROMETRY (LC-ESI-LTQ-ORBITRAP-MS)

Paola Quifer-Rada[1,2], Anna Vallverdu-Queralt[1,2], Miriam Martinez-Huelamo [1,2], Olga Jauregui [3], Gemma Chiva-Blanch[2,4], Ramon Estruch [2,4], Rosa M Lamuela-Raventos [1,2].

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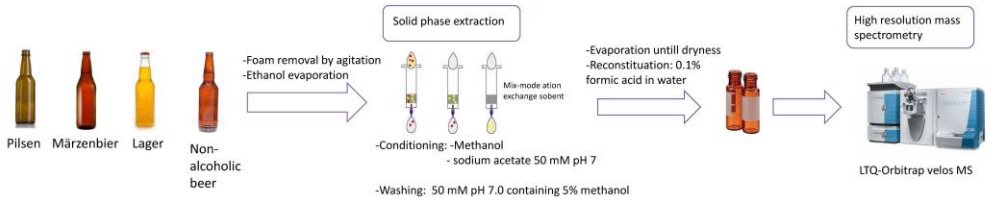
Introduction

Beer is the second most consumed alcoholic beverage in Europe, according to the Spirits Europe organization, beer accounts for 37% of the total EU alcohol consumption. Particularly, in Spain beer consumption is higher than wine and it is part of the traditional Mediterranean diet.

Beer is an alcoholic beverage rich in nutrients including carbohydrates, minerals (potassium, magnesium), vitamins (niacin, riboflavin, folate, cobalamin, pyrodoxine) and amino acids. Moreover, beer contains polyphenols which about 70-80% come from malt, the rest 30-20% are hops polyphenols (1). Although various phenolic compounds have been described in beer using different detectors such as coulometric array (2), electrochemical(3), ultraviolet-visible spectrophotometry,(4), photodiode-array, (5) and low resolution mass spectrometry (6) a comprehensive identification of its phenolic profile by high resolution mass spectrometry is still lacking.

Materials and methods



Results

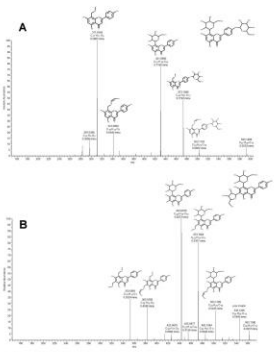


Figure 1. MS^2 spectrum of apigenin-*C*-hexoside-*O*-hexoside (A) and apigenin-*C*-hexoside-*C*-pentoside (B).

Compound	Rt (min)	Accurate mass [M-H-]	Fragments m/z	Error (mDa)	Molecular Formula
Gallic acid*	1.16	169.0142	125.0241	0.60	$C_7H_6O_5$
4-vinylguiacol	1.46	149.0607	134.0362	0.80	$C_9H_{10}O_2$
Caffeic acid-*O*-hexoside I	1.55	341.0877	179.0346 , 135.0400	0.40	$C_{15}H_{18}O_9$
Caffeic acid-*O*-hexosideII	2.23	341.0877	179.0346 , 135.0400	0.43	$C_{15}H_{18}O_9$
Protocatechuic acid-*O*-hexoside	2.3	315.0710	153.0190, 109.0291	1.10	$C_{13}H_{16}O_9$
Protocatechuic acid*	2.45	153.0193	109.0292	0.06	$C_7H_6O_4$
Catechin*	2.96	289.0717	245.0814, 205.0502,179.0345	0.12	$C_{15}H_{14}O_6$
Catechin-*O*-hexoside I	3.09	451.1245	289.0710 , 245.0817	0.13	$C_{21}H_{24}O_{11}$
Neochlorogenic acid 3-caffeoylquinic acid	3.24	353.0877	191.0557, 179.0346 , 135.0449	0.13	$C_{16}H_{18}O_9$
Hydroxyphenil acetic acid I	3.29	151.0395	107.0499	0.05	$C_8H_8O_3$
Catechin-*O*-dihexoside	3.33	613.1773	451.1242, 289.0714	0.12	$C_{27}H_{34}O_{16}$
Coumaric acid-*O*-hexoside	3.5	325.0928	163.0402	0.10	$C_{15}H_{18}O_8$
Hydroxybenzoic acid*	3.54	137.0241	93.0343	0.20	$C_7H_6O_3$
Hydroxyphenil acetic acid II	3.62	151.0395	107.0498	0.05	$C_8H_8O_3$
Epicatechin*	4.19	289.0717	245.0814, 205.0502,179.0345	0.12	$C_{15}H_{14}O_6$
1-caffeoylquinic acid	4.27	353.0877	191.0555, 179.0343	0.11	$C_{16}H_{18}O_9$
Vanillicacid*	4.5	167.0349	152.0108, 123.0447, 108.0211	0.07	$C_8H_8O_4$
Chlorogenic acid 5-caffeoylquinic acid*	4.53	353.0877	191.0557, 179.0346, 135.0449	0.09	$C_{16}H_{18}O_9$
Catechin-*O*-hexoside II	4.66	451.1245	289.0710, 245.0818	0.12	$C_{21}H_{24}O_{11}$
Caffeic acid*	4.8	179.0349	134.0675	0.06	$C_9H_8O_4$
Feruloylquinic acid	4.91	367.1034	193.0502 , 191.0557	0.10	$C_{17}H_{20}O_9$
Cryptochlorogenic acid (4-caffeoylquinic acid)	4.91	353.0877	191.0557, 179.0344	0.11	$C_{16}H_{18}O_9$
Hydroxyphenil acetic acid II	5.01	151.0395	107.0498	0.05	$C_8H_8O_3$
Coumaric acid	5.94	163.0400	119.0498	0.05	$C_9H_8O_3$
Sinapic acid-*O*-hexoside I	6.39	385.1139	223.0610 , 208.0400 , 179.0698	0.11	$C_{17}H_{22}O_{10}$
Ferulic acid-*O*-hexoside	6.42	355.1034	193.0507 , 178.0280	0.10	$C_{16}H_{20}O_9$
Indole-3-carboxilic acid	6.6	174.055	130.0657	0.06	$C_{10}H_9NO_2$
Sinapicacid-*O*-hexoside II	6.89	385.1136	223.0610 , 208.0400 , 179.0698	0.12	$C_{17}H_{22}O_{10}$
Ferulic acid	7.03	193.0506	149.0604, 178.0267 , 134.0370	0.06	$C_{10}H_{10}O_4$
Apigenin-*C*-hexoside-*O*-hexoside	7.39	593.1511	311.0598 , 431.0980, 473.1086 ,341.0662 , 297.0401	0.30	$C_{27}H_{30}O_{15}$
Apigenin-*C*-hexoside-*C*-pentoside	7.93	563.1406	443.0982, 473.1087 , 353.0664 , 383.0769, 503.1192, 545.1300	0.23	$C_{26}H_{28}O_{14}$
Sinapic acid	8.37	223.0611	208.0371, 179.0706, 164.0474	0.06	$C_{11}H_{12}O_5$
Apigenin-*C*-hexoside	9.29	431.0983	311.0561, 341.0656, 413.0871	0.2	$C_{21}H_{20}O_{10}$
Quercetin-3-*O*-glucoside*	9.57	463.0881	301.0346	0.12	$C_{21}H_{20}O_{12}$
Kaempferol-3-*O*-glucoside*	11.1	447.0932	285.0403	0.05	$C_{21}H_{20}O_{11}$
3,7-dimethylquercetin	14.3	329.0666	314.0432, 299.0200	0.09	$C_{17}H_{14}O_7$
Isoxanthohumol*	14.4	353.1394	233.0814, 119.0499	0.07	$C_{21}H_{22}O_5$
8-prenylnaringenin*	14.6	339.1237	219.0659 , 245.0811	0.22	$C_{20}H_{20}O_5$
6-prenylnaringenin	14.7	339.1237	219.0656 , 245.0812	0.13	$C_{20}H_{20}O_5$
Cohumulone I	14.8	347.1864	235.1337	0.3	$C_{20}H_{28}O_5$
ad-Humulone	14.9	361.2020	235.1337 , 262.1313	1	$C_{21}H_{30}O_5$
Cohumulone II	15.3	347.1864	278.1156	0.14	$C_{20}H_{28}O_5$
n-Humulone	15.5	361.2020	262.1312, 343.1910	1.11	$C_{21}H_{30}O_5$
iso-a-cohumulone	16	347.1864	251.1288, 329.1754	0.3	$C_{20}H_{28}O_5$
iso-a-adh-humulone	16.2	361.2020	265.1440 , 343.1916, 235.1339	0.1	$C_{21}H_{30}O_5$
iso-a-adh-humulone	16.4	361.2020	265.1442, 343.1910, 235.1336	0.2	$C_{21}H_{30}O_5$

Table 1. Phenolic compounds identified in beer.

Conclusions

Using a high resolution mass spectrometer LTQ-Orbitrap we were able to identify **47 phenolic compounds in beer**, seven of which, as far as we know, were reported for the first time. Most of these compounds are hexosides, dihexosides, pentosides and quinic conjugates :

-Feruoylquinic acid
-Caffeic acid-*O*-hexoside
-Coumaric acid-*O*-hexoside
-Sinapic acid-*O*-hexoside
-Catechin-*O*-dihexoside
-Kaempferol-*O*-glucoside
-Apigenin-*C*-hexoside-pentoside.

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http://polyphenolresearch.com

8.2.23. Comunicación 23. Póster

Título: Improved characterization of naringenin metabolites from mice stomach using two analytical techniques.

Autores: Naiara Orrego, Elvira Escribano, **Miriam Martinez-Huélamo**, Anna Vallverdú-Queralt, and Rosa M. Lamuela-Raventós.

Congreso: 9th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical technology. Lisbon, Portugal, 2014.

IMPROVED CHARACTERIZATION OF NARINGENIN METABOLITES FROM MICE STOMACH USING TWO ANALYTICAL TECHNIQUES

Naiara Orrego[1], Elvira Escribano[1], Miriam Martinez[2], Anna Vallverdú-Queralt[2] and Rosa M. Lamuela-Raventos[2,3]

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1. Introduction

Naringenin (NAR), an antioxidant found in high concentration in tomato, is strongly associated with a reduced risk of chronic and cardiovascular diseases and cancer (Zygmunt K, 2010).

Liquid chromatography/electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) is useful for identification and quantification of NAR and its metabolites (Martinez-Huelamo, M, 2012).
The mass spectrometry (MS) techniques, liquid chromatography /electrospray ionization linear ion trap quadrupole-Orbitrap-mass spectrometry (LC-ESI-LQT-Orbitrap-MS) are used for identification of all compounds with the most accurate precision, and allow to identify lower concentration metabolites, because of its greatest sensitivity (Vallverdú-Queralt, 2010).

Aim: Combine both analytical techniques to enhance the knowledge about the metabolic fate of NAR in stomach. Samples of gastric content were obtained after an *in situ* single-pass perfusion technique in mice.

2. Material and methods

Perfusion experiments

Briefly, a solution of 3.5 µg/mL of NAR in pH 2 (267mOsm/kg) with a 0.1mg/mL of Phenol Red at 37ºC was perfunded (0.16 mL/min) into the stomach of anesthetized CD1 mice (30-35g). Samples were collected at 10 min intervals for 1h 40 min.

Analytical techniques

@ **HPLC analysis**: Identification and quantification

Liquid chromatograph

Agilent series 1100 (Agilent, Waldbronn, Germany) equipped with a quaternary pump and a thermostated autosampler.

Analytical conditions

- **Column:** 5 µm Luna C_{18} (Phenomenex. USA)
- **Injection volume**: 20 µL
- **Flow**: 0.6 mL / min
- **Column temperature** : 30 ºC
- **Mobil phases**: - A : H_2O with 0.1% formic acid
  - B : AcN with 0.1% formic acid
- **Gradient (t (min), %B (v/v))**: (0, 5); (2, 25); (10, 90); (11,100); (12,100); (17, 5);(20, 5).

- **Turbo ion spray (negative mode) :**
  - ☑ **Capillary voltage** = - 4500 V
  - ☑ **Nebulizer gas (N2)**= 10 au
  - ☑ **Curtain gas (N2)** = 12 au
  - ☑ **Entrance potencial (EP)** = -11V
  - ☑ **Drying gas (N2)** heated to 400ºC
  - ☑ **Declustering potential (DP)***
  - ☑ **Focusing potential (FP)***
  - ☑ **Collision energy (CE)***

* Optimized to detect phenolics with the highest signals (Table 1)

Table 1. List of metabolites identified in perfusion samples by LC-MS/MS

Compound	rt (min)	MS/MS	DP	FP	CE	LD (µg/mL)	LQ (µg/mL)
Naringenin*	5,22	271->151	-50	-190	-30	$2,99x10^{-4}$	$9,99x10^{-4}$
Naringenin-4,7-glucuronide*	4,2	447->271	-50	-190	-30	$2,51x10^{-4}$	$8,37x10^{-4}$
Naringenin-sulfate	7,14	351->271	-50	-190	-30	—	—
3,4-Hydroxyphenyl propionic acid*	2,97	165->121	-30	-200	-20	0,0338	0,1128

* Identified with standard

@ **LTQ-Orbitrap:** Identification

LTQ Orbitrap

Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK)

- **Equiped** → ESI Source in **negative mode** was used to acquire mass spectra in profile mode with a setting of 30000 resolution at m/z 400
- **Mass range** → From m/z 100 to 1000
- **Data analysis** → Xcalibour software
- **Operation parameters** →
  - ☑ Source voltage =4kV
  - ☑ Sheath gas= 20 (arbitrary units (au))
  - ☑ Auxiliary gas= 10 (au)
  - ☑ Sweep gas, 2 (au)
  - ☑ Capillary temperature, 275 8 C.

Liquid chromatograph

Accela (Thermo Scientific, Hemel Hempstead, UK) equipped with a quaternary pump, a photodiode array detector and a thermostated autosampler.

Analytical conditions

- **Column:** reversed-phase column Atlantis T3 C18 from Waters (USA)
- **Injection volume:** 5 µL
- **Flow: 0.6350mL / min**
- **Column temperature :** 25 ºC
- **Mobil phases:** - A : H2O with 0.1% formic acid
  - B : AcN with 0.1% formic acid
- **Gradient (t (min), %B (v/v)): (**0, 10); (1, 10); (15, 30); (22,50); (28,100); (34, 100);(36, 10).

3. Results

- LTQ-Orbitrap confirmed the presence of NAR, NAR-GLU, NAR-SULF and 3, 4-HPPA and enable us to identify low-intensity signals corresponding to naringenin glutation, 4-hydroxyhippuric acid, phloroglucinol and naringenin oxidated.

