UNIVERSITAT DE BARCELONA

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Abreviaturas y acrónimos

ANOVA	Análisis de varianza para medidas repetidas
AOAC	Asociación Oficial de Químicos Analíticos
BHT	Butil-hidroxi-tolueno
CXCL10	CXC quimoquina 10
DAD	Detector de diodos
DA	Dosis alta de zumo de tomate
DB	Dosis baja de zumo de tomate
DER	Desviación estándar relativa
ECV	Enfermedades cardiovasculares
FDA	Administración de Alimentos y Medicamentos
HDL	Lipoproteínas de alta densidad
HPLC	Cromatografía líquida de alta resolución
ICAM-1	Moléculas de adhesión intercelular 1
IDL	Lipoproteínas de densidad intermedia
IFN-γ	Interferón gamma
IL	Interleuquina
IMC	Índice de masa corporal
LDC	Límite de cuantificación
LDD	Límite de detección
LDL	Lipoproteínas de baja densidad
Mt	Millones de toneladas
OMS	Organización Mundial de la Salud
oxLDL	Lipoproteínas de baja densidad oxidadas
PCR	Proteína C reactiva
ROS	Especies reactivas de oxígeno
SMC	Células musculares lisas
SPSS	Paquete Estadístico para Ciencias Sociales
SR	*Scavenger*
TLR	Receptores tipo *Toll*
TMBE	*tert*-Metil butil éter
TNF-α	Factor de necrosis tumoral
UV	Ultravioleta
VCAM-1	Moléculas de adhesión vascular 1
VLDL	Lipoproteínas de muy baja densidad

Índice

Resumen

I. Resumen

El tomate es una de las hortalizas más consumidas en todo el mundo y tanto su demanda como su producción han aumentado progresivamente en los últimos 20 años. Europa se encuentra entre los principales productores mundiales, siendo España uno de los diez países con mayor producción de tomates tanto para la industria, como para el consumo en fresco.

Además de sus propiedades sensoriales y de su versatilidad culinaria, que lo convierten en un alimento apto para consumo en crudo, así como en diferentes preparaciones, el tomate es considerado una fuente importante de compuestos bioactivos, entre los que destacan los carotenoides.

Los carotenoides son una familia de compuestos de más de 600 pigmentos vegetales liposolubles que se encuentran tanto en organismos fotosintéticos, como no fotosintéticos y son responsables de las coloraciones amarillas, anaranjadas y rojas que se observan en la naturaleza.

El licopeno es uno de los carotenoides más abundantes del tomate, el cual es responsable de su coloración roja. En los alimentos crudos, el licopeno se encuentra principalmente en la configuración *trans*, mientras que en el plasma y en los tejidos, la configuración predominante es la *cis*.

El estudio de los carotenoides con relación a la salud, y muy especialmente del licopeno, ha cobrado mucha relevancia en los últimos años, ya que se ha visto que su consumo regular se relaciona con un menor riesgo de desarrollar enfermedades crónico-degenerativas, tales como las enfermedades neurodegenerativas, distintas formas de cáncer, así como de enfermedades cardiovasculares. Entre las enfermedades cardiovasculares, la aterosclerosis es una de las responsables de más muertes y comorbilidades en todo el mundo y tanto en su inicio como en su progresión, se encuentran implicados múltiples procesos inmunoinflamatorios.

La determinación de los efectos de los carotenoides del tomate sobre las moléculas inflamatorias y quimiotácticas implicadas en el desarrollo y la progresión de la aterosclerosis, así como el esclarecimiento del papel biológico que cumplen los distintos isómeros del licopeno en la modulación de estas moléculas es de suma importancia para la implementación de estrategias dietéticas y nutricionales que contribuyan con la prevención del desarrollo de estas enfermedades.

I. Summary

The tomato is one of the most consumed vegetables worldwide, and both demand and production have steadily increased over the past 20 years. Europe is among the world's leading producers of this fruit, being Spain one of the ten countries with the largest crop of tomatoes for industry and for fresh consumption.

Besides its sensory properties and its culinary versatility, making it a food suitable for raw consumption as well as in different preparations, tomato is considered an important source of nutrients, due to their high content of bioactive compounds, among which carotenoids are include.

Carotenoids are a family of more than 600 soluble plant pigments found in both photosynthetic and non-photosynthetic organisms and are responsible for the yellow, orange, and red colorations observed in nature.

Lycopene is one of the most abundant carotenoid in tomatoes, which is responsible for its red coloration. On raw foods, lycopene is found mainly in the *trans* configuration, whereas in plasma and tissues, the predominant configuration is the *cis*.

The study of carotenoids in relation to health, and particularly of lycopene, has become increasingly important in recent years as it has seen that its regular consumption is associated with a lower risk of developing chronic degenerative diseases, such as neurodegenerative diseases, various types of cancer, and also cardiovascular diseases. Among cardiovascular diseases, atherosclerosis is one of those that cause more deaths and morbidities worldwide, and both its beginning and its progression, are involved multiple immune-inflammatory processes.

Determining the effects of tomato carotenoids on inflammatory and chemotactic molecules involved in the development and progression of atherosclerosis, as well as the elucidation of the biological role played by the various isomers of lycopene in the modulation of these molecules is of utmost importance for the implementation of dietary and nutritional strategies that contribute to the prevention of the development of these diseases.

Hipótesis y objetivos

II. Hipótesis y objetivos

Hipótesis

El consumo habitual de tomate, un alimento rico en carotenoides, principalmente licopeno, mejora los parámetros de riesgo cardiovascular al disminuir el estado de inflamación generalizada y al actuar como inmunomodulador, previniendo el desarrollo de aterosclerosis en individuos de una población de edad avanzada, sin enfermedad cardiovascular diagnosticada, pero con alto riesgo de desarrollarla.

Objetivos

Objetivo General

El objetivo general de esta tesis doctoral es determinar la asociación existente entre el consumo habitual de zumo de tomate, las concentraciones plasmáticas de carotenoides y las concentraciones de moléculas inflamatorias, así como de moléculas de adhesión celular como marcadores de riesgo cardiovascular.

Objetivos Específicos

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

1. Desarrollar un método analítico sensible y específico que permita la identificación y cuantificación de carotenoides del tomate, así como sus principales isómeros en plasma humano.

2. Estudiar la relación dosis-respuesta entre los niveles de carotenoides, en especial de licopeno y sus isómeros, tras la ingesta crónica de zumo de tomate durante 4 semanas a diferentes dosis.

3. Determinar la asociación existente entre los carotenoides del tomate y la concentración de moléculas inflamatorias, como marcadores de riesgo

cardiovascular, en pacientes sin enfermedad cardiovascular, pero con alto riesgo de desarrollarla.

Introducción

III. Introducción

1. El tomate

1.1. Origen, distribución y clasificación botánica

El tomate es un fruto originario de América, proveniente de la región andina que actualmente comprende las zonas de Chile, Bolivia, Ecuador, Colombia y Perú, donde crecía de forma silvestre. Se cree que su cultivo como hortaliza comestible comenzó en México y que fue Hernán Cortés quien lo introdujo a España entre los siglos XV y XVI, tras la conquista de Tenochtitlán (actual Ciudad de México). Tanto españoles como portugueses, durante el período de conquistas expandieron el cultivo del tomate a países como Italia, Francia e Inglaterra, así como a Oriente Medio, África y Filipinas, desde donde se extendió a otros países asiáticos. Con la colonización inglesa, se reintrodujo a América, llegando a los Estados Unidos y Canadá[1,2].

En Europa, el tomate fue cultivado para consumo humano posiblemente a partir del siglo XVII[2], aunque en algunos países, como Italia, Francia y Alemania, se utilizaba únicamente con fines ornamentales, por ser considerado un fruto tóxico y no fue sino hacia finales del siglo XVIII y principios del siglo XIX que lo introdujeron a la dieta[2,3].

Teniendo en cuenta su uso y su forma de cultivo, es considerado una hortaliza, pero desde un punto de vista botánico, el tomate (*Solanum Lycopersicum*) es una fruta, específicamente una baya, debido a que es indehiscente, de textura pulposa y tiene una o más semillas que son comestibles. Pertenece a la familia de las Solanáceas, la cual está compuesta por más de 3000 especies[1]. El género *Solanum* es uno de los más destacados dentro de las Solanáceas, abarcando unas 1700 especies de gran interés económico, entre las que se encuentran el tomate, la berenjena, el pimiento, el tabaco y la patata[2]. Las especies del género *Solanum* se caracterizan por una amplia variedad morfológica y ecológica, pudiendo cultivarse en zonas tanto templadas como tropicales. Los tomates pueden tener diversas formas y tamaños, como ser redondos, asurcados, oblongos o de tipo cherry (cóctel), y pueden pesar entre 5 y 500 g.

1.2. Importancia económica

El tomate es uno de los frutos más consumidos en todo el mundo. Tanto su demanda, como su producción y comercio aumentan cada año, alcanzando en el año 2014 una

producción de casi 164 millones de toneladas (Mt), convirtiéndolo en el séptimo cultivo más importante a nivel mundial[4].

En los últimos 20 años, tanto la producción como las áreas dedicadas al cultivo del tomate han aumentado (**Figura 1**) y Asia se ha convertido en el principal continente productor de este fruto, con un 51,6% de la producción mundial, superando a América (18,9%) y Europa (17,1%) (**Figura 2**), que hasta aquel momento eran los continentes que lideraban la producción del mismo.

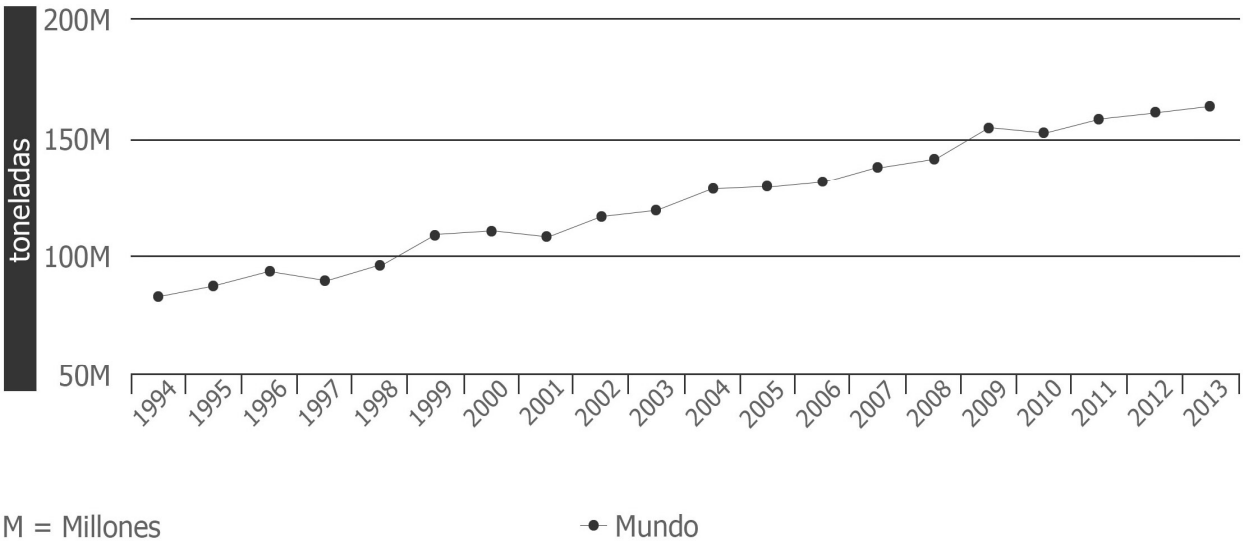

Figura 1. Promedio mundial de cultivo de tomate desde el año 1994 hasta el 2014.