Table 2. List of compound indentified in perfusion samples by Orbitrap

Compound	rt (min)	[M-H]-	Acc.mass	mDa	MF
Naringenin	17,37	271	271,0611	0,2	$C_{15}H_{11}O_5$
Naringenin-4,7-glucuronide	7,65	447	447,0933	>2	$C_{21}H_{19}O_{11}$
Naringenin-sulfate	12,2	351	351,018	0,9	$C_{15}H_{11}O_8S$
3,4-Hydroxyphenyl propionic acid	4,72	165	165,0557	0,9	$C_9H_{10}O_3$
Naringenin glutation	8,18	577	577,1371	0,8	$C_{25}H_{26}N_3O_{11}S$
4-Hidroxyhippuric acid	20,83	194	194,0458	0,04	$C_9H_8NO_4$
Phloroglucinol	8,19	125	125,0244	0,7	$C_6H_5O_3$
Naringenin oxidated	18	269	269,0611	0,3	$C_{15}H_9O_5$

- The chromatogram in MRM mode obtained in the triple quadrupole system revealed the presence of NAR, NAR-GLU, Naringenin sulfate (NAR-SULF) and 3, 4-HPPA, however low-intensity compounds had not been detected.

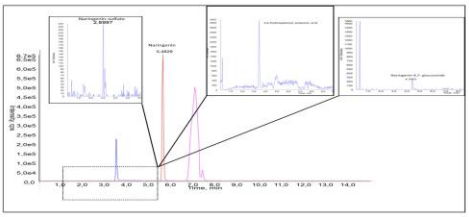

Figure 1. Chromatogram of perfusion sample at 1h 40min after the administration of naringenin solution.

4. Conclusions

1. LC/ESI-LTQ-Orbitrap-MS and allowed the identification of 8 metbolites from naringenin digestion, showed 4 metabolites more than using only LC-ESI-MS.
2. The use of the LTQ-Orbitrap was crucial for the identification of naringenin glutation, 4-hydroxyhippuric acid, phloroglucinol and naringenin oxidated, which could not be identified in triple quadrupole system probably due to their low concentration.

5. References

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Acknowledgments

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9th World meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology. Lisbon, Portugal

8.2.24. Comunicación 24. Póster

Título: High resolution LTQ-Orbitrap-MS improves the identification of polyphenols and their metabolites in grape extracts as functional ingredients and human urine.

Autores: **Miriam Martínez-Huélamo**, Anna Vallverdú-Queralt, Gemma Sasot, Mireia Roig, Jorge Regueiro, Ramón Estruch, Mercè Mercader-Martí, Rosa M. Lamuela-Raventós.

Congreso: Oxygen Club of California World Congress. Davis, California, EEUU, 2014.

High resolution LTQ-Orbitrap-MS improves the identification of polyphenols and their metabolites in grape extracts as functional ingredients and human urine

Miriam Martínez-Huélamo[1,2], Anna Vallverdú-Queralt[1,2], Gemma Sasot[1], Mireia Roig[1], Jorge Regueiro[3], Ramón Estruch[2,4], Mercè Mercader-Martí[5], Rosa M. Lamuela-Raventós[1,2]

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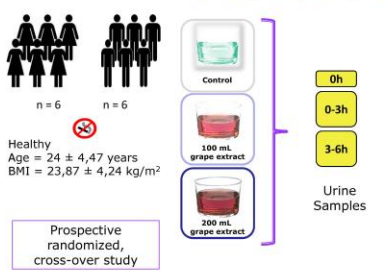
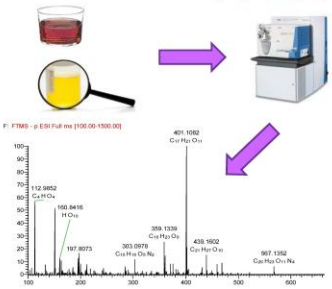
INTRODUCTION

Grape pomace contains many polyphenols with biological activities which may be used as functional ingredients for new or functional foods. However, an exhaustive identification of the polyphenols present in this functional ingredient, grape extract, and in biological samples obtained after the consumption of this novel food is necessary. The application of high resolution mass spectrometry (HR-MS) allows the identification of phenolics using accurate mass measurements.

STUDY DESIGN



IDENTIFICATION



RESULTS

Table 1. Phenolic compounds identified in the grape pomace

Compounds	Rt (min)	Accurate Mass [M-H]⁻	Fragments m/z (% intensity)	Error (mDa)	Molecular formula
Procyanidin trimer type B isomer 1	1.50	865.1985	695 (100), 739 (70), 577 (65), 407 (40), 287 (20)	4	$C_{45}H_{38}O_{18}$
Gallic acid	1.57	169.0142	125 (100)	1.1	$C_7H_6O_5$
Galloylglucose	1.6	331.0665	169 (100), 125 (10)	3.1	$C_{13}H_{16}O_{10}$
Protocatechuic acid-O-hexoside	1.86	315.0721	153 (100), 109 (20)	2.2	$C_{13}H_{16}O_9$
Genistic acid	2.70	153.0188	123 (100), 109 (10)	0.4	$C_7H_6O_5$
Protocatechuic acid	2.79	153.0193	153 (40), 109 (90)	0.9	$C_7H_6O_4$
Methyl gallate	3.09	183.0293	139 (100), 95 (40)	1.3	$C_8H_8O_5$
Caftaric acid	3.22	311.0408	149 (100)	2	$C_{13}H_{12}O_9$
Procyanidin dimer type B isomer 1	3.40	577.1346	425 (100), 451 (60), 407 (50), 559(30), 289 (30)	2.9	$C_{30}H_{26}O_{12}$
Caffeic acid-C-hexoside 1	3.54	341.0877	281 (90), 251 (100), 221 (40), 179 (60), 135 (10)	2.1	$C_{15}H_{18}O_9$
Procyanidin dimer type B isomer 2	4.09	577.1346	425 (100), 451 (60), 407 (50), 559(30), 289 (30)	2.9	$C_{30}H_{26}O_{12}$
Procyanidin trimer type B monogalloylate 1	4.16	1,017.2034	729 (100), 891 (80), 865 (60), 999 (50), 407 (30)	6.4	$C_{52}H_{42}O_{21}$
Caffeic acid-C-hexoside 2	4.19	341.0877	281 (90), 251 (100), 221 (40), 179 (60), 135 (10)	2	$C_{15}H_{18}O_9$
Coumaric-O-hexoside	4.36	325.0928	163 (100), 119 (12)	1.2	$C_{15}H_{18}O_8$
Catechin	4.43	289.0718	245 (100)	1.7	$C_{15}H_{14}O_6$
p-Hydroxybenzoic acid	4.49	137.0244	93 (100)	0.7	$C_7H_6O_3$
Procyanidin trimer type B isomer 2	4.75	865.1985	695 (100), 739 (70), 577 (65), 407 (40), 287 (20)	4	$C_{45}H_{38}O_{18}$
Procyanidin trimer type B isomer 3	5.30	865.1985	695 (100), 739 (70), 577 (65), 407 (40), 287 (20)	4	$C_{45}H_{38}O_{18}$
Procyanidin trimer type B monogalloylate 2	5.60	1,017.2034	729 (100), 891 (80), 865 (60), 999 (50), 407 (30)	6.4	$C_{52}H_{42}O_{21}$
Caffeic acid	5.72	179.0349	135 (100)	1.1	$C_9H_8O_4$
Procyanidin trimer type B monogalloylate 3	6.04	1,017.2034	729 (100), 891 (80), 865 (60), 999 (50), 407 (30)	6.4	$C_{52}H_{42}O_{21}$
Epicatechin	6.47	289.0718	245 (100)	1.9	$C_{15}H_{14}O_6$
Eriodictyol-O-hexoside	6.64	449.1089	287 (100), 259 (40), 269 (38)	2.6	$C_{21}H_{22}O_{11}$
Procyanidin dimer type B monogalloylate 1	6.85	729.1461	711 (20), 577 (80), 559 (60), 407 (100), 289 (40)	3.8	$C_{37}H_{30}O_{16}$
Procyanidin trimer type B isomer 4	7.29	865.1985	695 (100), 739 (70), 577 (65), 407 (40), 287 (20)	4	$C_{45}H_{38}O_{18}$
Procyanidin dimer type B monogalloylate 2	7.83	729.1461	711 (20), 577 (80), 559 (60), 407 (100), 289 (40)	3.8	$C_{37}H_{30}O_{16}$
Coumaric acid	8.24	163.0400	119 (100)	0.9	$C_9H_8O_3$
Myricetin-O-hexoside	8.49	479.0826	316 (100), 317 (50)	2.2	$C_{21}H_{20}O_{13}$
Kaempferol-3-O-rutinoside	8.79	593.1511	285 (100)	1.6	$C_{27}H_{30}O_{15}$
Ferulic acid	9.17	193.0506	178 (70), 149 (100), 134 (50)	1.2	$C_{10}H_{10}O_4$
Ploeid acid isomer 1	9.50	389.1241	227 (100), 193 (80), 341 (70)	2	$C_{20}H_{22}O_8$
Procyanidin dimer type B digalloylated	9.53	881.1520	729 (100), 711 (30) , 559 (20), 441 (10), 407 (20), 289 (5)	2.1	$C_{44}H_{34}O_{18}$
Epicatechin-O-gallate	9.8	441.0822	289 (100), 331 (20), 271 (19), 169 (30)	1.8	$C_{22}H_{18}O_{10}$
Kaempferol-3-O-glucoside	10.20	447.0932	285 (100)	2.4	$C_{21}H_{20}O_{11}$
Quercetin-O-hexoside	10.20	463.0881	301 (100), 293 (40), 271 (15), 169 (10)	2.2	$C_{21}H_{20}O_{12}$
Procyanidin trimer type B monogalloylate 4	10.26	1,017.2034	729 (100), 891 (80), 865 (60), 999 (50), 407 (30)	6.4	$C_{52}H_{42}O_{21}$
Quercetin-O-rhamnoside	11.67	447.0932	301 (100), 284 (20), 285 (20)	1.9	$C_{21}H_{20}O_{11}$
Ploeid acid isomer 2	12.23	389.1241	227 (100)	2.2	$C_{20}H_{22}O_8$
Quercetin	16.30	301.0353	179 (100), 151 (90), 107 (10)	1.5	$C_{15}H_{10}O_7$
Resveratrol	16.58	227.0713	185 (100), 143 (30)	2.5	$C_{14}H_{12}O_3$
Naringenin	17.88	271.0611	151 (100), 177 (20), 119 (10)	1.7	$C_{15}H_{12}O_5$

Table 2. Phenolic compounds identified in urine after the administration of the grape pomace

Compound	Rt (min)	Accurate Mass [M-H]⁻	Fragments m/z (% intensity)	Error (mDa)	Molecular formula
Pyrogallol	1.08/1.38	125.0246	97 (100)	0.4	$C_6H_6O_3$
2-*O*-Methylpyrogallol	4.99	139.03987	124 (100)	0.2	$C_7H_8O_3$
Gallic acid	1.53	169.01396	125 (100)	0.3	$C_7H_6O_5$
O-Methylgallic acid	1.81/3.15/3.44	183.02962	168 (100), 139 (70), 124 (20)	0.3	$C_8H_8O_5$
Hydroxyhippuric acid	2.47/3.11	194.04587	180 (100)	0.3	$C_9H_9NO_4$
3,4-*O*-Dimethylgallic acid / Gallic acid ethyl ester	1.69/2.62/5.91	197.0451	137 (100), 182 (10)	0.4	$C_9H_{10}O_5$
5-(3,4-Dihydroxyphenyl)-γ-valerolactone	6.96/9.09	207.06627	181(100), 179 (40), 162 (40), 161(40)	0.4	$C_{11}H_{12}O_4$
4-*O*-Methylgallic acid-3-*O*-sulfate	2.67/3.17	262.98642	219 (40), 183 (100)	0.3	$C_8H_8O_8S$
Dihydroferulic acid 4-sulfate	5.42/5.58/7.33/12.26	273.02222	195 (100), 177 (90)	0.9	$C_{10}H_{10}O_7S$
Catechin	5.56	289.0712	245 (100), 205 (60)	0.6	$C_{15}H_{14}O_6$
Epicatequin	7.08	289.07096	245 (100), 207 (70)	0.9	$C_{15}H_{14}O_6$
Quercetin	11.46	301.03537	265 (100), 243 (65)	0.0	$C_{15}H_{10}O_7$
Dihydroquercetin	11.53	303.5319	223 (100)	2.2	$C_{15}H_{12}O_7$
trans-Resveratrol-3-*O*-sulfate	12.97	307.02817	227 (100)	0.3	$C_{14}H_{12}O_6S$
trans-Resveratrol-4'-*O*-sulfate	14.41	307.02817	227 (100)	0.6	$C_{14}H_{12}O_6S$
cis-Resveratrol-3-*O*-sulfate	17.21	307.02817	227 (100)	0.9	$C_{14}H_{12}O_6S$
cis-Resveratrol-4'-*O*-sulfate	18.68	307.02817	227 (100)		$C_{14}H_{12}O_6S$
Protocatechuic-*O*-glucoside	1.84	315.07147	153 (100)	0.7	$C_{13}H_{16}O_9$
Dihydrocaffeic acid 3-*O*-glucuronide	9.53	357.08270	181 (100), 113 (45)	0.9	$C_{15}H_{18}O_{10}$
Catechin sulfate	5.56	369.02795	289 (100), 245 (10)	0.6	$C_{15}H_{14}O_9S$
Epicatechin sulfate	7.08	369.02798	290 (100), 245 (10)	0.6	$C_{15}H_{14}O_9S$
Dihydroferulic acid 4-*O*-glucuronide	4.73/9.89/10.35	371.0983	195 (60), 175 (60), 113 (100)	0.1	$C_{16}H_{20}O_{10}$
Methyl-catechin sulfate	6.56	383.04391	303 (100), 217 (80), 137 (30)	0.3	$C_{16}H_{16}O_9S$
Methyl-epicatechin sulfate	7.12/8.19/8.76	383.04364	303 (100), 243 (30), 217 (60), 137 (30)	0.6	$C_{16}H_{16}O_9S$
Epigallocatechin-*O*-glucuronide	11.7	481.09874	305 (100)	1.7	$C_{21}H_{22}O_{13}$
Catechin glucuronide	4.17	465.10297	289 (100)	0.9	$C_{21}H_{22}O_{12}$
Epicatechin glucuronide	5.84	465.10355	289 (100), 245 (10)	0.3	$C_{21}H_{22}O_{12}$
Methyl-catechin glucuronide	3.85	479.11971	303 (100)	0.8	$C_{22}H_{24}O_{12}$
3'-*O*-Methyl-epicatechin-7-*O*-glucuronide	5.73	479.1189	303 (100)	0.6	$C_{22}H_{24}O_{12}$
4'-*O*-Methyl-epicatechin-3'-*O*-glucuronide	11.76	479.11633	461(20), 303 (100)	3.2	$C_{22}H_{24}O_{12}$
4'-*O*-Methyl-epigallocatechin-*O*-glucuronide	8.98	495.11439	319 (100)		$C_{22}H_{24}O_{13}$
Catechin glucuronide sulfate	2.52	545.06042	465 (45), 369 (100)	0.2	$C_{21}H_{22}O_{15}S$
Epicatechin glucuronide sulfate	5.10	545.06073	465 (45), 369 (100)	0.1	$C_{21}H_{22}O_{15}S$
trans-Resveratrol-3-*O*-glucuronide	12.49	403.10344	227 (20), 175 (100)	0.2	$C_{20}H_{20}O_9$
trans-Resveratrol-4'-*O*-glucuronide	15.45	403.10344	227 (100)	0.8	$C_{20}H_{20}O_9$
cis-Resveratrol-3-*O*-glucuronide	17.62	403.10344	227 (90), 175 (100)	1.4	$C_{20}H_{20}O_9$
cis-Resveratrol-4'-*O*-glucuronide	18.79	403.10344	341 (100), 227 (30)	1.6	$C_{20}H_{20}O_9$
Dihydroresveratrol-3-*O*-sulfate	12.87	309.04382	229 (100), 123 (15)	0.8	$C_{14}H_{14}O_6S$
Dihydroresveratrol-4-*O*-sulfate	11.55	309.04382	229 (100), 123 (15)	1	$C_{14}H_{14}O_6S$
Dihydroresveratrol-glucuronide-sulfate	8.88	485.07598	309 (100), 233(20), 175 (15)	0.4	$C_{20}H_{22}O_{12}S$
3-Hydroxybenzoic acid	2.99	137.02441	93(100)	0.33	$C_7H_6O_3$
4-Hydroxybenzoic acid	4.66	137.02441	93(100)	0.43	$C_7H_6O_3$
2-Hydroxyphenylacetic acid	4.9	151.04006	107 (100)	0.4	$C_8H_8O_3$
3-Hydroxyphenylacetic acid	7.05	151.04006	107 (100)	0.3	$C_8H_8O_3$
2-Hydroxy-2-phenylacetic acid	7.1	151.04006	107 (100)	0.4	$C_8H_8O_3$
4-Hydroxyphenylacetic acid	10.3	151.04006	107 (100)	0.5	$C_8H_8O_3$
2-Hydroxy-2-phenylacetic acid	10.3	151.04006	107 (70), 93 (100)	0.3	$C_8H_8O_3$
3,4-Dihydroxybenzoic acid (Protocatechuic acid)	2.8	153.01932	109 (100)	0.5	$C_7H_6O_4$
3,4-Dihydroxyphenylacetic acid (Homoprotocatechuic acid)	2.43	167.03497	123 (100)	0.4	$C_8H_8O_4$
Hippuric acid	4.92	178.05096	134 (100)	0.01	$C_9H_9NO_3$
Caffeic acid	3.18	179.03497	135 (100)	2.2	$C_9H_8O_4$
3,4-Dihydroxyphenylpropionic acid (Dihydrocaffeic acid)	3.5	181.05062	163 (100), 135 (40)	0.6	$C_9H_{10}O_4$
Homovanillic acid	3.52	181.05062	137 (100)	0.5	$C_9H_{10}O_4$
4-Hydroxy-5-(3,4-dihydroxyphenyl)valeric acid	13.14	225.07683	207 (30), 181 (40), 137 (100), 83 (20)	0.5	$C_{11}H_{14}O_4$
Vanillic acid 4-sulfate	3.36	246.99179	203 (80), 167 (100), 123 (100)	0.05	$C_8H_8O_6S$
Caffeic acid 3-sulfate	4.56	258.99179	179 (100)	0.9	$C_9H_8O_6S$
Caffeic acid 4-sulfate	6.03	258.99179	179 (100)	0.8	$C_9H_8O_6S$
Homovanillic acid 4-sulfate	3.93	261.00744	181 (100)	0.4	$C_9H_{10}O_6S$
Isoferulic acid 3-sulfate	6.32	273.00677	229 (20), 193 (100)	0.7	$C_{10}H_{10}O_6S$
Ferulic acid 4-sulfate	6.36	273.00744	193 (100)	0.9	$C_{10}H_{10}O_6S$
Epigallocatechin	6.42	305.06666	225 (100)	2.2	$C_{15}H_{14}O_7$
p-Coumaric acid	8.44	163.03969	119 (100)	0.4	$C_9H_8O_3$
3-(3-Hydroxyphenyl)propionic acid / 3-(4-Hydroxyphenyl)propionic acid	8.78	165.05571	147 (20), 121 (100)	0.4	$C_9H_{10}O_3$
3,5-Dimethoxy-4-hydroxybenzoic acid (Syringic acid)	1.69	197.04553	137 (100)	0.3	$C_9H_{10}O_5$

CONCLUSIONS

In conclusion, grape extract is a rich source of bioative compounds since 41 phenolic compounds were identified in the grape extract and 64 metabolites in urine after the administration of the grape pomace by HR-MS that enhances the identification of large variety of phenolic compounds and their metabolites with very good mass accuracies for all molecular ions.

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Boto-Ordóñez, *et al.* ***J Agric Food Chem*** 2013; 61(38):9166-75

8.2.25. Comunicación 25. Póster

Título: Early changes in malondialdehyde and lipid profile after the consumption of tomato products suggest an improvement on oxidative stress.

Autores: Palmira Valderas-Martínez, Rosa Casas, Gemma Chiva-Blanch, Sara Arranz, **Miriam Martínez-Huélamo**, Xavier Torrado, Dolores Corella, Rosa M. Lamuela-Raventós, and Ramon Estruch.

Congreso: Oxygen Club of California World Congress. Davis, California, EEUU, 2014.

Early changes in malondialdehyde and lipid profile after the consumption of tomato products suggest an improvement on oxidative stress

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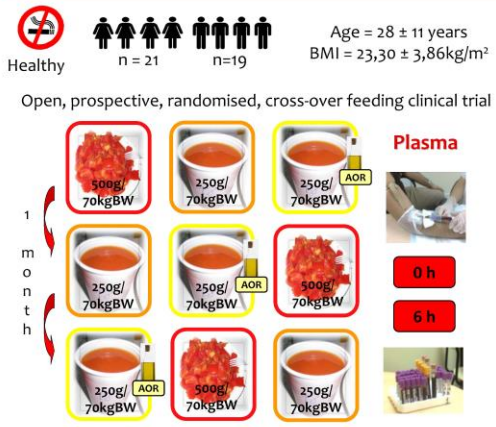
INTRODUCTION

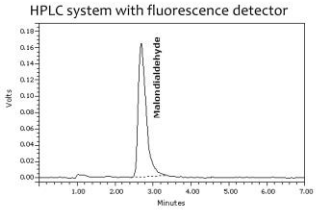
Malondialdehyde (MDA) is a biomarker of lipid peroxidation and progression of atherosclerosis that correlates with oxidative stress, one of the main mechanisms responsible of cardiovascular lesions. Between different foods, epidemiological studies have pointed out that tomato intake may reduce cardiovascular risk due to its functional components such as carotenoids, vitamin C and polyphenols. Some researchers have suggested that cooking and addition of a fatty matrix may increase the bioavailability of bioactive compounds. However, to our knowledge, no previous studies have analyzed the effects of a single dose of raw and processed tomato products on oxidative status.

OBJECTIVE

The aim of this study was to examine the postprandial effects of a single dose of raw tomato (RT), tomato sauce (TS) and tomato sauce with refined olive oil (TSOO) on oxidative stress parameters.

METHOD AND MATERIAL



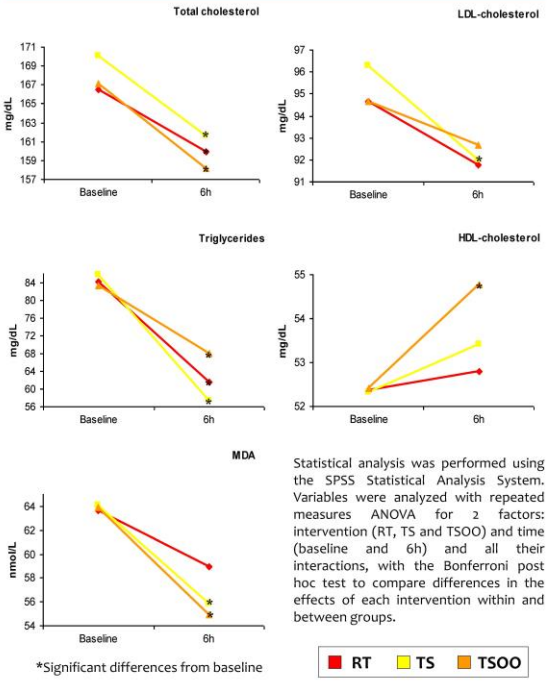


LIPID PROFILE

Total cholesterol
Triglycerides
by enzymatic procedures

HDL cholesterol after precipitation with phosphotungstic acid and magnesium chloride

RESULTS



Statistical analysis was performed using the SPSS Statistical Analysis System. Variables were analyzed with repeated measures ANOVA for 2 factors: intervention (RT, TS and TSOO) and time (baseline and 6h) and all their interactions, with the Bonferroni post hoc test to compare differences in the effects of each intervention within and between groups.

*Significant differences from baseline

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CONCLUSIONS

We concluded that tomato intake have beneficial effects on oxidative status, especially when it is consumed cooked and/or enriched with olive oil, possibly due to the better bioavailability of its functional compounds in a fatty matrix.

Oxygen Club of California World Congress 2014 May 7-10, 2014 University of California, Davis

8.2.26. Comunicación 26. Póster

Título: A novel high resolution LTQ-ORBITRAP-MS enhances the identification of polyphenols and their metabolites in a functional food made with grape extract, and in human urine.

Autores: Gemma Sasot, **Miriam Martínez-Huélamo**, Anna Vallverdú-Queralt, Mireia Roig, Jorge Regueiro, Ramón Estruch, Gemma Chiva-Blanch, Mercè Mercader-Martí, and Rosa M. Lamuela-Raventós.

Congreso: Segona Jornada de Recerca Enologia i Viticultura a Catalunya. Tarragona, Spain, 2014.

A novel high resolution LTQ-ORBITRAP-MS enhances the identification of polyphenols and their metabolites in a functional food made with grape extract, and in human urine

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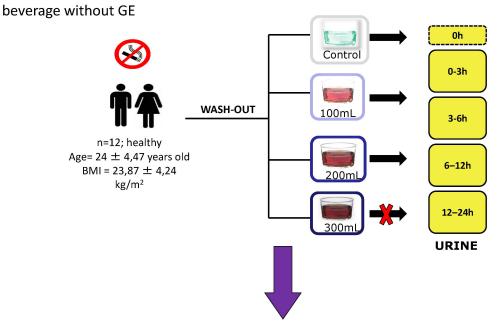
Introduction

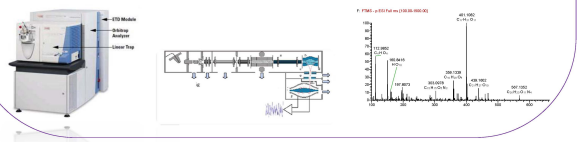
Grape pomaces are known to be a rich source of polyphenols with biological activity, which may be used as functional ingredients for the development of new interesting products. Numerous studies have reported that bioactive compounds may act through multiple mechanisms, like antioxidant, anticarcinogen or anti-inflammatory (1).
However, an exhaustive identification of the polyphenols present in this functional compound, grape extract (GE) and, in biological samples obtained after the consumption of this novel food is required. In last years, there has been an increased interest in metabolites formed by the intestinal microbiota to understand the benefits derived from consumption (2). The application of high resolution mass spectrometry (HR-MS) allows the identification of phenolics using accurate mass measurements.

Study design and Method

In order to verify the oral absorption and metabolism of the polyphenols content, we performed a prospective randomized, cross-over study in 12 healthy volunteers with three interventions: 500 mL of a functional beverage enriched with 100 mL of grape extract, 500 mL of the same beverage with 200 mL of GE and 500 mL of a control beverage without GE



LTQ-Orbitrap-MS enabled the identification of phenolics in GE and in human urine collected