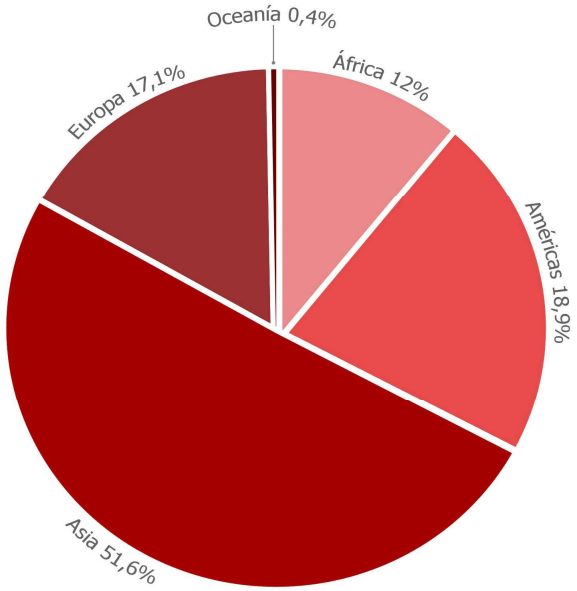

Figura 2. Producción mundial de tomate.

De acuerdo con datos de la División de Estadística de la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAOSTAT), en el 2013 los países con mayor producción de tomate fueron China (50,5 Mt), seguido de India (18,2 Mt), EE.UU. (12,5 Mt), Turquía (11,8 Mt) y Egipto (8,5 Mt) (**Figura 3**).

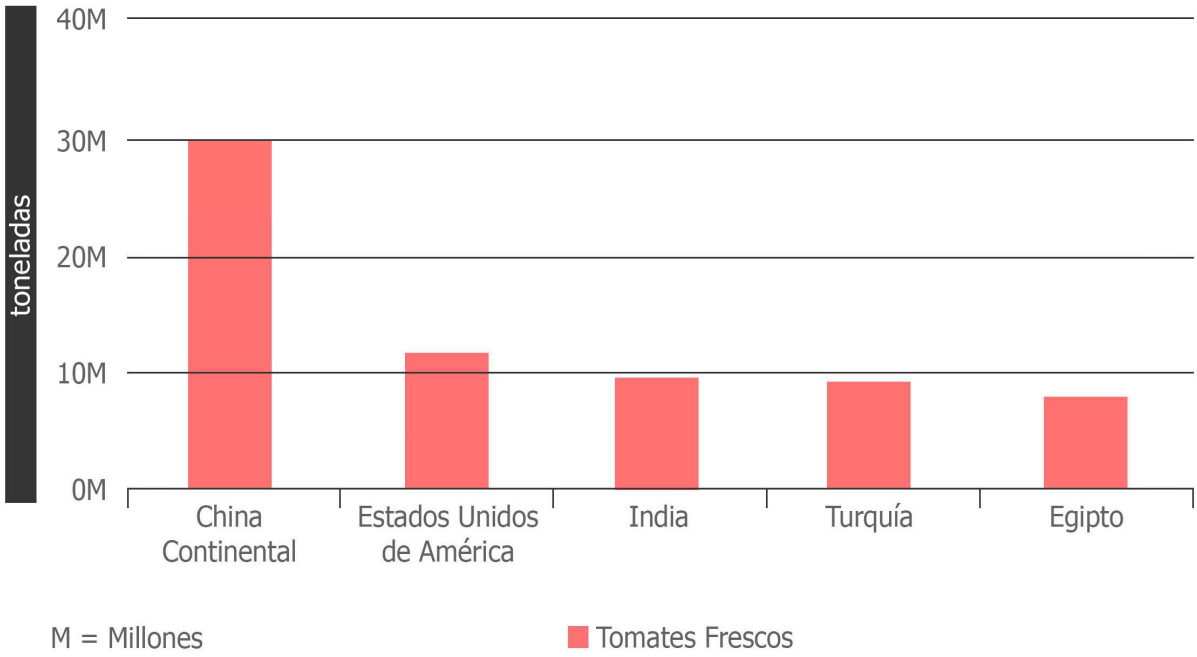

Figura 3. Mayores países productores de tomate en el año 2013.

España se encuentra en la novena posición a nivel mundial con una producción de 3,7 Mt.[4], destacando Extremadura como principal comunidad autónoma productora de tomates destinados a la industria y Andalucía, en la producción de tomates para consumo en fresco[5].

1.3. Productos derivados del tomate

El tomate es un fruto muy apreciado por sus cualidades sensoriales, así como por su versatilidad culinaria, pudiendo consumirse tanto crudo como cocinado en diferentes preparaciones. Más del 80% de los tomates cultivados se consumen en forma de productos procesados, como tomate enlatado entero pelado o en dados, concentrado líquido de tomate, concentrado seco, salsas, purés, pastas, sopas y zumos[6].

1.4. Composición y valor nutricional

La composición físico-química y nutricional del tomate puede verse afectada por diversos motivos, tales como la variedad, las técnicas de cultivo, el grado de madurez del fruto, el grado de exposición a la luz, la temperatura, la disponibilidad de agua, entre otros[7]. La piel y las semillas constituyen el 1% del fruto y entre la materia seca, cabe destacar los azúcares (25% de fructosa y 22% de glucosa), los ácidos orgánicos (principalmente los ácidos málico y cítrico), los elementos minerales y los sólidos insolubles (la celulosa, la hemicelulosa y la pectina)[8]. Teniendo en cuenta su composición en macronutrientes, podría decirse que el tomate es un fruto de bajo valor nutricional; sin embargo es considerado una fuente de nutrientes importante para la salud humana, debido a su alto contenido en compuestos bioactivos.

1.4.1. En macronutrientes

El tomate está compuesto principalmente por agua (aproximadamente 94% en el tomate fresco y casi 96% en el zumo de tomate) y entre los macronutrientes, el mayoritario corresponde a los hidratos de carbono (3,5% en el tomate fresco y 3% en el zumo de tomate), seguido de las proteínas (aproximadamente 1% tanto en el tomate fresco como en el zumo) y por último los lípidos (0,1% en el tomate fresco y la mitad en zumo de tomate)[4].

1.4.2. En micronutrientes

Entre las vitaminas, cabe destacar la vitamina C, con un contenido medio de entre 15 y 19 mg/100 g de producto, seguida de la vitamina E, con un contenido aproximado de 1 mg/100 g. En lo que respecta a los minerales, el potasio es el elemento predominante, con un contenido medio de 236 mg/100 g, tanto en el tomate fresco como en el zumo de tomate[4]. En la **Tabla 1** se muestra la composición nutricional del tomate, así como del zumo de tomate por cada 100 g de producto.

Tabla 1. Composición nutricional del tomate y del zumo de tomate por cada 100 g[4]

	Por 100 g	
	Tomate	***Zumo de tomate***
Macronutrientes		
Energía (Kcal)	19	16
Hidratos de Carbono (g)	3,5	3,0
Proteínas (g)	0,9	0,8
Lípidos totales (g)	0,1	0,05
AG Saturados (g)	Tr	Tr
AG Monoinsaturados (g)	Tr	Tr
AG Poliinsaturados (g)	0,1	0,02
Fibra alimentaria (g)	1,1	0,6
Agua (g)	93,9	95,6
Micronutrientes		
Vitaminas		
Vitamina C (mg)	19	15
Vitamina E: Equivalentes de alfa tocoferol (mg)	0,9	1,0
Vitamina A: Equivalentes de retinol (µg)	82	90
Tiamina (mg)	0,06	0,05
Riboflavina (mg)	0,04	0,02
Equivalentes niacina totales (mg)	0,8	0,8
Vitamina B_6 (mg)	0,1	0,1
Folato total (µg)	29	13
Minerales		
Potasio (mg)	236	236
Fósforo (mg)	22	16
Sodio (mg)	18	4,8
Magnesio (mg)	10	9,5
Hierro (mg)	0,5	0,6
Calcio (mg)	11	15
Zinc (mg)	0,2	0,1
Yodo (µg)	2,2	2,0
Selenio (µg)	0,9	0,6

Tr: Trazas; AG: Ácidos grasos

2. Compuestos bioactivos del tomate

Los compuestos bioactivos son sustancias presentes en los alimentos a una concentración muy baja pero que, no obstante, presentan una actividad biológica en el organismo que contribuye con la preservación de la salud.

De acuerdo con su estructura química, estos compuestos pueden clasificarse en vitaminas, minerales, fibra dietética y fitoquímicos, que a su vez engloban a los compuestos fenólicos, los alcaloides, los compuestos nitrogenados, los compuestos organosulfurados, los fitoesteroles y los carotenoides[9].

Los compuestos bioactivos no pueden ser sintetizados por el cuerpo, por lo que deben ser ingeridos a través de la dieta. En los últimos años se han publicado numerosos estudios epidemiológicos que relacionan el consumo de estos compuestos con una disminución del riesgo de padecer diferentes enfermedades crónico-degenerativas, tales como el cáncer, enfermedades neurodegenerativas, enfermedades cardiovasculares (ECV), entre otras[9–14]. Entre estos compuestos, los carotenoides son uno de los más estudiados y se estima que más del 30% del total de los carotenoides de la dieta son aportados por el tomate y los productos derivados del tomate[15,16].

2.1. Carotenoides. Descripción, estructura química y clasificación

Los carotenoides son una familia de compuestos de más de 600 pigmentos vegetales liposolubles que se encuentran tanto en tejidos fotosintéticos, como no fotosintéticos (animales, bacterias y hongos) y son responsables de las coloraciones amarillas, anaranjadas y rojas que se observan en la naturaleza. Como se ha mencionado anteriormente, los mamíferos no son capaces de sintetizarlos, por lo que necesariamente deben incorporarlos a través de la dieta.

Químicamente, la mayoría de los carotenoides son tetraterpenoides compuestos por 40 átomos de carbono, derivados de la unión de 8 unidades de isoprenoides, unidas de forma que en el centro de la molécula la secuencia se invierte; es decir, la unión de las unidades isoprenoides es de forma “cabeza-cola”, excepto en el centro de la molécula, donde la unión es “cabeza-cabeza”.

Los carotenoides pueden presentar una estructura acíclica, como el licopeno, o poseer estructuras cíclicas de cinco o seis carbonos en uno o ambos extremos de la molécula, como el caso del *β*-caroteno[17].

Dado el gran número de dobles enlaces de la cadena polienoica central, los carotenoides pueden existir en diversas conformaciones *cis/trans* (*Z*/*E*), aunque la más estable y por lo tanto, presente en la naturaleza es la *all-trans* (*all-E*)[18]. Las reacciones

químicas, la exposición a la luz o al calor, también pueden ocasionar isomerización de los carotenoides, pasando de una configuración *trans* a una mono o poli-*cis*. Los isómeros del licopeno que han sido identificados son: *all-trans*, 5-*cis*, 9-*cis*, 13-*cis* y 15-*cis*[19]. Tanto en humanos como en animales, la configuración *cis* parece ser la más biodisponible[20–23].

Considerando los elementos químicos presentes en sus moléculas, los carotenoides pueden dividirse en dos grandes grupos:

- Carotenos: que son propiamente hidrocarburos (compuestos únicamente por hidrógeno y carbono), y
- Xantófilas: derivados de los carotenos que contienen, además, átomos de oxígeno en su molécula. Éstos pueden estar presente en forma de grupo hidroxilo, metoxilo, epóxido, carbonilo o carboxilo, principalmente. Otros grupos oxigenados presentes en los carotenoides pueden ser acetatos, lactonas y sulfatos[17].

En el tomate, las xantófilas predominantes son las que contienen oxígeno en la forma de grupos hidroxilo, como la luteína, la *β*-criptoxantina o la zeaxantina.

La configuración química que adopten los carotenoides será la responsable de su reactividad química, así como de las propiedades de absorción de la luz, y por tanto de su color.

Otra forma de clasificar a los carotenoides es por su capacidad para ser convertidos en vitamina A (retinol) en el organismo; así encontramos a los carotenoides pro vitamínicos y no pro vitamínicos. El número de carotenoides precursores de vitamina A oscila entre 50 y 60, destacando los carotenos (*α*, *β* y *ɣ*-caroteno) y algunas xantófilas (*β*-criptoxantina)[24].

En la **Figura 4** se representa la estructura de los carotenoides más habituales del tomate y de mayor importancia biológica.

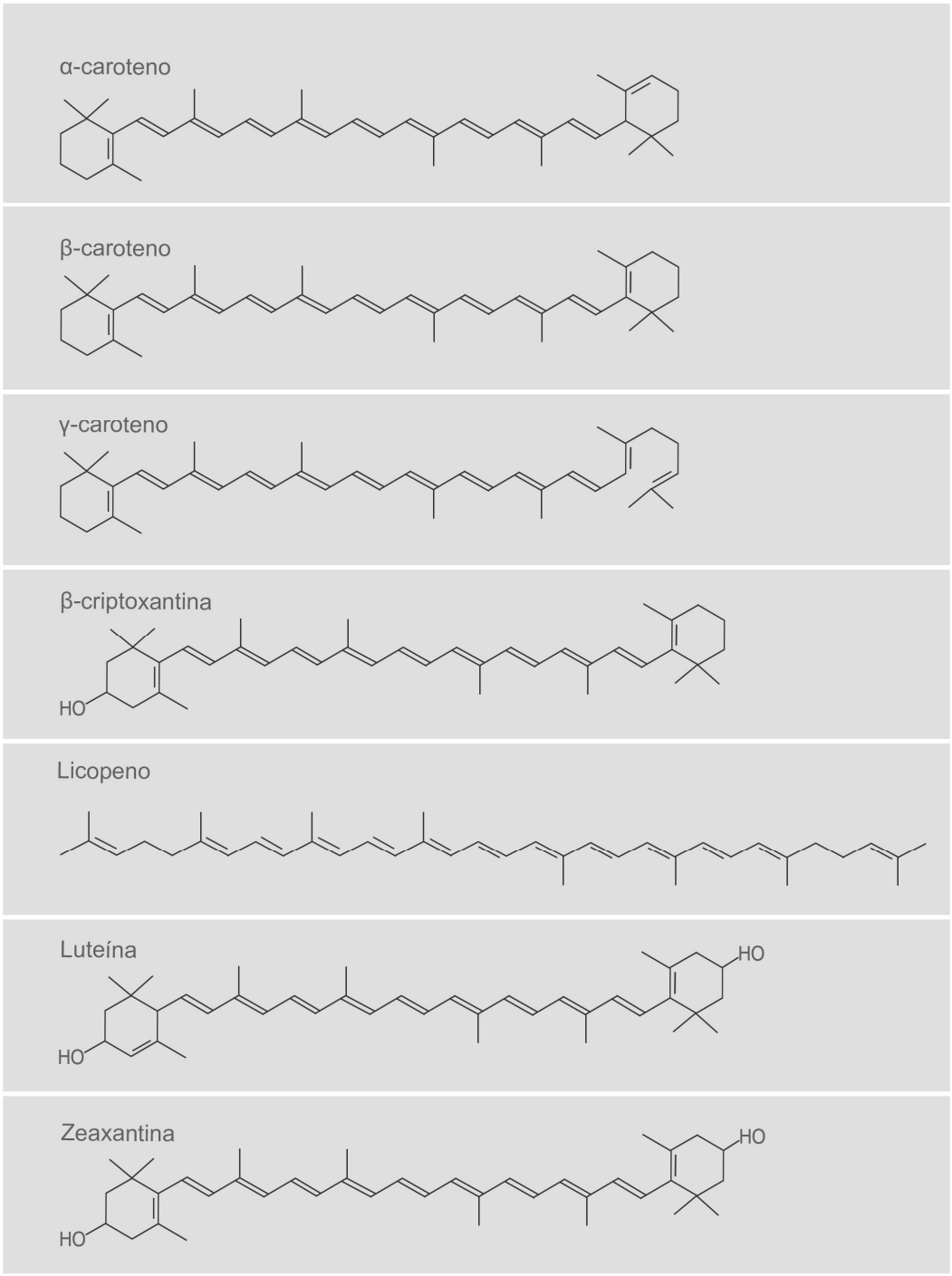

Figura 4. Principales carotenoides del tomate.

2.2. Función y actividad biológica de los carotenoides

Los carotenoides no sólo son responsables del color de muchas flores y frutos, favoreciendo la polinización y dispersión de semillas, sino que desempeñan funciones esenciales en la fotosíntesis y la fotoprotección en las plantas. El papel de

fotoprotección de los carotenoides en las plantas se debe a su capacidad para inactivar las especies reactivas de oxígeno (ROS), especialmente el oxígeno singlete, que se forma a partir de la exposición de la luz y la radiación. Los carotenoides pueden reaccionar con los radicales libres y convertirse en radicales a sí mismos. Como se ha expuesto, su reactividad química se ve influenciada principalmente por la longitud de la cadena de dobles enlaces conjugados y las características de los grupos funcionales finales.

Debido a su naturaleza hidrofóbica, los carotenoides normalmente se encuentran en ambientes lipófilos, como las membranas, aunque los carotenoides más polares, como las xantófilas que contienen oxígeno en su estructura, pueden encontrarse también en medios acuosos, al estar unidos a proteínas o tras haber sufrido procesos de glicosilación o metilación[25,26].

La presencia de carotenoides en las membranas celulares afecta algunas de sus propiedades tales como la rigidez, el grosor, la permeabilidad, la fluidez o la resistencia mecánica[27], lo cual es fundamental para el funcionamiento de éstas.

Aparte de las funciones descritas, a nivel fisiológico y dietético, los carotenoides son importantes por su actividad como pro vitamina A, nutriente esencial para la visión, así como para el mantenimiento de la epidermis , y además de desempeñar un importante rol en el crecimiento y la función reproductiva[28]. La actividad como pro vitamina A depende de la presencia de un anillo *β*-ionona en la molécula. Los carotenoides que contienen como mínimo un anillo de *β*-ionona pueden convertirse en retinol en los animales. De esta forma, el más importante es el *β*-caroteno, que contiene dos de estos anillos. La actividad biológica del anillo de *β*-ionona en los carotenoides cesa por la introducción de un grupo hidroxilo en el mismo y así, la *β*-criptoxantina, por ejemplo, con un anillo de *β*-ionona sustituido por un hidroxilo y el otro intacto, tiene una menor actividad pro vitamínica que el *β*-caroteno. La zeaxantina tiene dos anillos de *β*-ionona hidroxilados, por lo que no actúa como provitamina A.

Sin embargo, el interés que los carotenoides han suscitado en los últimos años se debe a una serie de estudios que demuestran su actividad antioxidante[19,27,29–31]. Desde un punto de vista nutricional, los antioxidantes son sustancias presentes en los alimentos que disminuyen significantemente los efectos adversos de los radicales libres siendo los más comunes, los derivados del oxígeno y del nitrógeno.

En condiciones fisiológicas normales, los radicales libres son producidos en el organismo debido al metabolismo celular; pero una producción excesiva de éstos ha sido implicada en la etiopatogenia y en la progresión de varias enfermedades crónico-degenerativas, tales como el cáncer, las enfermedades neuro-degenerativas y las ECV, entre otras.

Para contrarrestar la producción de radicales libres, nuestro organismo cuenta con un sistema de enzimas antioxidantes que son capaces de neutralizarlos, como por ejemplo la catalasa, la superóxido dismutasa o la glutatión peroxidasa[32], que actúan de manera sinérgica junto con los antioxidantes que consumimos a través de la dieta, entre ellos, los carotenoides. Las propiedades antioxidantes de los carotenoides están asociadas principalmente a sus propiedades de captación y neutralización de radicales libres y su excepcional habilidad para eliminar oxígeno singlete[27].

Entre los carotenoides más representativos del tomate, destacan el licopeno, que ha demostrado ser un potente antioxidante tanto *in vitro* como en animales y humanos[27,33–35] y el *β*-caroteno, cuya función más importante es la de ser un precursor de la vitamina A[36].