Results

Compound	R_t (min)	Accurate mass [M-H]	MS/MS ions (%intensity)	mDa	Molecular formula
Pyrogallol	1,08 / 1,38	125,02406	97 (100)	0,4	$C_6H_6O_3$
2-O-Methylpyrogallol	4,99	139,03987	124 (100)	0,2	$C_7H_8O_3$
Gallic acid	1,53	169,01396	125 (100)	0,3	$C_7H_6O_5$
O-Methylgallic acid	1,81 /3,15 / 3,44	183,02962	168 (100), 139 (70), 124 (20)	0,3	$C_8H_8O_5$
Hydroxyhippuric acid	2,47 / 3,11	194,04587	150 (100), 106 (100)	0,3	$C_9H_9NO_4$
3,4-O-Dimethylgallic acid / Gallic acid ethyl ester	1,68 / 2,62 / 5,91	197,0451	182 (100), 153 (40), 137 (100)	0,4	$C_9H_{10}O_5$
5-(3,4-Dihydroxyphenyl)-γ-valerolactone	6,96 / 9,09	207,06627	181 (100), 179 (100), 163 (100), 141 (40)	0,4	$C_{11}H_{12}O_4$
4-O-Methylgallic acid-3-O-sulfate	2,67 / 3,17	262,98642	219 (45), 183 (100)	0,3	$C_8H_8O_8S$
Dihydroferulic acid 4-sulfate	5,42 / 5,58 / 7,33/ 12,25	271,02222	195 (100), 177 (90)	0,9	$C_{10}H_{12}O_7S$
Catechin	5,56	289,0712	245 (100), 205 (60)	0,6	$C_{15}H_{14}O_6$
Epicatequin	7,08	289,07086	245 (100), 207 (70)	0,9	$C_{15}H_{14}O_6$
Quercetin	11,46	301,03537	265 (100), 243 (65)	0,0	$C_{15}H_{10}O_7$
Dihydroquercetin	11,53	303,5319	223 (100)	2,2	$C_{15}H_{12}O_7$
trans-Resveratrol-3-O-sulfate	12,07	307,02817	227 (100)	0,3	$C_{14}H_{12}O_6S$
trans-Resveratrol-4-O-sulfate	14,41	307,02817	227 (100)	0,6	$C_{14}H_{12}O_6S$
cis-Resveratrol-3-O-sulfate	17,21	307,02817	227 (100)	0,9	$C_{14}H_{12}O_6S$
cis-Resveratrol-4-O-sulfate	18,68	307,02817	227 (100)		$C_{14}H_{12}O_6S$
Protocatechuic-O-glucoside	1,84	315,07147	153 (100)	0,7	$C_{13}H_{16}O_9$
Dihydrocaffeic acid 3-O-glucuronide	9,53	357,08270	181 (100), 113 (45)	0,9	$C_{15}H_{18}O_{10}$
Catechin sulfate	5,56	369,02795	289 (100), 245 (10)	0,6	$C_{15}H_{14}O_9S$
Epicatechin sulfate	7,08	369,02798	290 (100), 245 (10)	0,6	$C_{15}H_{14}O_9S$
Dihydroferulic acid 4-O-glucuronide	4,73 / 9,89 / 10,35	371,09778	195 (60), 175 (60), 113 (100)	0,1	$C_{16}H_{20}O_{10}$
Methyl-catechin sulfate	6,56	383,04391	303 (100), 217 (80), 137 (30)	0,3	$C_{16}H_{16}O_9S$
Methyl-epicatechin sulfate	7,12 / 8,19 / 8,76	383,04364	303 (100), 245 (30), 217 (60), 137 (30)	0,6	$C_{16}H_{16}O_9S$
Epigallocatechin O-glucuronide	11,7	481,09874	305 (100)	1,7	$C_{21}H_{22}O_{13}$
Catechin glucuronide	4,17	465,10297	289 (100)	0,9	$C_{21}H_{22}O_{12}$
Epicatechin glucuronide	5,84	465,10355	289 (100), 245 (10)	0,3	$C_{21}H_{22}O_{12}$
Methyl-catechin glucuronide	3,85	479,11871	303 (100)	0,8	$C_{22}H_{24}O_{12}$
3'-O-Methyl-epicatechin-7-O-glucuronide	5,73	479,1189	303 (100)	0,6	$C_{22}H_{24}O_{12}$
4'-O-Methyl-epicatechin-3'-O-glucuronide	6,59 / 11,176	479,11902	461 (20), 303 (100)	0,5	$C_{22}H_{24}O_{12}$
4'-O-Methyl-epigallocatechin-O-glucuronide	8,98	495,11439	319 (100)		$C_{22}H_{24}O_{13}$
Catechin glucuronide sulfate	2,52	545,06042	465 (45), 369 (100)	0,2	$C_{21}H_{22}O_{18}S$
Epicatechin glucuronide sulfate	5,10	545,06073	465 (45), 369 (100)	0,1	$C_{21}H_{22}O_{18}S$
trans-Resveratrol-3-O-glucuronide	12,49	405,10344	227 (20), 175 (100)	0,2	$C_{20}H_{18}O_9$
trans-Resveratrol-4'-O-glucuronide	15,45	405,10344	227 (100)	0,8	$C_{20}H_{18}O_9$
cis-Resveratrol-3-O-glucuronide	17,62	405,10344	227 (90), 175 (100)	1,4	$C_{20}H_{18}O_9$
cis-Resveratrol-4'-O-glucuronide	18,79	405,10344	341 (100), 227 (30)	1,6	$C_{20}H_{18}O_9$
Dihydroresveratrol-3-O-sulfate	12,87	309,04382	229 (100), 123 (15)	0,8	$C_{14}H_{14}O_6S$
Dihydroresveratrol-4-O-sulfate	11,55	309,04382	229 (100), 123 (15)	1	$C_{14}H_{14}O_6S$
Dihydroresveratrol glucuronide-sulfate	8,88	485,07910	309 (100), 233(20), 175 (15)	0,4	$C_{20}H_{22}O_{12}S$
3-Hydroxybenzoic acid	2,99	137,02441	93(100)	0,33	$C_7H_6O_3$
4-Hydroxybenzoic acid	4,66	137,02441	93(100)	0,43	$C_7H_6O_3$
2-Hydroxyphenylacetic acid	4,9	151,04006	107 (100)	0,4	$C_8H_8O_3$
3-Hydroxyphenylacetic acid	7,05	151,04006	107 (100)	0,3	$C_8H_8O_3$
2-Hydroxy-2-phenylacetic acid	7,1	151,04006	107 (100)	0,4	$C_8H_8O_3$
4-Hydroxyphenylacetic acid	10,3	151,04006	107 (100)	0,5	$C_8H_8O_3$
2-Hydroxy-2-phenylacetic acid	10,3	151,04006	107 (70), 93 (100)	0,3	$C_8H_8O_3$
3,4-Dihydroxybenzoic acid (Protocatechuic acid)	2,8	153,01932	109 (100)	0,5	$C_7H_6O_4$
3,4-Dihydroxyphenylacetic acid (Homoprotocatechuic acid)	2,43	167,03497	123 (100)	0,4	$C_8H_8O_4$
Hispanic acid	4,92	178,05036	134 (100)	0,01	$C_9H_9NO_3$
Caffeic acid	3,18	179,03497	135 (100)	2,2	$C_9H_8O_4$
3,4-Dihydroxyphenylpropionic acid (Dihydrocaffeic acid)	3,5	181,05062	163 (100), 135 (40)	0,6	$C_9H_{10}O_4$
Homovanillic acid	3,52	181,05062	137 (100)	0,5	$C_9H_{10}O_4$
4-Hydroxy-5-(3,4-dihydroxyphenyl)valeric acid	13,14	225,07683	207 (30), 181 (40), 137 (100), 83 (20)	0,5	$C_{11}H_{14}O_5$
Vanillic acid 4-sulfate	3,36	246,99179	203 (80), 167 (100), 123 (100)	0,05	$C_8H_8O_7S$
Caffeic acid 3-sulfate	4,56	258,99179	179 (100)	0,9	$C_9H_8O_7S$
Caffeic acid 4-sulfate	6,03	258,99179	179 (100)	0,8	$C_9H_8O_7S$
Homovanillic acid 4-sulfate	3,93	261,00744	181 (100)	0,4	$C_9H_{10}O_7S$
Isoferulic acid 3-sulfate	6,32	273,00677	229 (20), 193 (100)	0,7	$C_{10}H_{10}O_7S$
Ferulic acid 4-sulfate	6,36	273,00744	193 (100)	0,9	$C_{10}H_{10}O_7S$
Epigallocatechin	6,42	305,06666	225 (100)	2,2	$C_{15}H_{14}O_7$
p-Coumaric acid	8,44 / 8,96	163,03969	119 (100)	0,4	$C_9H_8O_3$
3-(3-Hydroxyphenyl)propionic acid / 3-(4-Hydroxyphenyl)propionic acid	8,78	165,05571	147 (20), 121 (100)	0,4	$C_9H_{10}O_3$
3,5-Dimethoxy-4-hydroxybenzoic acid (Syringic acid)	1,69	197,04513	137 (100)	0,3	$C_9H_{10}O_5$

Table 1. List of compounds identified in urine samples

Compound	R_t (min)	Accurate mass [M-H]⁻	MS/MS ions (%intensity)	mDa	Molecular formula
Procyanidin trimer type B 1	1,50	865,1985	695 (100), 739 (70), 577 (65), 407 (40), 287 (20)	4	$C_{45}H_{38}O_{18}$
Gallic acid	1,57	169,0142	125 (100)	1,1	$C_7H_6O_5$
Galloylglucose	1,6	331,0665	169 (100), 125 (10)	3,1	$C_{13}H_{16}O_{10}$
Protocatechuic acid-O-hexoside	1,86	315,0721	153 (100), 109 (20)	2,2	$C_{13}H_{16}O_9$
Gensitic acid	2,70	153,0188	123 (100), 109 (10)	0,4	$C_7H_6O_4$
Protocatechuic acid	2,70	153,0193	153 (40), 109 (90)	0,9	$C_7H_6O_4$
Methyl gallate	3,09	183,0293	139 (100), 95 (40)	1,3	$C_8H_8O_5$
Caftaric acid	3,22	311,0408	149 (100)	2	$C_{13}H_{12}O_9$
Procyanidin dimer type B 1	3,40	577,1346	425 (100), 451 (60), 407 (50), 559(30), 289 (30)	2,9	$C_{30}H_{26}O_{12}$
Caffeic acid-C-hexoside 1	3,54	341,0877	281 (90), 251 (100), 221 (40), 179 (60), 135 (10)	2,1	$C_{15}H_{18}O_9$
Procyanidin dimer type B 2	4,09	577,1346	425 (100), 451 (60), 407 (50), 559(30), 289 (30)	2,9	$C_{30}H_{26}O_{12}$
Procyanidin trimer type B monogallloylate 1	4,16	1.017,2034	729 (100), 891 (80), 865 (60), 999 (50), 407 (30)	6,4	$C_{51}H_{46}O_{23}$
Caffeic acid-C-hexoside 2	4,19	341,0877	281 (90), 251 (100), 221 (40), 179 (60), 135 (10)	2	$C_{15}H_{18}O_9$
Coumaric-O-hexoside	4,36	325,0928	163 (100), 119 (12)	1,2	$C_{15}H_{18}O_8$
Catechin	4,43	289,0718	245 (100)	1,7	$C_{15}H_{14}O_6$
p-hydroxybenzoic acid	4,49	137,0244	93 (100)	0,7	$C_7H_6O_3$
Procyanidin trimer type B 2	4,75	865,1985	695 (100), 739 (70), 577 (65), 407 (40), 287 (20)	4	$C_{45}H_{38}O_{18}$
Procyanidin trimer type B 3	5,50	865,1985	695 (100), 739 (70), 577 (65), 407 (40), 287 (20)	4	$C_{45}H_{38}O_{18}$
Procyanidin trimer type B monogallloylate 2	5,60	1.017,2034	729 (100), 891 (80), 865 (60), 999 (50), 407 (30)	6,4	$C_{51}H_{46}O_{23}$
Caffeic acid	5,72	179,0349	135 (100)	1,1	$C_9H_8O_4$
Procyanidin trimer type B monogallloylate 3	6,04	1.017,2034	729 (100), 891 (80), 865 (60), 999 (50), 407 (30)	6,4	$C_{51}H_{46}O_{23}$
Epicatechin	6,47	289,0718	245 (100)	1,9	$C_{15}H_{14}O_6$
Eriodictyol-O-hexoside	6,64	449,1089	287 (100), 259 (40), 269 (38)	2,6	$C_{21}H_{22}O_{11}$
Procyanidin dimer type B monogallloylate 1	6,85	729,1461	711 (20), 577 (80), 559 (60), 407 (100), 289 (40)	3,8	$C_{37}H_{30}O_{16}$
Procyanidin trimer type B 4	7,29	865,1985	695 (100), 739 (70), 577 (65), 407 (40), 287 (20)	4	$C_{45}H_{38}O_{18}$
Procyanidin dimer type B monogallloylate 2	7,83	729,1461	711 (20), 577 (80), 559 (60), 407 (100), 289 (40)	3,8	$C_{37}H_{30}O_{16}$
Coumaric acid	8,24	163,0400	119 (100)	0,9	$C_9H_8O_3$
Myrectin-O-hexoside	8,49	479,0826	316 (100), 317 (50)	2,2	$C_{21}H_{20}O_{13}$
Kaempferol-3-O-rutinoside	8,79	593,1511	285 (100)	1,6	$C_{27}H_{30}O_{15}$
Ferulic acid	9,17	193,0506	178 (70), 149 (100), 134 (50)	1,2	$C_{10}H_{10}O_4$
Piceid acid 1	9,50	389,1241	227 (100), 193 (80), 341 (70)	2	$C_{20}H_{22}O_8$
Procyanidin dimer type B digalloylated	9,53	881,1520	729 (100), 711 (30) , 559 (20), 441 (10), 407 (20), 289 (5)	2,1	$C_{44}H_{34}O_{18}$
Epicatechin-O-gallate	9,8	441,0822	289 (100), 331 (20), 271 (19), 169 (30)	1,8	$C_{22}H_{18}O_{10}$
Kaempferol-3-O-glucoside	10,20	447,0932	285 (100)	2,4	$C_{21}H_{20}O_{11}$
Quercetin-O-hexoside	10,20	463,0881	301 (100), 293 (40), 271 (15), 169 (10)	2,2	$C_{21}H_{20}O_{12}$
Procyanidin trimer type B monogallloylate 4	10,26	1.017,2034	729 (100), 891 (80), 865 (60), 999 (50), 407 (30)	6,4	$C_{51}H_{46}O_{23}$
Quercetin-O-rhamanoside	11,67	447,0932	301 (100), 284 (20), 285 (20)	1,9	$C_{21}H_{20}O_{11}$
Piceid acid 2	12,23	389,1241	227 (100)	2,2	$C_{20}H_{22}O_8$
Quercetin	16,30	301,0353	179 (100), 151 (90), 107 (10)	1,5	$C_{15}H_{10}O_7$
Resveratrol	16,58	227,0713	185 (100), 143 (30)	2,5	$C_{14}H_{12}O_3$
Naringenin	17,88	271,0611	151 (100), 177 (20), 119 (10)	1,7	$C_{15}H_{12}O_5$

Table 2. List of compounds identified in grape extract

Conclusions

Forty one polyphenols were identified in extract mainly procyanidins, phenolic acids and flavonols.
In urine samples, more than sixty metabolites of phenolic compounds were detected including microbiota metabolites, glucuronides and sulfate derivatives.
In conclusion, the LTQ-Orbitrap-MS enhances the identification of a large variety of phenolic compounds from functional ingredients and their metabolites originated in the human body.

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2ª Jornada de Recerca en Enologia i Viticultura a Catalunya

8.2.27. Comunicación 27. Póster

Título: Study of the synergic effect of naringenin and quercetin in their permeability and metabolic fate.

Autores: Naiara Orrego-Lagarón, **Miriam Martínez-Huélamo**, Rosa M. Lamuela-Raventós, and Elvira Escribano-Ferrer.

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Study of the synergic effect of naringenin and quercetin in their permeability and metabolic fate

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1. Introduction

Naringenin (NAR) and quercetin (Q) are two of the major flavonoids in tomato with reported health-promoting properties against many diseases, in particular in cancer and oxidative stress-related chronic diseases (Lee Hilz et al. 2008), cardio protective and lipid metabolism effects (Erlund et al. 2004, Boots et al. 2008) and antioxidant, anti-inflammatory and anticarcinogenic activities (Xu et al. 2009). These benefits in human health are assumed to be related with their oral bioavailability. Low bioavailabilities of both flavonoids have been reported: 10% for NAR (Xu et al. 2009) and 0.04-0.1% for Q (Boonpawa et al. 2014) after oral administration. Bioavailability depends basically on the permeability of the compound but also on how extensive is the intestinal and hepatic metabolism, among others. Moreover, previous studies reported that flavonoids could interact together and these interactions could exhibit several properties, hence synergistic antioxidative properties or efficient competitions between them for metabolic enzymes (Silberberg et al. 2005).

Aim: study the intestinal permeability of NAR and Q, and to study the influence of their co-administration in their absorption and intestinal first pass metabolism.

2.Methods

Analytical methods: HPLC-MS/MS

- **Mobile phases** - A : H2O with 0.1% (v/v) formic acid.
  - B : Acetonitrile 0.1% (v/v) formic acid.
- **Method:** increasing linear gradient (v/v) of B.
- **Flow:** 600 µL min-1.
- **Turbo ion spray:** negative mode with the settings in Table 1.

Comp.	CV	GN (N2)	CG (N2)	DG (N2)	EP	DP	FP	CE
NAR	-4500	10 (u.a)	12 (u.a)	400ºC	- 11V	-50	-190	-30
Q	-4500	10 (u.a)	12 (u.a)	400ºC	- 10V	-50	-210	-20
NAR-GLU	-4500	10 (u.a)	12 (u.a)	400ºC	- 11V	-30	-200	-30
NAR-SULF	-4500	10 (u.a)	12 (u.a)	400ºC	- 11V	-50	-190	-30
Q-GLU	-4500	10 (u.a)	12 (u.a)	400ºC	- 11V	-30	-200	-30
Q-SULF	-4500	10 (u.a)	12 (u.a)	400ºC	- 11V	-50	-210	-20
ISOH-GLU	-4500	10 (u.a)	12 (u.a)	400ºC	- 11V	-60	-210	-30
ISOH-SULF	-4500	10 (u.a)	12 (u.a)	400ºC	- 11V	-60	-210	-30

Table 1. Settings of turbo ion spray source. CV: Collisium voltage; EP: Entrance Potencial; DP: Declustering Potential; FP: Focusing Potential; CE: Collision Energy. NAR-GLU: Naringenin-glucuronide; NAR-SULF: Naringenin-sulfate; Q-GLU: Quercetin-glucuronide; Q-SULF: Quercetin-sulfate; ISOH-GLU: Isorhamnetin-glucuronide; ISOH-SULF: Isorhamnetin-sulfate

Stability studies

The stability of the compounds was tested in the perfusion solutions (pH 6.2) at 37ºC for 1, 2, and 3h. The concentrations at a range of times were compared using an ANOVA-test (α 0.05).