2.3. Absorción, transporte y metabolismo de los carotenoides

Tras el consumo de alimentos ricos en carotenoides, la primera fase del proceso de digestión y absorción es la disolución de los mismos en pequeñas gotitas lipídicas, que ocurre principalmente en el estómago y en el duodeno, donde los carotenoides son liberados de la matriz alimentaria e incorporados a micelas –pequeñas moléculas formadas por ácidos biliares y productos de la digestión de los lípidos, tales como colesterol, ácidos grasos libres, mono glicéridos y fosfolípidos- en cantidades dependientes a diversos factores como la polaridad de los carotenoides[37], el pH o la concentración de ácidos biliares[38], así como la presencia de una mínima cantidad de grasas en la comida[39].

Aunque se especulaba que todos los carotenoides se introducían en los enterocitos mediante difusión pasiva, varios transportadores de lípidos han sido identificados en la absorción de carotenoides por la célula intestinal. También se ha sugerido que los carotenoides pueden asociarse a proteínas u otro tipo de estructuras lipídicas (como vesículas y liposomas) y de esta forma ser transportadas hasta el interior del enterocito[40].

Una vez dentro de los enterocitos, una importante cantidad de carotenoides no son metabolizados (puede llegar hasta un 40% de lo que se ha ingerido)[41]. Una parte de los carotenoides con actividad de pro vitamina A son convertidos a retinal, y a su vez el retinal puede convertirse en retinol y luego en ésteres de retinilo, o también pueden ser escindidos, junto con los carotenoides no provitamina A, en apocarotenoides[42].

Como se ha mencionado anteriormente, la actividad como pro vitamina A, viene dada por la presencia y cantidad de anillos *β*-ionona en la molécula del carotenoide, siendo en este caso el *β*-caroteno el más eficiente precursor de la vitamina A, seguido de la *β*-

criptoxantina y el *α*-caroteno, con efectividades de conversión del 50 y el 30% respectivamente. No se han observado isomerizaciones *cis-trans* dentro de los enterocitos, por lo que se cree que las isomerizaciones que puedan darse en los carotenoides se producen en el lumen intestinal. El licopeno por su parte, puede sufrir oxidación tanto por acción de las lipooxigenasas, así como por autooxidación, produciendo acicloretinoides y apolicopenales[43,44]. Los carotenoides se incorporan a quilomicrones para ser liberados al sistema linfático y por medio del sistema linfático mesentérico llegan a la circulación sistémica para distribuirse a los órganos y tejidos que los utilizan o almacenan, como el hígado, el tejido adiposo, las glándulas adrenales y la próstata. Finalmente son eliminados por medio de las heces.

2.4. Biodisponibilidad de los carotenoides

La biodisponibilidad de los carotenoides se refiere a la cantidad de carotenoides ingeridos que está disponible en la circulación sistémica para su utilización en las funciones fisiológicas normales o para almacenamiento en el cuerpo humano[45]. Existen diversas condiciones que pueden afectarla, como los factores intrínsecos y extrínsecos.

2.4.1. Factores intrínsecos

a. Matriz alimentaria y técnica de procesamiento

Con respecto a la matriz alimentaria, varios aspectos pueden influir en el contenido de carotenoides en un alimento, y por tanto en su posterior biodisponibilidad, como por ejemplo la exposición al sol, la accesibilidad al agua durante el cultivo, los diferentes tipos de cultivos, el grado de madurez, la variedad, entre otros[46,47]. En los alimentos, los carotenoides se encuentran almacenados en organelos celulares especializados llamados cloroplastos y cromoplastos, formando complejos que los ayudan en su papel estructural y funcional; por lo tanto la liberación de la matriz alimentaria es un paso clave en la biodisponibilidad de los carotenoides. En este sentido, el procesamiento de los alimentos puede jugar un papel importante, al romper las membranas celulares de la matriz y permitir la liberación de los carotenoides desde los organelos donde se almacenan. Numerosas investigaciones se han llevado a cabo para determinar la biodisponibilidad de carotenoides de los tomates tras la aplicación de distintas técnicas de procesamiento. Los distintos tratamientos aplicados modifican de manera significativa el contenido de fitoquímicos de los alimentos[48]; aunque los resultados observados han sido contradictorios, dependiendo del tratamiento aplicado,

así como del carotenoide estudiado[45,49–52]. Así también se ha visto que el almacenaje puede afectar el contenido de carotenoides de los alimentos, observándose pérdidas proporcionales al tiempo de almacenamiento[53,54].

b. Tipo de carotenoide y estructura química

La biodisponibilidad de los carotenoides puede verse afectada por su propia estructura química[38,49,55]. Por ejemplo, se ha observado que la absorción de los carotenos, tales como el *β*-caroteno, es relativamente menor que la de los carotenoides oxigenados, como la luteína y la zeaxantina. Debido a su naturaleza más polar, los carotenoides oxigenados pueden ser más fácilmente incorporados a la superficie exterior de las micelas de lípidos en el tracto gastrointestinal y por lo tanto, llegar con más facilidad a las membranas de los enterocitos y, finalmente, a los quilomicrones, aumentando su biodisponibilidad. Asimismo, la ubicación del carotenoide dentro del alimento puede afectar su biodisponibilidad, debido a las barreras físicas que dificultan la liberación y emulsión de los mismos[56].

Otro factor que influye en la biodisponibilidad de los carotenoides es su configuración *cis/trans*. Como se ha señalado previamente, en los vegetales crudos, la configuración *trans* del *β*-caroteno y del licopeno es la predominante; sin embargo, en el caso del licopeno la forma *cis* es mayoritaria tanto en el plasma humano como en los tejidos, sugiriendo esto una isomerización en el organismo y una mejor absorción que la forma *trans*[20,57,58]. En el caso del *β*-caroteno, la forma predominante en el plasma es la *trans*, pero en los tejidos, la proporción de la forma *cis* es mayor.

2.4.2. Factores extrínsecos

a. Interacción con otros componentes de la dieta

La co-ingestión de distintos carotenoides de la dieta puede llegar a afectar la absorción de los mismos por diferentes mecanismos. Los carotenoides pueden interactuar entre sí en cualquier momento durante la absorción, el metabolismo, y el proceso de transporte[38,59].

Otros factores que pueden afectar la biodisponibilidad de los carotenoides es la presencia de grasas y fibras en la dieta. Se ha sugerido que una mínima cantidad de entre 3 y 5 g de grasas en cada comida asegura la absorción de carotenoides, pero esto depende del tipo de grasa ingerida y de las características físico-químicas del carotenoide[60–64]. La fibra por su parte puede influir en la viscosidad de las emulsiones de carotenoides en el tracto gastrointestinal, así como modificar el tamaño y la distribución de las gotitas lipídicas de estas emulsiones; o bien, interferir con la

absorción al interaccionar con los carotenoides y ácidos biliares, resultando en un aumento de la excreción fecal de grasas y nutrientes solubles en grasa, como los carotenoides.

Por otro lado, el consumo de alcohol puede aumentar la absorción de ciertos carotenoides como el *β*-caroteno, al ser una sustancia pro oxidante; sin embargo se ha visto que disminuye la bioconversión del mismo en vitamina A[60].

Algunos otros condicionantes de la biodisponibilidad de los carotenoides son el estado nutricional de las personas[65,66], la dieta[67,68], así también como la edad[69].

b. Factores relacionados con el sistema digestivo

Entre las condiciones que pueden influir en la absorción y biodisponibilidad de los carotenoides, la posible existencia de variantes genéticas que codifican las proteínas de transporte de estos nutrientes, desempeña un rol fundamental. Las variaciones genéticas que conducen a modificaciones en la región promotora del gen o dentro de la secuencia de aminoácidos de la proteína pueden afectar a su expresión y/o actividad, y por lo tanto, su capacidad para absorber y transportar a sus ligandos, lo cual podría explicar la amplia variabilidad interindividual en la asimilación de carotenoides[70–73].

Como ya se ha explicado, los carotenoides necesitan ser emulsionados para poder ser transportados en forma de micelas hasta el borde del cepillo de los enterocitos, por lo que el contenido de ácidos biliares y el pH del intestino también juegan un papel importante en la absorción y posterior biodisponibilidad de los carotenoides[38].

2.5. Extracción y análisis de carotenoides

La identificación y cuantificación de carotenoides en alimentos y muestras biológicas es una tarea compleja debido a la alta variabilidad en sus estructuras químicas, la inestabilidad de estos compuestos, la falta de estándares disponibles en el mercado, las concentraciones generalmente bajas encontradas en las muestras biológicas, tales como el plasma y los tejidos humanos, y la presencia de compuestos potencialmente interferentes en las muestras. Para una mejor comprensión sobre las técnicas empleadas para la extracción y el análisis de los carotenoides, éstas se presentan a continuación agrupadas de acuerdo al tipo de muestra.

2.5.1. En alimentos

Para la extracción de carotenoides de matrices alimentarias, no existe una única técnica o método, debido a que su distribución en los distintos alimentos o partes de un mismo alimento varía considerablemente. Lo más común es la utilización de la técnica de extracción líquido-líquido y el uso de distintos solventes como acetona, etanol, tetrahidrofurano, hexano, tolueno, éter de dietilo, metanol, acetato de etilo, diclorometano, éter de petróleo, cloroformo y butanol, solos o aplicados en mezclas como por ejemplo, acetona/etanol[74,75], acetona/hexano[76], acetona/éter de petróleo[77], hexano/dietiléter[78,79], hexano/etanol/acetona[80], hexano/etanol/ acetona/tolueno[81], metanol/hexano/diclorometano[82], hexano/etil acetato[83], y metanol/THF[84]. Sin embargo, etanol/hexano (4:3) ha sido una de las combinaciones más comunes para las muestras de alimentos[55,85–88].

Otras técnicas descritas en la literatura son la extracción de fluidos supercrítica[89], extracción de líquido presurizado[90], extracción acelerada de solventes[91], extracción con agua caliente presurizada[92], extracción asistida por ultrasonido[93], extracción asistida por microondas[94], y extracción en fase sólida[95].

2.5.2. En muestras biológicas

Para la extracción de carotenoides, específicamente del plasma, el disolvente más comúnmente empleado es el *n*-hexano[96], o bien una mezcla de *n*-hexano y otros disolventes, como por ejemplo, hexano/éter[97], hexano/etanol/acetona/tolueno[98], hexano/diclorometano[99], hexano/cloroformo[100], mezclas de hexanoacetato de etilo[101], o también heptano[102] o tetrahidrofurano[95]. Para evitar la oxidación de los carotenoides, es necesaria la adición de un antioxidante durante el proceso de extracción. El más utilizado es el butilhidroxitolueno (BHT), aunque también suelen usarse el pirogalol, el butilhidroxianisol, el ácido ascórbico y la butilhidroquinona. Para la precipitación de las proteínas del plasma por lo general se utiliza etanol, ya que las mismas pueden causar obstrucciones dentro de las columnas e interferir con la separación cromatográfica.

Para la identificación y cuantificación de carotenoides en las distintas matrices, varios métodos han sido utilizados, incluidos los métodos espectrofotométricos[103], la electroforesis capilar[104], la resonancia magnética nuclear[105], la espectroscopía de infrarrojos[106], y las técnicas cromatográficas tales como la cromatografía de fluidos supercríticos[107], la cromatografía de gases[108], la cromatografía líquida de alta resolución (HPLC)[109] y, más recientemente, la cromatografía líquida de ultra alta resolución[110].

La cromatografía líquida de alta resolución permite el acoplamiento con diferentes detectores, como el ultravioleta (UV)[111], la fluorescencia[112], índice de refracción[113], y espectrometría de masas[114]. Actualmente, la mejor herramienta de análisis para cuantificar y caracterizar los compuestos carotenoides es la cromatografía líquida de alta resolución combinada con detección ultravioleta.

Las columnas monoméricas de fase estacionaria octil (C_8) y octadecil (C_{18}) son las más utilizadas para la cromatografía de fase inversa, siendo especialmente buenas para la separación de analitos de cadena corta y bajo peso molecular. Sin embargo, tienen el inconveniente de presentar una pobre resolución de los isómeros geométricos *cis-trans*. No obstante, las columnas C_{30} con cadenas alquilo más largas, proporcionan una mejor forma y selectividad para los analitos de cadena larga, por lo que han demostrado una mejora significativa, y con frecuencia superior, en la separación y la selectividad que las columnas C_8 y la C_{18}, demostrando ser una mejor opción para la separación y resolución de los isómeros geométricos de los carotenos menos polares, principalmente el licopeno y el *β*-caroteno[115].

2.6. Carotenoides y enfermedad cardiovascular

En las últimas décadas, diversos estudios epidemiológicos, tanto observacionales, como de casos y controles, así como ensayos clínicos han sugerido que los niveles plasmáticos elevados de carotenoides se relacionan con un menor riesgo de desarrollar ECV[116–118]. Todos estos estudios poseen una elevada evidencia científica debido a la metodología empleada, el número de voluntarios incluidos, el tiempo de seguimiento de los mismos y la similitud de los resultados en distintas poblaciones como por ejemplo, en europeos, americanos o en asiáticos. En la **Tabla 2** se presenta un resumen de diversos estudios en los que se ha demostrado los efectos beneficiosos de los carotenoides sobre parámetros de riesgo cardiovascular.

Tabla 2. Estudios de los efectos beneficiosos de los carotenoides sobre la salud cardiovascular.

Autor	***Tipo de estudio***	***Características del estudio***	***Voluntarios***	***Carotenoide estudiado***	***Resultados obtenidos***
Zou *et al*., 2016[119]	Ensayo clínico randomizado	Determinar asociación entre los niveles plasmáticos tras una suplementación con luteína (20 mg), luteína (20 mg) + licopeno (20 mg) o placebo y el grosor de la íntima media de la carótida en una población china	144 voluntarios ♀ 62 ♀ 82 45-68 años	Luteína y licopeno	Disminución del grosor de íntima media de la carótida en ambos grupos suplementados, pero con una mejor respuesta en el grupo suplementado con la combinación de luteína y licopeno
Karppi *et al*., 2013[120]	Observación de una cohorte del *The Kuopio Ischaemic Heart Disease Risk Factor Study (KIHD)*	Determinar asociación entre los niveles plasmáticos de carotenoides y la progresión del grosor de la íntima de la carótida en hombres finlandeses	840 voluntarios ♀ de 46-65 años	Licopeno, α-caroteno, β-caroteno	Disminución del grosor de íntima media de la carótida en individuos que se encontraban en los tertiles más elevados de carotenoides plasmáticos
Xu *et al*., 2012[121]	Casos y controles de una cohorte proveniente de *The Beijing Atherosclerosis Study*	Determinar asociación entre los niveles plasmáticos de carotenoides y la expresión de moléculas de inflamación y de adhesión (Apolipoproteína E, MCP-1, IL-6, endotelina 1, IFN-ɣ, VCAM-1)	40 voluntarios con aterosclerosis subclínica y 40 controles de ♀ 30 ♀ 50 55-60 años	Luteína, zeaxantina, β-caroteno y licopeno	Correlación inversa entre la luteína y la IL-6; asociación inversa entre zeaxantina, licopeno y VCAM-1
Riccioni *et al*., 2011[122]	Casos y controles de una cohorte proveniente de *The Manfredonia Study*	Comparar los niveles de licopeno plasmático entre individuos con y sin evidencia ultrasónica de aterosclerosis subclínica	58 voluntarios con aterosclerosis asintomática ♀ 34 ♀ 24 62 voluntarios sanos 41-62 años	Licopeno	Bajos niveles plasmáticos de licopeno en pacientes con aterosclerosis asintomática, así como niveles plasmáticos elevados de LDL-colesterol, colesterol total y triglicéridos

Zou *et al.*, 2011[123]	Casos y controles de una cohorte proveniente de *The Beijing Atherosclerosis Study*	Determinar asociación entre los niveles plasmáticos de carotenoides, el grosor de la íntima de la carótida y la rigidez de la carótida	125 voluntarios con aterosclerosis subclínica y 107 voluntarios sanos 45-68 años	Luteína, zeaxantina y β-caroteno	Asociación inversa entre la luteína y el grosor de la íntima de la carótida; asociación inversa entre zeaxantina y β-caroteno y la rigidez de la carótida
Koh *et al.*, 2011[124]	Casos y controles anidados de una cohorte proveniente de *The Singapore Chinese Health Study*	Determinar la asociación entre niveles plasmáticos de carotenoides, vitaminas A y E y la incidencia de infarto agudo de miocardio (IAM) en una población china	280 voluntarios que han sufrido un IAM ♂ 188 ♀ 92 y 560 voluntarios sanos (2 por cada caso) 45-74 años	α-caroteno, β-caroteno, β-criptoxantina, luteína, zeaxantina, licopeno	Asociación inversa entre niveles plasmáticos de β-criptoxantina y luteína y riesgo de IAM
Riccioni *et al.*, 2010[125]	Casos y controles	Determinar asociación entre niveles de antioxidantes plasmáticos, inflamación sistémica y grosor de la íntima de la carótida en pacientes con enfermedad renal	40 voluntarios	Licopeno y β-caroteno	Asociación inversa entre niveles plasmáticos de licopeno, β-caroteno y PCR; asociación inversa entre niveles plasmáticos de licopeno y β-caroteno y grosor de la íntima de la carótida
Sesso *et al.*, 2004[118]	Casos y controles de una cohorte proveniente de *The Women's Health Study*	Examinar la asociación entre los niveles de licopeno plasmático y el riesgo de ECV en mujeres norteamericanas sanas	28345 voluntarias ♀ de 58-66 años	Licopeno	Las mujeres que se encontraban en los 3 cuartiles superiores de concentración de licopeno plasmático presentaron un 50% menos de riesgo de padecer una ECV que las que se encontraban en el cuartil más bajo
Rissanen *et al.*, 2001[126]	Transversal de una cohorte del *The Kuopio Schaemic Heart Disease Risk Factor Study*	Determinar asociación entre las concentraciones de licopeno plasmático y el grosor de íntima media de la carótida en hombres finlandeses	725 voluntarios ♂ de 46-64 años	Licopeno y β-caroteno	Disminución del grosor de íntima media de la carótida en individuos que se encontraban en los cuartiles más elevados de licopeno plasmático

3. Las enfermedades cardiovasculares

Las ECV engloban a un conjunto de patologías del corazón, enfermedades vasculares del cerebro y enfermedades de los vasos sanguíneos. Las mismas pueden sub clasificarse en:

a) ECV debidas a arteriosclerosis:
   - Cardiopatía isquémica o enfermedad de las arterias coronarias (como por ejemplo, infarto de miocardio),
   - Enfermedad cerebrovascular (por ejemplo, derrame cerebral o embolia), y
   - Enfermedades de la aorta y las arterias (hipertensión arterial y enfermedad vascular periférica).

b) Otras ECV:
   - Cardiopatía congénita
   - Cardiopatía reumática
   - Cardiomiopatías
   - Arritmias cardiacas

De acuerdo con el último informe de la Organización Mundial de la Salud (OMS), las ECV han sido responsables del 46,2% de todas las muertes por enfermedades no transmisibles ocurridas durante el año 2012, lo que equivale a un total de 17,5 millones de muertes en todo el mundo[127], de las cuales las ECV debidas a arteriosclerosis han sido responsables del 86% de muertes en hombres y 82% en mujeres[128].

3.1. La aterosclerosis. Etiopatogenia

La aterosclerosis es un proceso patológico bastante complejo en el que se desarrolla una placa de ateroma en las paredes de los grandes vasos sanguíneos a lo largo de los años, y en el cual están implicados múltiples procesos oxidativos, inflamatorios y de remodelación vascular[129].

En este proceso están involucrados diferentes tipos celulares, tanto de la propia pared vascular (células endoteliales y células musculares lisas); células sanguíneas (monocitos y linfocitos); así como distintas citoquinas, quimoquinas y factores de crecimiento. Todas las moléculas se comunican entre sí por intermedio de moléculas de adhesión que incluyen selectinas, integrinas e inmunoglobulinas que se expresan en la superficie de éstas células.

Existen diversos factores de riesgo que pueden promover el desarrollo de la aterosclerosis, entre los que se encuentran los factores no modificables, como la edad o ciertos condicionantes genéticos, y los factores modificables, como por ejemplo la hipertensión arterial, la obesidad y el sobrepeso, la diabetes mellitus, la dislipidemia, el estrés, el tabaquismo, la ausencia de actividad física regular o el consumo de una dieta inadecuada, alta en grasas saturadas, sal y un bajo consumo de frutas y verduras[130,131].

Actualmente, es aceptada la hipótesis de que una reacción inflamatoria crónica de los vasos sanguíneos en respuesta a la exposición continuada a uno o varios de estos factores de riesgo, junto con las lesiones comunes endoteliales representan el punto de partida en el desarrollo de la aterosclerosis.

A continuación se presentan las distintas fases de la aterogénesis o formación de la placa de ateroma, que deriva posteriormente en el establecimiento de la aterosclerosis.

3.1.1. Desequilibrio en el metabolismo lipídico y respuesta inflamatoria en la pared vascular

Dado que los lípidos son sustancias insolubles en agua, necesitan unirse a proteínas para ser transportados por el torrente sanguíneo, formando complejos denominados lipoproteínas.