Perfusion experiments

- **Animals:** Male CD1 mice (30-35g) divided in 3 groups of mice according to the perfusion solution (Cin) :
  - **Group 1:** Perfusion solution 3.5 µg/mL of NAR .
  - **Group 2:** Perfusion solution 0.003 µg/mL of Q.
  - **Group 3:** Perfusion solution 3.5 µg/mL of NAR and 0.003 µg/mL of Q.

  Every isoosmotic solution contained 0.1 mg/mL of phenol red.
- **Previous conditions:** mice were fed during one week with a diet absent of polyphenols.
- **Anesthesia:** i.p. ketamine/xylacin (100 mg/kg ; 10 mg/kg, respectively).
- **Assay conditions:**
  - ☑ The biliary duct was tied.
  - ☑ A segment of small intestine was isolated and cannulated (L).
- **Flow:** 0.20 mL/min (0in).
- **Samples :**
  - ☑ Perfusion samples; collected at 10 min intervals for 60 min.
  - ☑ Blood sample; at 60 min with cardiac puncture.
  - ☑ Bile sample: the gall bladder was collected at the end of the assay.

An image of the surgical technique is shown in figure 1:

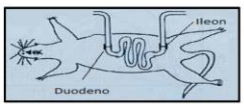

Figure 1. Intestinal surgical technique.

Data analysis

Effective permeability coefficients (Peff) and percentage of absorption (%abs) were calculated according to equations 1 and 2 respectively, after correcting the outlet concentration following the phenol red method (Zakeri-Milani 2007).

$$Peff = \frac{-\phi_{in}}{2\pi \cdot R \cdot L} \cdot Ln \frac{Cin}{Cout}$$

Equation 1

$$\%abs = \left[1 - e^{-\frac{2\pi \cdot R \cdot L}{\phi} \cdot Peff}\right] \cdot 100$$

Equation 2, where

ϕ_{in} is the flow rate, Cin and Cout.corr are the respective inlet and corrected outlet concentrations at steady state, R is radius of the intestine.

Area under the curve (AUC)

For the metabolites, the linear trapezoidal method was used to calculate the area under the lumen concentration curve (normalized by the inlet perfusion concentration of NAR and/or Q) from time zero until the last sample, using the WinNonlin® software v6.3 (2012 Certara, L.P.)

3.Results and discussion

- **Stability**: Q was stable for 3h (p= 0.129) and NAR was stable for 2h, time enough to perform the permeation experiments.
- **Permeability study:**
  - ☑ The permeability and % absorption of NAR did not change when administered alone or co-administered with Q.
  - ☑ Regarding Q, it showed a tendency to increase its permeability and percentage of absorption when administered together with NAR.

	Peff *10-4(cm/s)± SD	% Absorption	n
NAR Assay	7.80 ± 1.54	93.94 ± 5.90	4
NAR + Q assay	7.01 ± 2.22	91.92 ± 3.03	6
Q Assay	1.18 ± 0.47	29.57 ± 10.27 *	4
Q + NAR Assay	1.59 ± 0.38	47.46 ± 4.26	4

Table 2. Peff values and percentage of absorption of NAR and Q in the different assays (*P<0.05 t-test).

  - ☑ With regard to the intestinal first pass effect, the main metabolites found in the lumen were glucuronides and sulfates (tables 3 and 4). An increase in AUC (p<0.05) was observed for NAR-metabolites in co-administration with Q, and the opposite was observed for Q.

Metabolites from Naringenin	AUC (min)	
	NAR assay	NAR + Q assay
Naringenin-glucuronide	4.07 ± 1.47*	10.63 ± 2.03
Naringenin sulfate	48.31 ± 3.58*	70.04 ± 5.13

Table 3 . Mean AUC (± SD) of NAR metabolites in the two assays (*P<0.05 t-test).

Metabolites from Quercetin	AUC (min)	
	Q assay	NAR + Q assay
Quercetin-glucuronide	11.31 ± 4.53	ND
Quercetin-sulfate	152.70 ± 15.84 *	46.60 ± 9.83
Isohamentin-glucuronide	242.40 ± 43.92	145.20 ± 92.57
Isohamentin-sulfate	668.50 ± 342.60	8312 ± 6604

Table 4 . Mean AUC (± SD) of Q metabolites in the two assays (*P<0.05 t-test).

4. Conclusions

1. NAR showed a high intestinal permeability and percentage of absorption, and not affected by the co-administration of Q.
2. Q shows a medium intestinal permeability and percentage of absorption, and its administration together with NAR seems to increase its absorption. A thorough study is needed to confirm this trend.
3. The amount of sulfates in intestinal lumen was higher than the glucuronides for both polyphenols. For NAR the co-administration with Q led to an increase of these metabolites, and the opposite occurred for Q.
4. For Q, the mixed conjugate isorhamentin-sulfate was the main metabolite obtained in the intestinal lumen.

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8.2.28. Comunicación 28. Póster

Título: Estudio del efecto sinérgico en la permeabilidad de la naringenina y la quercetina.

Autores: Naiara Orrego-Lagarón, **Miriam Martínez-Huélamo**, Rosa M. Lamuela-Raventós, and Elvira Escribano-Ferrer.

Congreso: First European Conference on Pharmaceutics: Drug Delivery. Reims, France, 2015.

Estudio del efecto sinérgico en la permeabilidad de la naringenina y la quercetina

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1. Introducción

La **quercetina (Q)** y la **naringenina (NAR)** son polifenoles del tomate, con efecto sobre el metabolismo lipídico, cardioprotectores, anticancerígenos y antiinflamatorios (1-2). Efectos beneficiosos que suelen estar relacionados con su biodisponibilidad tras su ingesta. Estudios recientes han puesto de manifiesto su baja biodisponibilidad: 0.04-0.1% para Q (3) y 10% para NAR (4). La biodisponibilidad oral depende básicamente, de la permeabilidad intestinal del principio activo, de su solubilidad, y de los procesos de eliminación por secreción intestinal, y por efectos de primer paso intestinal y hepático. Hay pocos estudios acerca la permeabilidad de la NAR (4, 5) y/o de la Q (6,7) en intestino delgado. Por otro lado, estudios previos han puesto de manifiesto que al administrar dos flavonoides simultáneamente se potencia la actividad por efecto sinérgico entre ambos, como es el caso de la capacidad antioxidante (8).

Objetivo: determinar mediante un ensayo de perfusión intestinal *in situ* en ratón, la permeabilidad intestinal (Peff) de la NAR y la Q en administración separada y conjunta, con el fin de evaluar un potencial efecto sinérgico en la absorción.

2.Métodos

Técnica analítica: HPLC-MS/MS

HPLC-MS/MS (Agilent serie 1100):

- **Fases móviles:** - A : H_2O con 0.1% (v/v) ácido fórmico.
  - B : AcN con 0.1% (v/v) ácido fórmico.
- **Método:** a gradiente.
- **Turbo ion spray:** Modo negativo con los ajustes de la **tabla 1** (9).

Tabla 1. Ajustes de la fuente iónica. CV: Collisium voltage; EP: Entrance Potencial; DP: Declustering Potential; FP: Focusing Potential; CE: Collision Energy.

Comp.	CV	GN (N_2)	CG (N_2)	DG (N_2)	EP	DP	FP	CE
NAR	-4500	10 (u.a)	12 (u.a)	400ºC	- 11V	-50	-190	-30
Q	-4500	10 (u.a)	12 (u.a)	400ºC	- 10V	-50	-210	-20

Estudio de estabilidad

Se estudio la estabilidad de la Q y la NAR en solución de perfusiñon (pH 6.2) a 37ºC durante 3h y se guardaron a -20ºC hasta su análisis por HPLC-MS/MS.

Perfusión intestinal in situ en ratón.

Condiciones experimentales:

- 3 Grupos según la solución de perfusión (Cin) :
  - Grupo 1: Solución de perfusión 3.5 µg/mL de NAR (10).
  - Grupo 2: Solución de perfusión 0.003 µg/mL de Q (11).
  - Grupo 3: Solución de perfusión 3.5 µg/mL de NAR y 0.003 µg/mL de Q.

Todas soluciones isoosmóticas de 267mOsm/kg y con una concentración de 0.1mg/mL de Rojo de Fenol a 37ºC (12).

- Animales: 5 ratones macho CD1(30-35g) por grupo.
- Condiciones previas: dieta ausente de polifenoles (7 días) y ayunas las 12h previas al ensayo con acceso libre a agua.
- Anestesia: i.p. ketamina/xylacina (100 mg/kg ; 10 mg/kg, respectivamente).
- Condiciones del ensayo:
  - ☑ Obstrucción del conducto biliar.
  - ☑ Canulación del duodeno (orificio de entrada) y del íleon (orificio de salida).
  - ☑ Al final del experimento, el animal fue eutanasiado y se midió la longitud del intestino (L).
- Flujo: 0.20 mL/min (Qin).
- Muestras de perfusión: Recogida a los 5min y cada 10min hasta 1h (Cout). .(Centrifugación a 9000 r.p.m y almacenamiento a -20ºC hasta el análisis.

La **figura 1** nos muestras la cirugía utilizada:

Figura 1. Técnica quirúrgica para el ensayo de perfusión intestinal in situ.

Análisis de datos

- **Coeficiente de permeabilidad** (Peff) y **porcentaje de absorción** (%abs) se calcularon a partir de las Cout corregidas en el estado estacionario de acuerdo con la ecuación 1 y la ecuación 2, respectivamente(13).

$$Peff = \frac{-\phi_{in}}{2\cdot\pi\cdot R\cdot L} \cdot Ln\frac{Cin}{Cout}$$

Ecuación 1

$$\%abs = \left[1 - e^{\frac{-2\cdot\pi\cdot R\cdot L}{\phi}\cdot Peff}\right]\cdot 100$$

Ecuación 2, donde

Cout.corr son las concentraciones de salida del intestino corregidas por rojo de fenol.

3.Resultados y discusión

- **Estabilidad:** Las soluciones de Q a pH 6.2 resultaron ser estables durante 3 h (ANOVA, p= 0.129), sin embargo las soluciones de NAR a pH 6.2 solo fueron estables durante 2 h, tiempo suficiente para realizar el ensayo.
- **Coeficientes de permeabilidad y porcentaje absorbido:**
  - ☑ Los valores de Peff no sufrieron cambios significativos (p >0.05) cuando se administran por separado o de forma conjunta, sin embargo, se observa como la permeabilidad de la Q tiene una tendencia a aumentar cuando se administra conjuntamente con la NAR (**Tabla 2**).

a)

Peff *10-4(cm/s)		
	NAR	NAR+Q
R1	8.71	7.79
R2	7.95	7.58
R3	7.44	3.61
R4	6.75	10.07
R5	ND	5.71
R6	ND	7.27
Media	**7.80**	**7.01**
DS	**1.54**	**2.22**

b)

Peff *10-4(cm/s)		
E	Q	Q+NAR
R1	1.48	1.03
R2	1.48	1.86
R3	1.12	1.72
R4	0.49	1.77
Media	**1.18**	**1.59**
DS	**0.47**	**0.38**

Tabla 2. Valores de Peff para la NAR, administrada sola o en coadministración con Q (a); Valores de Peff para la Q sola o en administración conjunta con NAR (b).

  - ☑ Esta tendencia se traduce en un aumento significativo en el porcentaje de Q absorbido en coadministración con NAR (29.57 ± 10.27% vs. 47.46 ± 4.26%, respectivamente (Tabla 3).

a)

Absorción %		
E	NAR	NAR+Q
R1	97.15	93.99
R2	97.78	93.23
R3	91.27	87.01
R4	93.06	94.08
R5	ND	89.27
R6	ND	93.94
Media	**93.94**	**91.92**
DS	**5.90**	**3.03**

b)

Absorción %		
E	Q	Q+NAR
R1	31.30	26.79
R2	41.10	44.52
R3	29.75	43.18
R4	16.13	50.24
R5	ND	51.91
Media	**29.57***	**47.46**
DS	**10.27**	**4.26**

Tabla 3 .Porcentaje absorbido (%) para la NAR sola o en coadministración con Q (a); para la Q sola o en administración conjunta con NAR (b)* P<0.05

4. Conclusiones

1. La NAR muestra una elevada permeabilidad intestinal y porcentaje de absorción, con lo cual su baja biodisponibilidad debe atribuirse a otros factores como el metabolismo de primer paso intestinal o hepático (4).
2. La Q muestra una permeabilidad intestinal y porcentaje de absorción medios; su baja biodisponibilidad, puede atribuirse a su dificultad de atravesar la membrana intestinal unida a un potencial metabolismo de primer paso.
3. La administración conjunta de ambos compuestos, no producen cambios significativos en los coeficientes de permeabilidad intestinales. Pero en el caso de la Q se observa un incremento en el valor de este parámetro.
4. El porcentaje de Q absorbido cuando se administra conjuntamente con la NAR aumenta significativamente, respecto a cuando ésta se administra sola. Posteriores estudios realizados a una dosis más alta de Q son necesarios para confirmar esta tendencia.

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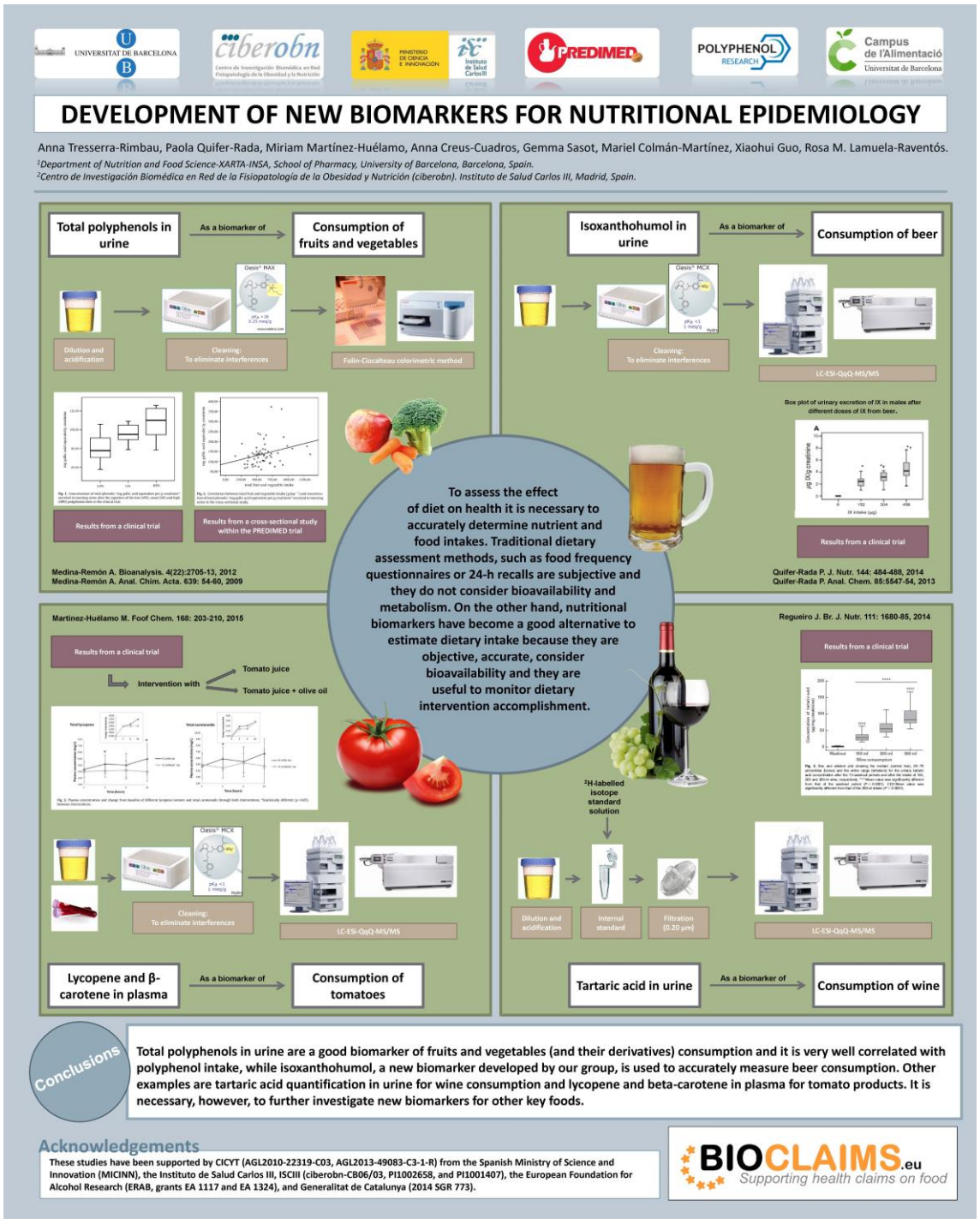
8.2.29. Comunicación 29. Póster

Título: Development of new biomarkers for nutritional epidemiology.