El colesterol que es ingerido a través de la dieta, viaja por el torrente sanguíneo hasta el hígado donde será metabolizado y empaquetado en quilomicrones. El hígado también produce colesterol, que se transporta en fracciones de lipoproteínas de muy baja densidad (VLDL) para llegar a los distintos tejidos que precisan del colesterol y otros sustratos grasos para su normal funcionamiento[132]. Estas partículas de VLDL llevan unida una apo proteína, llamada Apo B (B100 o B48 dependiendo de si proceden del hígado o del intestino respectivamente) que actúa como ligando en los receptores LDL de varias células[133]. Tras depositar su contenido graso en los distintos tejidos, las VLDL se vuelven más densas, denominándose lipoproteínas de densidad intermedia (IDL). A medida que va vaciándose de su contenido graso, estas partículas se vuelven aún más densas, pasando a llamarse lipoproteínas de baja densidad (LDL). Durante el metabolismo de las lipoproteínas, las fracciones LDL una vez vaciadas de su contenido, son transportadas de regreso al hígado, donde se incorporan a los hepatocitos, que también cuentan con receptores LDL, donde finalmente son degradados[134]. El ciclo metabólico de los lípidos se reinicia con la formación de nuevas lipoproteínas VLDL por el hígado.

Sin embargo, en estados de dislipidemia como ocurre en la hipercolesterolemia, las partículas LDL son infiltradas en gran cantidad al sub endotelio de la íntima vascular, causando una respuesta inflamatoria e iniciándose una serie de modificaciones oxidativas de estas lipoproteínas[135].

3.1.2. Modificación de las LDL y activación del endotelio

El proceso aterosclerótico continúa por interacciones entre las LDL, específicamente entre la fracción Apo B100 y Apo B48 de las LDL y las proteínas de la matriz extracelular de la pared vascular, o más concretamente los proteoglicanos[135]. En los vasos sanguíneos humanos, los proteoglicanos se encuentran principalmente en áreas donde existe hiperplasia de la íntima, que es un engrosamiento de la capa más interior de la pared vascular, debido a una acumulación de células del músculo liso vascular[136].

Las LDL infiltradas son propensas a ser oxidadas por enzimas y agentes oxidantes (como el anión superóxido, el peróxido de hidrógeno, los peroxinitritos, el ozono, entre otros), produciendo la liberación de fosfolípidos que a su vez desencadena la activación de las células endoteliales, proceso implicado en la expresión de ciertas moléculas de adhesión de leucocitos al endotelio vascular, tales como las moléculas de adhesión intercelular 1 (ICAM-1) y las moléculas de adhesión vascular 1 (VCAM-1)[137], así como las E-selectinas y las P-selectinas[138].

3.1.3. Agregación de monocitos y diferenciación a macrófagos

Ciertas células inmunológicas, como los monocitos y los linfocitos, cuentan con receptores para las moléculas de adhesión, lo que las hace propensas a adherirse al endotelio donde están expresadas. Particularmente, los monocitos van rodando a lo largo de la superficie del endotelio hasta adherirse a las zonas donde este está activado[139]. Una vez adheridos, la íntima produce quimoquinas que estimulan la migración de los mismos al espacio subendotelial, mediante un proceso denominado transmigración y diapédesis.

Cuando ya han transmigrado al subendotelio vascular, los factores estimulantes de colonias de macrófagos, estimulan la diferenciación de monocitos a macrófagos, lo cual es considerado un paso crítico en la aterosclerosis. Durante este proceso, existe una sobre expresión de receptores de reconocimiento de la inmunidad innata, como los receptores *scavenger* (SR) y los receptores tipo Toll (TLR), respectivamente[140]. Mediante la expresión de los SR, los macrófagos tienen la capacidad de unirse a

elementos potencialmente tóxicos para la célula, como por ejemplo bacterias, endotoxinas, fragmentos apoptóticos de otras moléculas, entre otros, e internalizarlas; y es por este mismo mecanismo por el cual las LDL oxidadas (oxLDL) son destruidas y eliminadas[139].

3.1.4. Formación de células espumosas

Una vez internalizadas por los macrófagos, las oxLDL son hidrolizadas y metabolizadas en los lisosomas por acción de distintas enzimas, dejando libres moléculas de colesterol o bien ésteres de colesterol. La acumulación de colesterol dentro de los macrófagos es lo que le concede una apariencia espumosa característica. El colesterol libre es luego modificado a una forma más soluble, o bien transportado fuera del espacio sub endotelial, donde las lipoproteínas de alta densidad (HDL) juegan un papel muy importante como receptoras de este colesterol para su transporte reverso al hígado[141].

El depósito de células espumosas en el endotelio deriva en la formación de estrías lipídicas, que son lesiones típicas de la aterosclerosis en estadios tempranos. Sin embargo, la continua captación de moléculas de oxLDL produce estrés en el retículo endoplásmico de los macrófagos, y finalmente las células espumosas mueren apoptóticamente, liberando colesterol, junto con sustancias pro inflamatorias y pro trombóticas, y formando así lo que se conoce como núcleo necrótico[142]. Dado que estas sustancias son extrañas en este compartimento vascular, se desencadena una serie de reacciones con el fin de proteger a la íntima; células musculares lisas (SMC), provenientes de la capa adventicia de la arteria, migran hasta la zona afectada y crean una capa fibrosa sobre este núcleo necrótico, de manera a encapsularlo y aislarlo del entorno[143].

3.1.5. Progresión de las lesiones y respuesta inmunológica

Para la progresión de las lesiones ateroscleróticas, los TLR juegan un papel muy importante, ya que son los que inician una señalización en cascada que produce la activación de varios tipos celulares como macrófagos, células dendríticas, mastocitos, células del endotelio, entre otras, produciendo citoquinas inflamatorias, proteasas y sustancias citotóxicas[140], desencadenándose así un estado inflamatorio generalizado. En este paso, también los neutrófilos contribuyen a la expansión de la lesión aterosclerótica, estimulando la adhesión de monocitos al endotelio vascular y su posterior migración a la íntima, retroalimentando todo el proceso.

Debido al carácter crónico de este proceso, se van formando lesiones focales o placas, que en fases avanzadas pueden llegar a ocluir la luz de los vasos sanguíneos de forma directa o bien por complicaciones aterotrombóticas.
Las complicaciones dependerán del tipo de placa y de su tamaño. Las placas más peligrosas son aquellas vulnerables a rupturas, que son las que contienen un núcleo lipídico mayor y una cubierta fibrosa fina[144], así como una elevada cantidad de células inflamatorias que liberan citoquinas, haciendo que las SMC, produzcan metaloproteasas, unas enzimas que degradan la matriz de la cubierta fibrosa, dando lugar a la ruptura de la placa, responsable de las manifestaciones clínicas de la aterosclerosis, como los síndromes coronarios agudos y el infarto agudo de miocardio[143,145].

3.2. Principales moléculas implicadas en la patogenia de la aterosclerosis

Como se ha observado, la aterosclerosis es una patología compleja en la que participan numerosos componentes tanto vasculares, como metabólicos e inmunológicos.
Como ya hemos señalado, los cambios más precoces que anteceden a la formación de la placa de ateroma, se sitúan en el endotelio. Esta alteración del endotelio aumenta la permeabilidad a las lipoproteínas, proceso mediado por múltiples sustancias tales como el óxido nítrico, las prostaciclinas, el factor de crecimiento derivado de las plaquetas, la angiotensina II y la endotelina.
Asimismo, hay una sobre regulación de moléculas de adhesión leucocitaria, tales como la L-selectina y las integrinas, así como de moléculas de adhesión del endotelio, tales como las selectinas E y P y las moléculas de adhesión intercelular. También hay un aumento en la migración de leucocitos dentro de la pared vascular, mediada por la LDL oxidada, las proteínas quimiotácticas para los leucocitos, la interleuquina 8 (IL-8), el factor derivado de las plaquetas y el factor estimulante de la colonia de macrófagos[145,146]. Otras moléculas quimiotácticas que están siendo estudiadas, dado que se ha observado que participan activamente en el reclutamiento y migración de leucocitos y en la modulación de la expresión de macrófagos en el proceso aterosclerótico son la eotaxina y la CXC quimoquina 10 (CXCL10)[147–149].
El progreso de la aterosclerosis, desde un estadio inicial de estrías lipídicas al establecimiento de lesiones avanzadas, implica un proceso de varias fases que incluye la migración de células musculares lisas, estimulada por el factor de crecimiento derivado de las plaquetas y de los fibroblastos, y por el factor de crecimiento β; la activación de células T, mediada por el factor de necrosis tumoral (TNF-α), la

interleuquina 2 (IL-2) y el factor estimulante de las colonias de macrófagos; la formación de células espumosas, mediante las LDL oxidadas, el factor estimulante de colonias de macrófagos, el TNF-*α* y la interleuquina 1 (IL-1); y finalmente, la adhesión y agregación plaquetaria, estimulada por las integrinas, la selectina P, la fibrina, el tromboxano A2, el factor tisular y los factores de adhesión y migración de leucocitos citados anteriormente.

A medida que las estrías lipídicas progresan hacia lesiones más avanzadas tienden a desarrollar una cápsula fibrosa que las separa de la luz, considerándose una forma de cicatrización o de respuesta al daño vascular[145]. Esta cápsula fibrosa es la que cubre el núcleo necrótico y va creciendo por sus bordes, estimulada por los mismos factores que han sido descritos. La cápsula fibrosa se produce por una actividad incrementada del factor de crecimiento derivado de las plaquetas, el factor de crecimiento *β*, el TNF-*α* y por una degradación del tejido conectivo. En las etapas avanzadas de la enfermedad, la rotura de la cápsula fibrosa puede precipitar la trombosis coronaria.

En todo el proceso de la aterogénesis, la inflamación desempeña un papel trascendental[150,151]. Diversos biomarcadores se han estudiado con el fin de determinar el estado inflamatorio, tanto a nivel local como sistémico, entre los que podemos citar a los reactantes de fase aguda, como la proteína C reactiva (PCR), las citoquinas pro y antiinflamatorias (interleucinas), las metaloproteinasas, los marcadores de activación plaquetaria y leucocitaria, entre otros.

Esta tesis doctoral se ha focalizado en evaluar el posible efecto cardioprotector de los carotenoides del tomate, como modulador de la PCR, importante marcador de inflamación sistémica, así como de las moléculas de adhesión ICAM-1, VCAM-1, la citoquina interferón gamma (IFN-*γ)*, y las quimoquinas IL-8, eotaxina y CXCL10, implicadas en el desarrollo y la progresión de la aterosclerosis.

Materiales y métodos

IV. Materiales y métodos

1. Diseño del estudio

a) Validación del método cromatográfico: Estudio piloto realizado con ocho voluntarios de alto riesgo cardiovascular

Para poder interpretar correctamente los resultados de estudios epidemiológicos y ensayos clínicos en los que se busca establecer una relación entre alimentación y salud, es importante contar con herramientas sensibles, capaces de realizar una correcta identificación y cuantificación de los compuestos alimentarios de interés. Es por esto, que en esta tesis doctoral se ha desarrollado y validado un método cromatográfico que permite la identificación y cuantificación de carotenoides en plasma humano y, para corroborar su aplicabilidad a ensayos clínicos con muestras reales, posteriormente se utilizó este método para el análisis de muestras de plasma de voluntarios procedentes de un estudio piloto realizado con 8 voluntarios adultos, de alto riesgo cardiovascular, quienes consumieron 250 mL de zumo de tomate durante 4 semanas.

Antes de iniciar el estudio, los voluntarios debieron abstenerse del consumo de tomate y productos derivados de tomate, así como de alimentos ricos en carotenoides durante 1 semana, de modo que las concentraciones plasmáticas de carotenoides pudieran estar en su nivel más bajo al momento de iniciar el estudio y evitar de esta forma errores por sesgo en la interpretación de los resultados del estudio.

Durante las 4 semanas de intervención dietética, todos los voluntarios fueron instruidos de consumir una dieta equilibrada, siguiendo los lineamientos de una dieta mediterránea y tanto al inicio como al final del estudio, se recogieron muestras de sangre para analizar las concentraciones plasmáticas de carotenoides con el método validado.

b) Estudio de intervención con pacientes de alto riesgo cardiovascular

Diversos estudios han demostrado que la configuración *cis* es la más biodisponible y que por lo tanto, es la responsable de los efectos beneficiosos del consumo de tomate sobre la salud cardiovascular. Sin embargo, no existen estudios en los que se analice la implicancia de los diferentes isómeros sobre las moléculas inflamatorias y de adhesión vascular, relacionadas con la aterosclerosis.

Por ello, se llevó a cabo un estudio abierto, prospectivo, aleatorizado, cruzado y controlado para evaluar los efectos del consumo de diferentes dosis diarias de zumo

de tomate durante 4 semanas, sobre biomarcadores inflamatorios de la aterosclerosis en pacientes sin ECV diagnosticada, pero con alto riesgo de desarrollarla y, de esta forma, poder determinar una dosis óptima de consumo que permita la modulación de las moléculas inflamatorias relacionadas con el desarrollo y la progresión de la aterosclerosis y, a su vez, elucidar el efecto de los isómeros *cis/trans* sobre estas moléculas.

Después de un periodo de 3 semanas, en el que se pidió a los participantes evitar el consumo de tomate o productos derivados del mismo, así como alimentos ricos en carotenoides que pudieran interferir con los resultados del estudio, los voluntarios consumieron: a) dosis baja (DB), 200 mL de zumo de tomate/día consumidos con la comida; o b) dosis alta (DA), 400 mL de zumo de tomate/día, repartidos entre comida y cena; o c) la misma cantidad de agua como intervención control, de acuerdo con la aleatorización correspondiente. Entre cada intervención hubo un período de lavado de 21 días, para asegurar que los niveles plasmáticos de carotenoides volvieran a las condiciones basales antes de iniciar la siguiente intervención.

Un total de 28 pacientes, con una edad media de 69,7 ± 3,1 años y una media de Índice de masa corporal (IMC) de 31,5 ± 3,6 kg/m^2 participó en el estudio. Los criterios de inclusión fueron: edad comprendida entre 55 y 80 años, hipertensión arterial diagnosticada, sin ECV establecida al momento de iniciar el estudio (enfermedad isquémica del corazón -angina o infarto de miocardio reciente o antiguo, accidente cerebrovascular, enfermedad vascular periférica), pero con alto riesgo de desarrollarla; es decir, que haya presentado dos o más de los siguientes factores de riesgo: a) tabaquismo, b) hipercolesterolemia (LDL-colesterol >160 mg/dL), c) bajos niveles de HDL-colesterol (valores <40 mg/dL), d) obesidad o sobrepeso (IMC >25 kg/m^2), e) diabetes tipo II, y/o f) antecedentes familiares de enfermedad cardíaca temprana (parientes de primer orden, hombres <55 años de edad o mujeres <65 años). Se excluyeron aquellos voluntarios con antecedentes de ECV, cualquier enfermedad crónica grave, alcoholismo u otras adicciones, o alergia/intolerancia al tomate.

Se recogieron muestras de sangre en ayunas (10 mL) al iniciar el estudio y después de cada intervención. Todas las muestras obtenidas fueron inmediatamente centrifugadas a 1500 g durante 15 min a 4 ºC para separar el plasma. Finalmente, el plasma se alicuotó y se almacenó a -80 ºC hasta el momento del análisis.

En la **Tabla 3** se presentan las características de los voluntarios que participaron en el estudio.

Tabla 3. Características de los voluntarios al inicio del estudio *(n=28)*.

Variables	***Total (n=28)***
Sexo	
Masculino *(n)*	11
Femenino *(n)*	17
Edad (años)	69,7 ± 3,1[a]
Hábitos tabáquicos	
Nunca han fumado *(n)*	15
Ex fumadores *(n)*	8
Fumadores activos *(n)*	5
IMC (kg/m^2)	31,5 ± 3,6[a]
25-30 *(n)*	13
>30 *(n)*	15
Hipertensión *(n)*	28
Diabetes tipo II *(n)*	11
Dislipemia *(n)*	21
Historia familiar de ECV prematura *(n)*	4
Medicación utilizada	
Antihipertensivos *(n)*	8
Insulina *(n)*	4
Hipoglucemiantes orales *(n)*	13
Antiagregantes plaquetarios *(n)*	8
Agentes hipolipemiantes *(n)*	22

[a]Los valores están expresados como medias ± DE; DE: Desviación estándar

2. Preparación y recepción del zumo de tomate

El zumo de tomate utilizado en el estudio consistía en una mezcla de tomate triturado y aceite de oliva refinado (5%), envasado individualmente en packs Tetra brik de 200 mL. Al momento de la recepción, los mismos fueron almacenados protegidos de la luz y del calor hasta su análisis y posterior distribución a los voluntarios. En la **Figura 5** se esboza el proceso de preparación de los zumos utilizados durante el estudio.

Figura 5. Diagrama de flujo de la preparación de las muestras de zumo de tomate.

3. Métodos analíticos e instrumentación utilizada

3.1. Extracción líquido-líquido

a) En muestras de zumo de tomate

Para evitar la oxidación de los carotenoides por exposición a la luz, el oxígeno y el calor, el procedimiento de extracción se realizó en frío y en una habitación protegida de la luz. En primer lugar, se tomaron 0,5 g de muestra fresca y se homogenizaron con 5 mL de una solución etanol:hexano (4:3 v/v). A continuación, la mezcla se sonicó y centrifugó (4000 rpm a 4 ºC) durante 5 y 15 minutos, respectivamente. El sobrenadante fue transferido a un tubo de recolección y la extracción se repitió una segunda vez. Ambos sobrenadantes se combinaron y se evaporaron bajo una corriente de N_2 hasta sequedad. Finalmente, el residuo se reconstituyó en 1 mL de *tert*-metil-butil-éter (TMBE) y se almacenó a -80 ºC hasta su análisis[152].

b) En plasma

Para la extracción de carotenoides del plasma, el procedimiento también se llevó a cabo en frío y protegido de la luz para evitar la oxidación de los compuestos. En primer lugar se procedió a la desproteinización de las muestras; 800 µL de plasma se mezclaron con 800 µL de etanol y se agitaron con un vórtex durante 1 minuto. Una vez precipitadas las proteínas, se procedió a la extracción de los carotenoides añadiendo 2 mL de hexano/BHT (100 mg/L) como agente antioxidante. Seguidamente la mezcla se agitó por 1 minuto y se centrifugó a 2062 g por 5 minutos a 4 ºC. El sobrenadante fue transferido a un tubo de recolección y la extracción se repitió. Ambos sobrenadantes se combinaron y se evaporaron bajo una corriente de N_2 hasta sequedad. Por último, el residuo se reconstituyó en 300 µL de TMBE y se almacenó a -80 ºC hasta el día de su análisis[153].

3.2. Cromatografía líquida de alta resolución acoplada a un detector ultravioleta de diodos: *HPLC/UV-DAD.*

Para la identificación y cuantificación de carotenoides de las muestras de zumo de tomate y plasma se utilizó la técnica de HPLC acoplada a un detector UV de diodos (DAD G1315B). El cromatógrafo de líquidos utilizado fue un HP1100 HPLC (Hewlett-Packard, Waldbronn, Alemania), equipado con una bomba cuaternaria y un inyector automático. Las determinaciones analíticas de los carotenoides se realizaron utilizando una columna C_{30} de 250 mm de largo, 4,6 mm de diámetro y 5 µm de medida de

partícula interna (YMC Carotenoid S-5, Waters Co, Milford, MA, EUA), mantenida a 25 ºC y unida a una pre-columna de 20 mm de largo, 4 mm de diámetro y 5 µm de medida de partícula interna (YMC Guard Cartridge Carotenoid S-5, Waters Co, Milford, MA, EUA). El volumen de inyección de las muestras fue de 20 µL a un flujo de 0,6 mL/min. La separación cromatográfica se completó en 72 min y las fases móviles utilizadas consistieron en 3 solventes distintos: Agua Mili-Q (A), metanol (B) y TMBE (C). El solvente A se utilizó isocráticamente a 4%, mientras que los demás solventes se utilizaron con un gradiente en las siguientes condiciones: 0 min, 90% B; 40 min, 40% B; 60 min, 6% B; 62 min, 90% B; 72 min, 90% B. El TMBE se utiliza como un modificador, de modo a facilitar la elución del licopeno y sus isómeros, que tienden a ser fuertemente retenidos con el metanol. En la **Figura 6** se presenta la instrumentación utilizada para la identificación y cuantificación de los carotenoides.

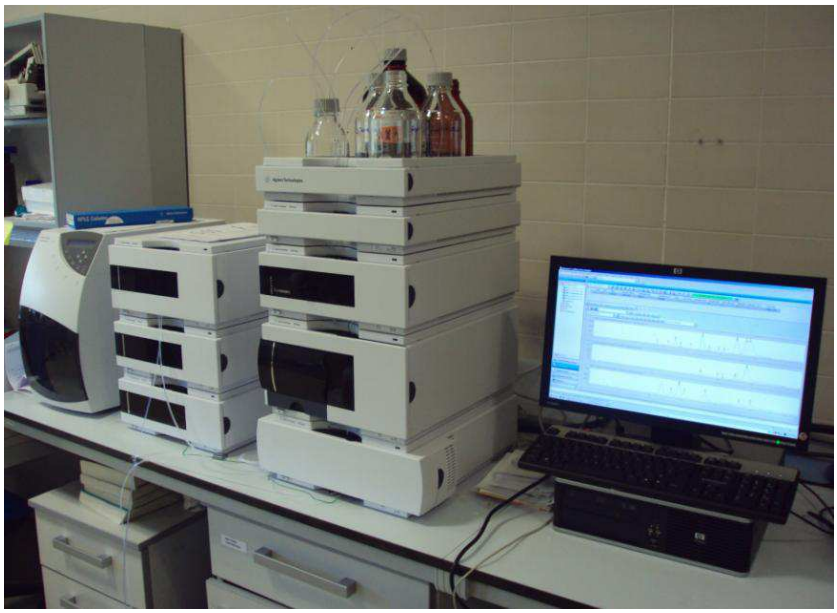

Figura 6. Cromatógrafo líquido de alta resolución (HPLC).