Autores: Anna Tresserra-Rimbau, Paola Quifer-Rada, **Miriam Martínez-Huélamo**, Anna Creus-Cuadros, Gemma Sasot, Mariel Colmán-Martínez, Xiaohui Guo, Rosa M. Lamuela-Raventós.

Congreso: Biomarkers and Health Claims on Food: BIOCLAIMS Meeting with stakeholders. Palma de Mallorca, Spain, 2015.


DEVELOPMENT OF NEW BIOMARKERS FOR NUTRITIONAL EPIDEMIOLOGY
Anna Tresserra-Rimbau, Paola Quifer-Rada, Miriam Martínez-Huélamo, Anna Creus-Cuadros, Gemma Sasot, Mariel Colmán-Martínez, Xiaohui Guo, Rosa M. Lamuela-Raventós.
[1]Department of Nutrition and Food Science-XARTA-INSA, School of Pharmacy, University of Barcelona, Barcelona, Spain.
[2]Centro de Investigación Biomédica en Red de la Fisiopatología de la Obesidad y Nutrición (ciberobn). Instituto de Salud Carlos III, Madrid, Spain.
Total polyphenols in urine
As a biomarker of
Consumption of fruits and vegetables
Dilution and acidification
Cleaning: To eliminate interferences
Folin-Ciocalteau colorimetric method
Results from a clinical trial
Results from a cross-sectional study within the PREDIMED trial
Medina-Remón A. Bioanalysis. 4(22):2705-13, 2012
Medina-Remón A. Anal. Chim. Acta. 639: 54-60, 2009
Isoxanthohumol in urine
As a biomarker of
Consumption of beer
Cleaning: To eliminate interferences
LC-ESI-QqQ-MS/MS
Box plot of urinary excretion of IX in males after different doses of IX from beer.
Results from a clinical trial
Quifer-Rada P. J. Nutr. 144: 484-488, 2014
Quifer-Rada P. Anal. Chem. 85:5547-54, 2013
To assess the effect of diet on health it is necessary to accurately determine nutrient and food intakes. Traditional dietary assessment methods, such as food frequency questionnaires or 24-h recalls are subjective and they do not consider bioavailability and metabolism. On the other hand, nutritional biomarkers have become a good alternative to estimate dietary intake because they are objective, accurate, consider bioavailability and they are useful to monitor dietary intervention accomplishment.
Martínez-Huélamo M. Food Chem. 168: 203-210, 2015
Results from a clinical trial
Intervention with
Tomato juice
Tomato juice + olive oil
Cleaning: To eliminate interferences
LC-ESI-QqQ-MS/MS
Lycopene and β-carotene in plasma
As a biomarker of
Consumption of tomatoes
Regueiro J. Br. J. Nutr. 111: 1680-85, 2014
Results from a clinical trial
[2]H-labelled isotope standard solution
Dilution and acidification
Internal standard
Filtration (0.20 µm)
LC-ESI-QqQ-MS/MS
Tartaric acid in urine
As a biomarker of
Consumption of wine
Conclusions
Total polyphenols in urine are a good biomarker of fruits and vegetables (and their derivatives) consumption and it is very well correlated with polyphenol intake, while isoxanthohumol, a new biomarker developed by our group, is used to accurately measure beer consumption. Other examples are tartaric acid quantification in urine for wine consumption and lycopene and beta-carotene in plasma for tomato products. It is necessary, however, to further investigate new biomarkers for other key foods.
Acknowledgements
These studies have been supported by CICYT (AGL2010-22319-C03, AGL2013-49083-C3-1-R) from the Spanish Ministry of Science and Innovation (MICINN), the Instituto de Salud Carlos III, ISCIII (ciberobn-CB06/03, PI1002658, and PI1001407), the European Foundation for Alcohol Research (ERAB, grants EA 1117 and EA 1324), and Generalitat de Catalunya (2014 SGR 773).
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8.2.30. Comunicación 30. Póster

Título: A new method for simultaneous identification of carotenoids, xanthophylls and fat soluble vitamins in human plasma samples.

Autores: Mariel Colmán-Martinez, **Miriam Martínez-Huélamo**, and Rosa M. Lamuela-Raventós.

Congreso: Young Research Fellow Meeting. Romainville, France, 2015.

A NEW METHOD FOR SIMULTANEOUS IDENTIFICATION OF CAROTENOIDS, XANTHOPHYLLS AND FAT SOLUBLE VITAMINS IN HUMAN PLASMA SAMPLES

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INTRODUCTION

Epidemiologic studies have shown that oxidative stress plays an essential role in the pathogenesis of many degenerative diseases, such as cancer, diabetes and cardiovascular diseases (1-3) and it has been suggested that antioxidants plays a protective role against these chronic diseases by defending against oxidative damage (4). There is an increasing interest in the analysis of carotenoids and some fat soluble vitamins due to their antioxidant properties and their relationship with the development of chronic diseases. The characterization and quantification of carotenoids and fat-soluble vitamins in the human plasma is essential for best interpretation of epidemiologic studies linking diet and health. High-performance liquid chromatography (HPLC) is the most used technique for the identification and quantification of carotenoids and fat-soluble vitamins.

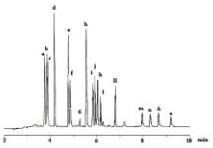
The aim of this word was validate a method for the identification of mainly carotenoids, xantophylls and fat soluble vitamins in human plasma, capable of be useful for dietary habit studies and for antioxidants status investigations.

MATERIALS AND METHOD

Extraction of carotenoids and fat soluble vitamins

Validation Method parameters

-Sensitivity:
LoD= 3*S/N
LoQ= 10*S/N
-Accuracy
-Recoveries
-Precision: RSD=SD/X*100



CONCLUSIONS

The HPLC method was completely validated, showing a sensitive analysis for carotenoids, xantophylls and fat soluble vitamins detection in plasma samples.

Due to the good results obtained in all parameters tested, this method can be applied to dietary habits studies and/or antioxidants status investigations.

RESULTS

The HPLC method was completely validated, providing a sensitive analysis for carotenoids, xantophylls and fat soluble vitamins detection and showing satisfactory data for all the parameters tested.

Table 1. Method validation parameters: correlation coefficients (R2), limit of detection (LOD), limit of quantification (LOQ), recovery and accuracy.

Analytes	R^2	LOD (µg/mL)	LOQ (µg/mL)	Recovery (%)	RSD (%)	Accuracy (%)
Retinol	0,9905	0,2	0,7	96	3,4	106
25-OH-Cholecalciferol	0,9904	0,5	1,7	99	7,8	107
α-tocotrienol	0,9947	0,6	2,0	70	5,0	108
Astaxanthin	0,9929	0,1	0,3	113	6,5	112
Lutein	0,9908	0,4	1,3	112	9,1	118
Zeaxanthin	0,991	0,1	0,3	107	5,2	115
Apo-8'-Carotenal	0,9914	0,2	0,7	94	3,3	107
Cryptoxanthin	0,9905	0,2	0,7	96	3,5	116
15Z-β-carotene	0,9969	1,3	4,3	101	2,1	141
13Z-β-carotene	0,9938	0,4	1,3	92	5,2	115
α-carotene	0,9932	0,5	1,7	89	4,6	112
β-carotene	0,9967	0,2	0,7	96	2,4	107
9Z-β-carotene	0,9918	0,2	0,7	93	3,8	123
Lycopene	0,9904	0,1	0,3	91	3,3	116

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22nd Young Research Fellow Meeting 2015

8.2.31. Comunicación 31. Póster

Título: A novel high resolution LTQ-ORBITRAP-MS enhances the identification of polyphenols and their metabolites in a functional food made with grape extract, and in human urine.

Autores: Gemma Sasot, **Miriam Martínez-Huélamo**, Anna Vallverdú-Queralt, Mireia Roig, Jorge Regueiro, Ramón Estruch, Gemma Chiva-Blanch, Mercè Mercader-Martí, and Rosa M. Lamuela-Raventós.

Congreso: Young Research Fellow Meeting. Romainville, France, 2015.

A NOVEL HIGH RESOLUTION LTQ-ORBITRAP-MS ENHANCES THE IDENTIFICATION OF POLYPHENOLS AND THEIR METABOLITES IN A FUNCTIONAL FOOD MADE WITH GRAPE EXTRACT AND, IN HUMAN URINE

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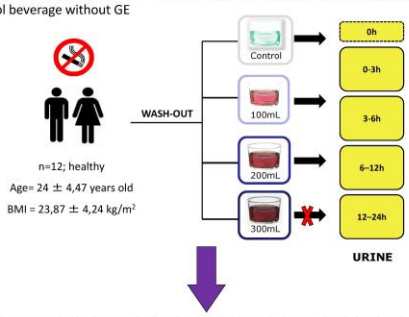
Introduction

Grape pomaces are known to be a rich source of polyphenols with biological activity, which may be used as functional ingredients for the development of new interesting products. Numerous studies have reported that bioactive compounds may act through multiple mechanisms, like antioxidant, anticarcinogen or anti-inflammatory (1).
However, an exhaustive identification of the polyphenols present in this functional compound, grape extract (GE) and, in biological samples obtained after the consumption of this novel food is required. In last years, there has been an increased interest in metabolites formed by the intestinal microbiota to understand the benefits derived from consumption (2). The application of high resolution mass spectrometry (HR-MS) allows the identification of phenolics using accurate mass measurements.

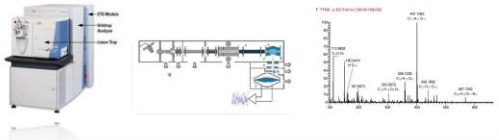
Study design and Method

In order to verify the oral absorption and metabolism of the polyphenols content, we performed a prospective randomized, cross-over study in 12 healthy volunteers with three interventions: 500 mL of a functional beverage enriched with 100 mL of grape extract, 500 mL of the same beverage with 200 mL of GE and 500 mL of a control beverage without GE



n=12; healthy
Age= 24 ± 4,47 years old
BMI = 23,87 ± 4,24 kg/m²

LTQ-Orbitrap-MS enabled the identification of phenolics in GE and in human urine collected



Results

Compound	R_t (min)	Accurate mass [M-H]	MS/MS ions (%Intensity)	mDa	Molecular formula
Pyrogallol	1,08 / 1,38	125,02406	97 (100)	0,4	$C_6H_6O_3$
2-O-Methylpyrogallol	4,99	139,03987	124 (100)	0,2	$C_7H_8O_3$
Gallic acid	1,53	169,01396	125 (100)	0,3	$C_7H_6O_5$
O-Methylgallic acid	1,81 /3,15 / 3,44	183,02962	168 (100), 139 (70), 124 (20)	0,3	$C_8H_8O_5$
Hydroxyhippuric acid	2,47 / 3,11	194,04587	150 (100), 100 (100)	0,3	$C_9H_9NO_4$
3,4-O-Dimethylgallic acid / Gallic acid ethyl ester	1,68 / 2,62 / 5,91	197,0451	182 (100), 153 (40), 137 (100)	0,4	$C_9H_9O_5$
5-(3,4-Dihydroxyphenyl)-γ-valerolactone	6,96 / 9,09	207,06627	181 (100), 179 (100), 163 (100), 141 (40)	0,4	$C_{11}H_{11}O_4$
4-O-Methylgallic acid 3-O-sulfate	2,67 / 3,17	262,98642	219 (40), 183 (100)	0,3	$C_8H_8O_8S$
Dihydroferulic acid 4-sulfate	5,42 / 5,59 / 7,33/ 12,25	273,02222	193 (100), 178 (90)	0,9	$C_{10}H_{10}O_7S$
Catechin	5,56	289,0712	245 (100), 205 (60)	0,6	$C_{15}H_{14}O_6$
Epicatequin	7,08	289,07086	245 (100), 207 (70)	0,9	$C_{15}H_{14}O_6$
Quercetin	11,46	301,03537	265 (100), 243 (65)	0,0	$C_{15}H_{10}O_7$
Dihydroquercetin	11,53	303,5319	223 (100)	2,2	$C_{15}H_{12}O_7$
trans-Resveratrol-3-O-sulfate	12,97	307,02817	227 (100)	0,3	$C_{14}H_{12}O_6S$
trans-Resveratrol-4-O-sulfate	14,41	307,02817	227 (100)	0,6	$C_{14}H_{12}O_6S$
cis-Resveratrol-3-O-sulfate	17,21	307,02817	227 (100)	0,9	$C_{14}H_{12}O_6S$
cis-Resveratrol-4-O-sulfate	18,68	307,02817	227 (100)		$C_{14}H_{12}O_6S$
Protocatechuic-O-glucoside	1,84	315,07147	153 (100)	0,7	$C_{13}H_{16}O_9$
Dihydrocaffeic acid 3-O-glucuronide	5,53	357,09270	181 (100), 113 (45)	0,9	$C_{15}H_{18}O_{10}$
Catechin sulfate	5,56	369,02795	289 (100), 245 (10)	0,6	$C_{15}H_{14}O_9S$
Epicatechin sulfate	7,08	369,02798	290 (100), 245 (10)	0,6	$C_{15}H_{14}O_9S$
Dihydroferulic acid 4-O-glucuronide	4,73 / 9,89 / 10,35	371,09778	195 (60), 175 (60), 113 (100)	0,1	$C_{16}H_{20}O_{10}$
Methyl-catechin sulfate	6,56	383,04393	303 (100), 217 (80), 137 (30)	0,3	$C_{16}H_{16}O_9S$
Methyl-epicatechin sulfate	7,12 / 9,19 / 8,76	383,04364	303 (100), 245 (90), 217 (60), 137 (30)	0,6	$C_{16}H_{16}O_9S$
Epigallocatechin-O-glucuronide	11,7	481,08874	305 (100)	1,7	$C_{21}H_{22}O_{13}$
Catechin glucuronide	4,17	465,10297	289 (100)	0,9	$C_{21}H_{22}O_{12}$
Epicatechin glucuronide	5,84	465,10355	289 (100), 245 (10)	0,3	$C_{21}H_{22}O_{12}$
Methyl-catechin glucuronide	3,85	479,11871	303 (100)	0,8	$C_{22}H_{24}O_{12}$
3'-O-Methyl-epicatechin-7-O-glucuronide	5,73	479,1189	303 (100)	0,6	$C_{22}H_{24}O_{12}$
4'-O-Methyl-epicatechin-3'-O-glucuronide	6,59 / 11,176	479,11902	461 (20), 303 (100)	0,5	$C_{22}H_{24}O_{12}$
4'-O-Methyl-epigallocatechin-O-glucuronide	8,98	495,11439	319 (100)		$C_{22}H_{24}O_{13}$
Catechin glucuronide sulfate	2,52	545,06042	465 (45), 369 (100)	0,2	$C_{21}H_{22}O_{15}S$
Epicatechin glucuronide sulfate	5,10	545,06073	465 (45), 369 (100)	0,1	$C_{21}H_{22}O_{15}S$
trans-Resveratrol-3-O-glucuronide	12,49	403,10344	227 (20), 175 (100)	0,2	$C_{20}H_{20}O_9$
trans-Resveratrol-4'-O-glucuronide	15,45	403,10344	227 (100)	0,6	$C_{20}H_{20}O_9$
cis-Resveratrol-3-O-glucuronide	17,62	403,10344	227 (90), 175 (100)	1,4	$C_{20}H_{20}O_9$
cis-Resveratrol-4'-O-glucuronide	18,79	403,10344	341 (100), 227 (30)	1,6	$C_{20}H_{20}O_9$
Dihydroresveratrol-3-O-sulfate	12,87	309,04382	229 (100), 123 (15)	0,8	$C_{14}H_{14}O_6S$
Dihydroresveratrol-4-O-sulfate	11,55	309,04382	229 (100), 123 (15)	1	$C_{14}H_{14}O_6S$
Dihydroresveratrol glucuronide-sulfate	8,88	485,07590	309 (100), 293(20), 175 (15)	0,4	$C_{20}H_{22}O_{12}S$
3-Hydroxybenzoic acid	2,99	137,02441	93(100)	0,33	$C_7H_6O_3$
4-Hydroxybenzoic acid	4,66	137,02441	93(100)	0,43	$C_7H_6O_3$
2-Hydroxyphenylacetic acid	4,9	151,04006	107 (100)	0,4	$C_8H_8O_3$
3-Hydroxyphenylacetic acid	7,05	151,04006	107 (100)	0,3	$C_8H_8O_3$
2-Hydroxy-2-phenylacetic acid	7,1	151,04006	107 (100)	0,4	$C_8H_8O_3$
4-Hydroxyphenylacetic acid	10,3	151,04006	107 (100)	0,5	$C_8H_8O_3$
2-Hydroxy-2-phenylacetic acid	10,3	151,04006	107 (70), 93 (100)	0,3	$C_8H_8O_3$
3,4-Dihydroxybenzoic acid (Protocatechuic acid)	2,8	153,01932	109 (100)	0,5	$C_7H_6O_4$
3,4-Dihydroxyphenylacetic acid (Homoprotocatechuic acid)	2,43	167,03497	123 (100)	0,4	$C_8H_8O_4$
Hippuric acid	4,92	178,05096	134 (100)	0,01	$C_9H_9NO_3$
Caffeic acid	5,38	179,03497	135 (100)	2,2	$C_9H_8O_4$
3,4-Dihydroxyphenylpropionic acid (Dihydrocaffeic acid)	3,5	181,05062	163 (100), 135 (40)	0,6	$C_9H_{10}O_4$
Homovanillic acid	5,52	181,05062	137 (100)	0,5	$C_9H_{10}O_4$
4-Hydroxy-5-(3,4-dihydroxyphenyl)valeric acid	13,14	225,07683	207 (30), 181 (40), 137 (100), 83 (20)	0,5	$C_{11}H_{14}O_5$
Vanillic acid 4-sulfate	3,36	246,99179	203 (80), 167 (100), 123 (100)	0,05	$C_8H_8O_7S$
Caffeic acid 3-sulfate	4,56	258,99179	179 (100)	0,9	$C_9H_8O_7S$
Caffeic acid 4-sulfate	6,03	258,99179	179 (100)	0,8	$C_9H_8O_7S$
Homovanillic acid 4-sulfate	3,93	261,00744	181 (100)	0,4	$C_9H_{10}O_7S$
Isoferulic acid 3-sulfate	6,32	273,00677	229 (20), 193 (100)	0,7	$C_{10}H_{10}O_7S$
Ferulic acid 4-sulfate	6,36	273,00744	193 (100)	0,9	$C_{10}H_{10}O_7S$
Epigallocatechin	6,42	305,06666	225 (100)	2,2	$C_{15}H_{14}O_7$
p-Coumaric acid	8,44 / 8,96	163,03969	119 (100)	0,4	$C_9H_8O_3$
3-(3-Hydroxyphenyl)propionic acid / 3-(4-Hydroxyphenyl)propionic acid	9,78	165,05571	147 (20), 121 (100)	0,4	$C_9H_{10}O_3$
3,5-Dimethoxy-4-hydroxybenzoic acid (Syringic acid)	1,69	197,04553	137 (100)	0,3	$C_9H_{10}O_5$

Table 1. List of compounds identified in urine samples

Compound	R_t (min)	Accurate mass [M-H]	MS/MS ions (%Intensity)	mDa	Molecular formula
Procyanidin trimer type B 1	1,50	865,1985	695 (100), 739 (70), 577 (65), 407 (40), 287 (20)	4	$C_{45}H_{38}O_{18}$
Gallic acid	1,57	169,0142	125 (100)	1,1	$C_7H_6O_5$
Galloylglucose	1,6	331,0665	169 (100), 125 (10)	3,1	$C_{13}H_{16}O_{10}$
Protocatechuic acid-*O*-hexoside	1,86	315,0721	153 (100), 109 (20)	2,2	$C_{13}H_{16}O_9$
Gensitic acid	2,70	153,0188	123 (100), 109 (10)	0,4	$C_7H_6O_4$
Protocatechuic acid	2,79	153,0193	153 (40), 109 (90)	0,9	$C_7H_6O_4$
Methyl gallate	3,09	183,0293	139 (100), 95 (40)	1,3	$C_8H_8O_5$
Caftaric acid	3,22	311,0408	149 (100)	2	$C_{13}H_{12}O_9$
Procyanidin dimer type B 1	3,40	577,1346	425 (100), 451 (60), 407 (50), 559(30), 289 (30)	2,9	$C_{30}H_{26}O_{12}$
Caffeic acid-*C*-hexoside 1	3,54	341,0877	281 (90), 251 (100), 221 (40), 179 (60), 135 (10)	2,1	$C_{15}H_{18}O_9$
Procyanidin dimer type B 2	4,09	577,1346	425 (100), 451 (60), 407 (50), 559(30), 289 (30)	2,9	$C_{30}H_{26}O_{12}$
Procyanidin trimer type B monogallolyate 1	4,16	1.017,2034	729 (100), 891 (80), 865 (60), 999 (50), 407 (30)	6,4	$C_{51}H_{46}O_{22}$
Caffeic acid-*C*-hexoside 2	4,19	341,0877	281 (90), 251 (100), 221 (40), 179 (60), 135 (10)	2	$C_{15}H_{18}O_9$
Coumaric-*O*-hexoside	4,36	325,0928	163 (100), 119 (12)	1,2	$C_{15}H_{18}O_8$
Catechin	4,43	289,0718	245 (100)	1,7	$C_{15}H_{14}O_6$
p-hydroxybenzoic acid	4,49	137,0244	93 (100)	0,7	$C_7H_6O_3$
Procyanidin trimer type B 2	4,75	865,1985	695 (100), 739 (70), 577 (65), 407 (40), 287 (20)	4	$C_{45}H_{38}O_{18}$
Procyanidin trimer type B 3	5,50	865,1985	695 (100), 739 (70), 577 (65), 407 (40), 287 (20)	4	$C_{45}H_{38}O_{18}$
Procyanidin trimer type B monogallolyate 2	5,60	1.017,2034	729 (100), 891 (80), 865 (60), 999 (50), 407 (30)	6,4	$C_{51}H_{46}O_{22}$
Caffeic acid	5,72	179,0349	135 (100)	1,1	$C_9H_8O_4$
Procyanidin trimer type B monogallolyate 3	6,04	1.017,2034	729 (100), 891 (80), 865 (60), 999 (50), 407 (30)	6,4	$C_{51}H_{46}O_{22}$
Epicatechin	6,47	289,0718	245 (100)	1,9	$C_{15}H_{14}O_6$
Eriodictyol-*O*-hexoside	6,64	449,1089	287 (100), 259 (40), 269 (38)	2,6	$C_{21}H_{22}O_{11}$
Procyanidin dimer type B monogallolyate 1	6,85	729,1461	711 (20), 577 (80), 559 (60), 407 (100), 289 (40)	3,8	$C_{37}H_{30}O_{16}$
Procyanidin trimer type B 4	7,29	865,1985	695 (100), 739 (70), 577 (65), 407 (40), 287 (20)	4	$C_{45}H_{38}O_{18}$
Procyanidin dimer type B monogallolyate 2	7,83	729,1461	711 (20), 577 (80), 559 (60), 407 (100), 289 (40)	3,8	$C_{37}H_{30}O_{16}$
Coumaric acid	8,24	163,0400	119 (100)	0,9	$C_9H_8O_3$
Myrectin-*O*-hexoside	8,49	479,0826	316 (100), 317 (50)	2,2	$C_{21}H_{20}O_{13}$
Kaempferol-3-*O*-rutinoside	8,79	593,1511	285 (100)	1,6	$C_{27}H_{30}O_{15}$
Ferulic acid	9,17	193,0506	178 (70), 149 (100), 134 (50)	1,2	$C_{10}H_{10}O_4$
Piceid acid 1	9,50	389,1241	227 (100), 193 (80), 341 (70)	2	$C_{20}H_{22}O_8$
Procyanidin dimer type B digallolyated	9,53	881,1520	729 (100), 711 (30) , 559 (20), 441 (10), 407 (20), 289 (5)	2,1	$C_{44}H_{34}O_{18}$
Epicatechin-*O*-gallate	9,8	441,0822	289 (100), 331 (20), 271 (19), 169 (30)	1,8	$C_{22}H_{18}O_{10}$
Kaempferol-3-*O*-glucoside	10,20	447,0932	285 (100)	2,4	$C_{21}H_{20}O_{11}$
Quercetin-*O*-hexoside	10,20	463,0881	301 (100), 293 (40), 271 (15), 169 (10)	2,2	$C_{21}H_{20}O_{12}$
Procyanidin trimer type B monogallolyate 4	10,26	1.017,2034	729 (100), 891 (80), 865 (60), 999 (50), 407 (30)	6,4	$C_{51}H_{46}O_{22}$
Quercetin-*O*-rhamnoside	11,67	447,0932	301 (100), 284 (20), 285 (20)	1,9	$C_{21}H_{20}O_{11}$
Piceid acid 2	12,23	389,1241	227 (100)	2,2	$C_{20}H_{22}O_8$
Quercetin	16,30	301,0353	179 (100), 151 (90), 107 (10)	1,5	$C_{15}H_{10}O_7$
Resveratrol	16,58	227,0713	185 (100), 143 (30)	2,5	$C_{14}H_{12}O_3$
Naringenin	17,88	271,0611	151 (100), 177 (20), 119 (10)	1,7	$C_{15}H_{12}O_5$

Table 2. List of compounds identified in grape extract

Conclusions

Forty one polyphenols were identified in extract mainly procyanidins, phenolic acids and flavonols.
In urine samples, more than sixty metabolites of phenolic compounds were detected including microbiota metabolites, glucuronides and sulfate derivatives.
In conclusion, the LTQ-Orbitrap-MS enhances the identification of a large variety of phenolic compounds from functional ingredients and their metabolites originated in the human body.

References

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(2) Boto-Ordóñez, *et al.* ***J Agric Food Chem*** 2013; 61(38):9166-75

XXIInd Young Research Fellow Meeting 2015

8.2.32. Comunicación 32. Póster

Título: Naringenin-*O*-glucuronide as a biomarker of tomato consumption.

Autores: **Miriam Martínez-Huélamo**, Paola Quifer-Rada, Palmira Valderas-Martínez, Sara Arranz-Martínez, Ramon Estruch and Rosa M. Lamuela-Raventós.

Congreso: 7th International Conference on Polyphenols and Health. Tours, France, 2015.

Naringenin-*O*-glucuronide as a biomarker of tomato consumption

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INTRODUCTION

Naringenin, a characteristic polyphenol of citrus fruits, is also one of the most abundant flavonoids found in tomato and tomato products. Previous studies have demonstrated that the administration of a single dose of tomato sauce enriched with refined olive oil enhanced the bioavailability of naringenin and especially its metabolite, naringenin-*O*-glucuronide in plasma and urine.

AIM

The aim of the present work was to study the correlations between long-term consumption of different portions of tomato juices enriched with refined olive oil and the concentrations of naringenin and its metabolite, naringenin-*O*-glucuronide, in spot urine samples of participants at high cardiovascular risk.

STUDY DESIGN

High cardiovascular risk
Age = 70.2 ± 3.7 years
BMI = 30.7 ± 3.8 kg/m²

Open, randomized, crossover and controlled clinical trial

Baseline	Z0:0 mL	Washout	Z1:200 mL	Washout	Z2:400 mL
Baseline	Z1:200 mL	Washout	Z0:0 mL	Washout	Z2:400 mL
Baseline	Z2:400 mL	Washout	Z0:0 mL	Washout	Z1:200 mL
Baseline	Z0:0 mL	Washout	Z2:400 mL	Washout	Z1:200 mL

28 days | 28 days | 28 days | 28 days | 28 days

SAMPLE PREPARATION

Acidification / Addition internal standard → Solid phase extraction → Evaporation and reconstitution → Filtration

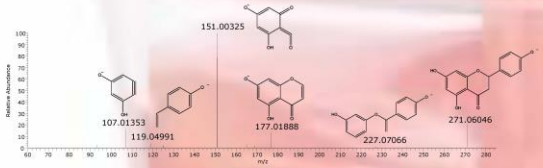
RESULTS

IDENTIFICATION

HPLC-LTQ-Orbitrap-MS

- ✓ HRMS (≤ 150.000 FWHM)
- ✓ Exact mass measurements (< 5 ppm)
- ✓ High m/z range
- ✓ Excellent sensitivity
- ✓ Multi-stage mass analysis (MS^n)

NARINGENIN (m/z 271.060650)



NARINGENIN-*O*-GLUCURONIDE (m/z 447.092740)

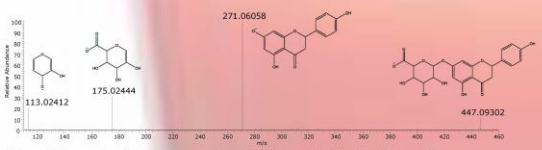


Figure 1. Mass spectra of naringenin and naringenin-O-glucuronide by HPLC-LTQ-Orbitrap-MS.

QUANTIFICATION

Table 1. Naringenin and naringenin-*O*-glucoside content in the tomato juice enriched with refined olive oil.

	Z0	Z1 (µg/200 mL)	Z2 (µg/400 mL)
Naringenin	–	420 ± 33	841 ± 66
Naringenin-*O*-glucoside	–	85 ± 11	170 ± 21

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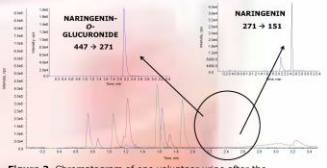

Figure 2. Chromatogram of one volunteer urine after the consumption of 400 mL of tomato juice enriched with refined olive oil.

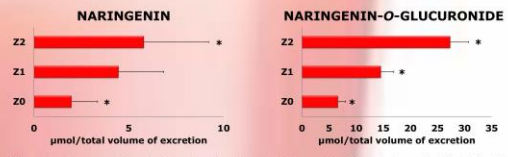

Figure 3. Urinary excretion of naringenin and naringenin-O-glucuronide of the volunteers after the intake of different doses of tomato juice enriched with refined olive oil. * significantly different.

CONCLUSIONS

There was a clear dose-response of naringenin and naringenin-*O*-glucuronide with tomato juice doses consumption. Naringenin-*O*-glucuronide can be used as a valid biomarker of tomato and tomato products consumption; however the interferences from citrus fruit consumption have to be considered.

FINANCIAL SUPPORT

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8.2.33. Comunicación 33. Póster

Título: Absorption of chlorogenic acid in the stomach of mice.

Autores: Naiara Orrego-Lagarón, Elvira Escribano-Ferrer, **Miriam Martínez-Huélamo**, Anna Vallverdú-Queralt, and Rosa M. Lamuela-Raventós.

Congreso: 7th International Conference on Polyphenols and Health. Tours, France, 2015.

7th International Conference on Polyphenols and Health

Absorption of chlorogenic acid in the stomach of mice

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1. Introduction

Chlorogenic acid (5-caffeoylquinic acid) is a phenolic compound present in almost every plant being the coffee the major source. The daily intake between coffee drinks and other dietary sources can be higher than 100 mg/d (Olthoff 2001). Chlorogenic acid presents antioxidant properties *in vitro*, and to evaluate the possible health effects is important to evaluate its absorption and disposition *in vivo*.

In spite of the absorption is not the main function of the stomach, some substances like glucose, simple sugars, amino acids, fat soluble substances and ethanol are readily absorbed. Moreover, the pH of the gastric contents controls the absorption of certain ionisable substances such as aspirin and ranitidine which are readily absorbed in its unionized form.

Aim: study the absorption and metabolism of chlorogenic acid in the stomach of mice using an *in situ* gastric perfusion technique.

2.Methods

Analytical methods:

1. HPLC-ESI-MS/MS: Identification and quantification of the aglycone and its metabolites.

Compound	rt (min)	MS/MS	DP	FP	CE	LOQ (mcg/ml)
Chlorogenic acid	2.81	353-191	-50	-190	-20	0.000148
Caffeic acid	2.97	179-135	-40	-170	-20	0.00056
Quinic acid	----	191-147	-40	-180	-30	----

Table 1. Settings of turbo ion spray source. CV: Collisium voltage; EP: Entrance Potencial; DP: Declustering Potential; FP: Focusing Potential; CE: Collision Energy.

- **Liquid cromatograph:** Agilent series 1100 equipped with a quaternary pump and a thermostated autosampler.
- **Column:** 5 µm Luna C18 (Phenomenex. USA)
- **Mobile phases** - A : H2O with 0.1% (v/v) formic acid.
  - B : Acetonitrile 0.1% (v/v) formic acid.
- **Method**: increasing linear gradient (v/v) of B.
- **Flow:** 600 µL min-1.
- **Turbo ion spray:** negative mode with the settings in Table 1.

2. LTQ-Orbitrap: Identification of chlorogenic acid metabolites.

- **LTQ-Orbitrap:** Velos mass spectrometer equiped with ESI Source in negative mode was used to acquire mass spectra in profile mode with a setting of 30000 resolution at m/z 400.
- **Liquid chromatograph:** Accela HPLC instrument.
- **Column:** reversed-phase column Atlantis T3 C18 from Waters (USA).
- **Mass range**: From m/z 100 to 1000
- **Data analysis:** Xcalibur software
- **Operation parameters:**
  - ☑Source voltage =4kV
  - ☑Sheath gas = 20 (au)
  - ☑Auxiliary gas= 10 (au)
  - ☑Sweep gas = 2 (au)
  - ☑Capillary temperature = 275 º C.
- **Mobil phases:** - A : H2O with 0.1% formic acid
  - B : Acetonitrile with 0.1% formic acid
- **Method:** increasing linear gradient (v/v) of B.
- **Flow:** 0.635 mL / min

Perfusion experiments

- Animals: 6 male CD1 mice (30-35g)
- Perfusion solution: Isotonic solution (pH 2) containing 32 ng/ml of chlorigenic acid and 0.1 mg/ml of phenol red.
- Previous conditions: mice fed for one week with a diet absent of polyphenols.
- Anesthesia: i.p. ketamine/xylacin (100 mg/kg ; 10 mg/kg, respectively).
- Assay conditions:
  - The biliary duct was tied.
  - The stomach was isolated and cannulated.
  - Flow: 0.16 mL/min (Øin).

- Samples :
  - Perfusion samples; collected at 10 min intervals for 60 min.
  - Blood sample; at 60 min with cardiac puncture.
  - Bile sample: the gall bladder was collected at the end of the assay.

The *in situ* perfusion model surgical technique is shown in figure 1.

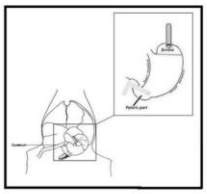

Figure 1. Gastric surgical technique.

Data analysis

- ***Percentage of absorption*** was calculated according to equation 1 (Varma and Panchagnula 2005).

$$\%abs = \left[1 - e^{-\frac{2\cdot\pi\cdot R\cdot L}{\phi}\cdot Peff}\right]\cdot 100$$

Equation 1 *where*

$$Peff = \frac{-\phi_{in}}{2\cdot\pi\cdot R\cdot L}\cdot Ln\frac{Cin}{Cout}$$

ϕ_{in} is the flow rate, Cin and Cout are the respective inlet and corrected outlet concentrations (phenol red method) at steady state, L is the length of the stomach and R its radius calculated assuming a cylinder shape with a volume of 0.4mL and according to equation 2.

$$R = \sqrt{\frac{0.4}{\pi\cdot L}}$$

Equation 2

3.Results and conclusions

- Chlorogenic acid was absorbed in 55.54 ± 9.24% and neither caffeic acid nor quinic acid was not found in the perfusion samples using HPLC-MS/MS .
- LTQ-Orbitrap enabled us to identify low-intensity signals corresponding to chlorogenic glutation, dihydrocaffeic acid-3-O-sulfate, carboxylated quinic acid, hydroxylated quinic acid and ferulic acid (Table 2) .

Compound	rt (min)	[M-H]-	Acc.mass	mDa	MF
Chlorogenic glutation	15.97	966	966. 2473	1	$C_{36}H_{51}N_6O_{21}S_2$
Dihydrocaffeic-O-sulfate	20.22	256	256. 9761	>2	$C_9H_6O_7S$
Quinic acid carboxilated	1.06	206	206. 0431	1.3	$C_7H_{11}O_7$
Quinic acid hidroxilated	20.01	207	207. 051	0.3	$C_7H_{12}O_7$
Ferulic acid	1.5	193	193. 0506	0.5	$C_{10}H_{10}O_4$

Table 2. List of compound indentified in perfusion samples by Orbitrap

4.References

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Acknowledgements

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POLYPHENOL RESEARCH

8.2.34. Comunicación 34. Póster

Título: Co-administration of naringenin and quercetin in mice: study on the effect in their absorption and metabolism.

Autores: Naiara Orrego-Lagarón, Elvira Escribano-Ferrer, **Miriam Martínez-Huélamo**, Paola Quifer-Rada, and Rosa M. Lamuela-Raventós.

Congreso: 7th International Conference on Polyphenols and Health. Tours, France, 2015.

7th ICPH International Conference on Polyphenols and Health

Co-administration of naringenin and quercetin in mice: study on the effect in their absorption and metabolism

UNIVERSITAT DE BARCELONA

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1. Introduction

Naringenin (NAR) and quercetin (QUER) are two of the major flavonoids in tomato with reported health-promoting properties against many diseases. The benefits in human health are assumed to be related with their oral bioavailability. However low bioavailability has been reported for both: 10% for NAR (1) and 0.04-0.1% for QUER (2) after oral administration. Bioavailability depends basically on the permeability of the compound but also on how extensive is the intestinal and hepatic metabolism. When there is a co-administration a synergistic effect could appear related to a competition between them for membrane transporters or metabolic enzymes, causing changes on their absorption and metabolism.

Aim: study the influence of their co-administration in their absorption and intestinal first pass metabolism using an *in situ* intestinal perfusion technique.

2. Methods

Analytical methods :

UPLC-ESI-MS/MS: Quantification of the aglycones and its metabolites.

- **Mobile phases** - A : H2O with 0.1% (v/v) formic acid.
  - B : Acetonitrile 0.1% (v/v) formic acid.
- **Method**: increasing linear gradient (v/v) of B.
- **Flow:** 600 µL min-1.
- **Turbo ion spray:** negative mode with the settings in Table 1.

Table 1. Settings of turbo ion spray source. CV: Collisium voltage; EP: Entrance Potencial; DP: Declustering Potential; FP: Focusing Potential; CE: Collision Energy. NAR-GLU: Naringenin-glucuronide; NAR-SULF: Naringenin-sulfate; QUER-GLU: Quercetin-glucuronide; QUER-SULF: Quercetin-sulfate; QUER-diGLU: Quercetin-diglucuronide; ISORH: Isorhamnetin; ISOHR-GLU: Isorhamnetin-glucuronide; ISOHR-SULF: Isorhamnetin-sulfate .* Quantified with standard.

Compound	RT(min)	MS/MS	DP (V)	FP (V)	CE (V)	EP (V)	LOD (µg/ml)	LOQ (µg/ml)
NAR*	3.51	271→151	-50	-190	-30	-11	0.00255	0.008513
NAR-GLU*	2.55	447→271	-50	-190	-30	-11	0.000715	0.00238
NAR-SULF	4.80	351→271	-50	-190	-30	-11	—	—
NAR-O-SULF-O-GLU	2.61	527→447	-50	-190	-30	-11	—	—
QUER*	3.12	301→151	-60	-210	-30	-11	0.0438	0.0163
QUER-GLU*	2.26	477→301	-30	-200	-30	-11	0.00342	0.01142
QUER-SULF	5.39	381→301	-60	-210	-30	-11	—	—
QUER-diGLU	2.39	653→477	-30	-200	-30	-11	—	—
ISORH *	3.66	315→151	-60	-210	-30	-11	0.172	0.574
ISORH-GLU	2.58	491→315	-60	-210	-30	-11	—	—
ISORH-SULF	5.07	395→315	-60	-210	-30	-11	—	—

Perfusion experiments

- Animals: Male CD1 mice (30-35g) divided in 3 groups of mice according to the perfusion solution (Cin) :
  - Group 1: Perfusion solution 3.5 µg/mL of NAR .
  - Group 2: Perfusion solution 2.36 µg/mL of QUER.
  - Group 3: Perfusion solution 3.5 µg/mL of NAR and 2.36 µg/mL of QUER.

  Every isoosmotic solution contained 0.1 mg/mL of phenol red.
- Previous conditions: mice fed for a week with a diet absent of polyphenols.
- Anesthesia: i.p. ketamine/xylacin (100 mg/kg ; 10 mg/kg, respectively).
- Assay conditions:
  - The biliary duct was tied.
  - A segment of small intestine was isolated and cannulated (L).
- Flow: 0.20 mL/min (Øin).
- Samples :
  - Perfusion samples: collected at 10 min intervals for 60 min.
  - Blood sample: at 60 min with cardiac puncture.
  - Bile sample: gall bladder collected at the end of the assay.

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An image of the surgical technique is shown in figure 1:

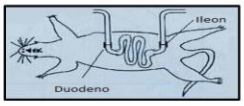

Figure 1. Intestinal surgical technique.

Data analysis

- ***Percentage of absorption :*** calculated according to equation 1 (3).

$$\%abs = \left[1 - e^{-\frac{2\cdot\pi\cdot R\cdot L}{\phi}\cdot Peff}\right]\cdot 100$$

Equation 1, *where*

$$Peff = \frac{-\phi_{in}}{2\cdot\pi\cdot R\cdot L}\cdot Ln\frac{Cin}{Cout}$$

ϕ_{in} is the flow rate, Cin and Cout are the respective inlet and corrected outlet concentrations (phenol red method) at steady state, and R is radius of the intestine.

3. Results and conclusions

- Both naringenin and quercetin showed a decrease on its absorption when were administered together (94.82 ± 3.15% to 74.04 ± 6.57% for NAR and 85.02 ± 1.37% to 65.48 ± 6.15% for QUER).
- The concentration of metabolites found in perfusion samples was higher in the co-administration.
- In plasma and bile samples the number of metabolites identified were also higher (Tables 2 and 3).

Table 2. Mean percentage (%) and standard deviation (SD) of NAR metabolites found in plasma and the bile in the NAR alone and co-administered assay.

Metabolites	PLASMA				BILE POOL	
	Naringenin		Naringenin + Quercetin		Naringenin	Naringenin + Quercetin
	mean (%)	SD	mean(%)	SD	%	%
NAR-GLU	23.15	8.57	43.78	7.21	-------	67.52
NAR-SULF	-------	-------	-------	-------	-------	34.22
NAR-O-SULF-O-GLU*	-------	-------	-------	-------	-------	75.24

**only analysed in the co-administration assay*

Table 3. Mean percentage (%) and standard deviation (SD) of QUER metabolites found in plasma and the bile in the QUER alone and co-administered assay.

Metabolites	PLASMA				BILE POOL	
	Quercetin		Quercetin + Naringenin		Quercetin	Quercetin + Naringenin
	mean (%)	SD	mean(%)	SD	%	%
QUER-GLU	-------	-------	26.77	8.02	-------	31.74
QUER-SULF	-------	-------	-------	-------	17.47	205.2
Isorhamnetin	-------	-------	89.38	45.17	-------	-------
ISORH-GLU	-------	-------	16.40	5.12	-------	88.18
ISORH-SULF	-------	-------	20.82	7.16	13.62	90.52

References

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3. Varma, M.V.S., Panchagnula, R., 2005. J. Pharm. Sci. 94, 1694–1704 (2005).

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