3.2.1. Validación del método cromatográfico

Para la caracterización e identificación de carotenoides en el plasma humano es fundamental contar con un método analítico que sea sensible, a la vez que selectivo y reproducible. Es por esto que se validó un nuevo método para la cuantificación de carotenos y xantófilas en plasma humano, en el cual también se incluyó la cuantificación de la vitamina A.

Los criterios de validación utilizados han sido los referenciados por la Association of Official Analytical Chemist (AOAC Internacional, http://www.aoac.org), y la Food and Drug Administration (FDA) (http://www.fda.gov), organismos americanos reconocidos a nivel mundial, encargados de establecer normas de validación de métodos analíticos aplicables a muestras biológicas, así como a la industria alimentaria.

- *Exactitud:* consiste en el grado de concordancia entre el valor medido y un valor de referencia. Fue establecida inyectando repetitivamente plasma blanco al que se le añadió 3 concentraciones conocidas de los analitos de interés: una concentración baja, una media y una alta, respecto a las rectas de calibrado, en 5 repeticiones. Los resultados se determinaron como el porcentaje de relación entre la concentración media observada y la concentración conocida que fue añadida a la matriz biológica. El valor medio debe estar comprendido entre 85 y 115%.

- *Precisión:* expresa el grado de concordancia (o grado de dispersión), entre una serie de mediciones obtenidas a partir de múltiples pruebas de una muestra homogénea en las condiciones establecidas del método. Se debe realizar con muestras de ensayo representantes de las matrices a las que se aplicará el método y estas muestras deben contener el rango esperado de concentraciones de analitos. Los resultados se miden en porcentaje de desviación estándar relativa (%DER) y no deben exceder de un 15%.

- *Recuperación:* se calcula mediante la preparación de rectas de calibrado tanto internas como externas, es decir, a las que se han añadido los analitos antes de realizar la extracción, y a las que se han añadido después de realizar la extracción, respectivamente. La respuesta obtenida de la cantidad de analitos añadida y extraída del plasma, se comparó con la respuesta obtenida por una recta de calibrado con las mismas concentraciones, pero inyectando estándares puros. Posteriormente se aplicó una regresión lineal entre la concentración de analitos y la concentración calculada anteriormente. El valor de la pendiente obtenida multiplicada por 100 corresponde a la recuperación del analito.

- *Límite de detección (LDD) y límite de cuantificación (LDC):* El LDD es la cantidad más pequeña de analito que se puede demostrar que es significativamente mayor que la medida de desviación de un blanco a un nivel

preestablecido de confianza (por lo general 95%); mientras que el LDC es la mínima cantidad de analito en una muestra que se puede determinar cuantitativamente con precisión y exactitud.

- *Linealidad:* la linealidad permite comprobar que la función matemática que vincula la señal instrumental al resultado analítico es efectivamente una recta para un intervalo definido de valores. Este estudio se efectúa comparando las desviaciones entre los puntos experimentales y el valor atribuido a los estándares puros, con una desviación máxima aceptada previamente definida.

3.3. Inmunofluorescencia

Para la determinación de moléculas inflamatorias y de adhesión celular, se utilizaron técnicas de inmunofluorescencia, las cuales forman parte de los ensayos basados en reacciones inmunológicas primarias; es decir, reacciones específicas entre un antígeno, que en este caso sería la molécula a analizar, y un anticuerpo específico, que corresponde al reactivo. Se consideran reacciones primarias porque se basan en el reconocimiento específico y la combinación antígeno-anticuerpo simplemente. La unión de un antígeno con un anticuerpo forma complejos macromoleculares, que debido a su tamaño, precipita, permitiendo así establecer la presencia de anticuerpos específicos. En la **Figura 7** se enseña resumidamente la reacción de unión antígeno-anticuerpo.

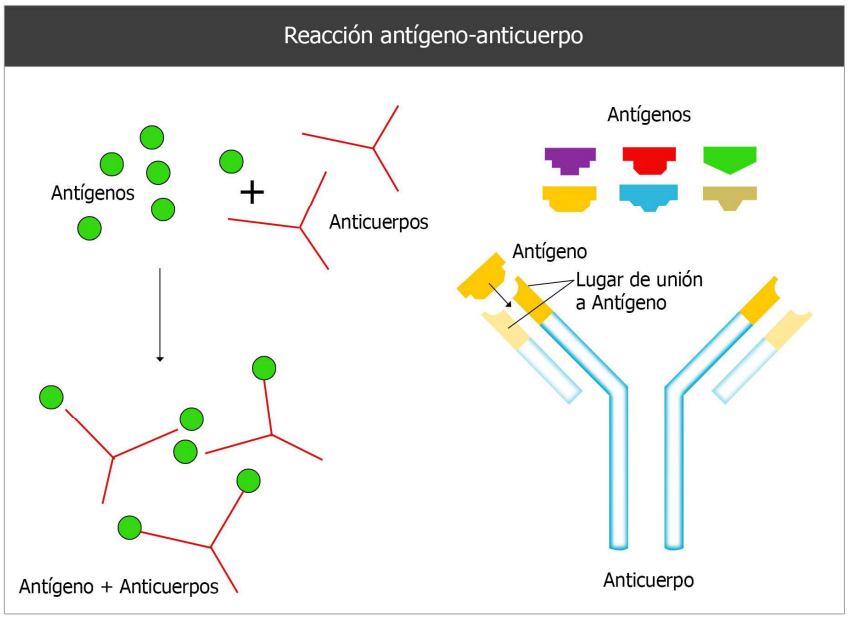

Figura 7. Reacción de unión de un antígeno con un anticuerpo.

Algunas sustancias químicas pueden absorber energía de ondas ultravioleta y emitirlas como ondas visibles de distinta longitud de onda, por ello se observan con distintas coloraciones en función de la longitud de onda de la luz recibida; a esta característica electroquímica se denomina fluorescencia. En particular, la técnica consiste en incubar el antígeno (parámetro) a valorar de la muestra, con un anticuerpo específico, luego se le añade un anti-anticuerpo marcado con el fluoróforo que se unirá al complejo y tras una nueva incubación y un lavado se visualiza la positividad o no del ensayo a través de un lector acoplado con lámpara ultravioleta.

Para la determinación de las moléculas ICAM-1, VCAM-1 y PCR se ha utilizado el Human ProcartaPlex® Inmunoassay Panel (Affymetrix, eBioscience Inc., CA, EUA) y la lectura se ha realizado en un equipo MAGPIX®. El análisis se ha realizado utilizando el software ProcartaPlex Analyst 1.0 de eBioscience. Para la determinación de las moléculas IL-8, eotaxina, IFN-γ y CXCL10, se ha utilizado un panel Bio-Plex Pro™ Assay, Bio-Rad (Bio-Rad Laboratories, Inc., CA, EUA). La lectura de las placas se ha realizado en un equipo Bio-Plex® 2200 System (Luminex Co., TX, EUA) y el análisis de los datos se ha realizado con el Bio-Plex Manager™ software.

La concentración de las muestras se calculó mediante la interpolación de la concentración de la curva de patrones con la curva generada por cada analito y la aplicación de un logaritmo 4PL o 5PL. Los resultados se muestran en ng/mL para la ICAM-1, VCAM-1 y la PCR; mientras que para la CXCL10, la IL-8, la eotaxina, y el IFN-γ, los resultados se expresan en pg/mL.

3.4. Análisis estadísticos

Para la realización de los análisis estadísticos se utilizó el paquete estadístico Statistical Package for Social Sciences (SPSS, versión 19.0; SPSS Inc., Chicago, IL, USA). La significancia estadística fue establecida como $p \leq 0,05$.

En primer lugar, se determinó la normalidad de las muestras mediante el test de *Kolmogorov-Smirnov*. A continuación, para establecer diferencias entre las distintas intervenciones se realizó el análisis de varianza para medidas repetidas (ANOVA). Los análisis de varianza permiten determinar si diferentes tratamientos muestran diferencias significativas ($p \leq 0,05$) o, por el contrario, puede suponerse que no existen diferencias entre las medias poblacionales. Seguidamente se determinaron las correlaciones existentes entre los carotenoides plasmáticos y las moléculas inflamatorias mediante el test de correlación de *Pearson* para variables paramétricas y, por último, para comprobar la causa de dichas correlaciones, es decir, la causalidad, se realizaron regresiones lineales simples.

Resultados

V. Resultados

1. Un nuevo método para la cuantificación simultánea de los antioxidantes: carotenos, xantófilas y vitamina A en plasma humano

De acuerdo con el primer objetivo propuesto, se ha desarrollado y validado un método analítico que permite la cuantificación de 8 carotenoides, 3 isómeros del *β*-caroteno, y retinol (vitamina A) en plasma humano, a través de HPLC-UV/DAD. Los parámetros que se consideraron para la validación del método fueron: exactitud, precisión, recuperación, LDD, LDC y linealidad, siguiendo los criterios de la AOAC International, así como de la FDA.

Para los ensayos de precisión y exactitud, se prepararon 3 diluciones de estándares en TMBE: una baja (1,5 µg/mL), una media (4 µg/mL) y una alta (9 µg/mL) de cada uno de los analitos. Para calcular la recuperación, se prepararon dos rectas de calibrado de 7 puntos cada una. A la primera se le añadieron los analitos estudiados antes del proceso de extracción (recta de calibrado), y a la otra, después de realizar la extracción (recta de externos). Las áreas de los analitos de la recta de calibración interna fueron introducidas en la recta de calibración externa, obteniéndose una concentración. Esta concentración calculada *versus* la concentración real utilizada se representó gráficamente; aplicando una regresión lineal y, multiplicando luego por 100 la pendiente de la recta, obtuvimos el valor de recuperación. Para obtener una buena recuperación, los valores deben estar comprendidos entre 100 ± 15%.

Tabla 4. Exactitud y recuperación de los compuestos estudiados.

Analito	***Exactitud (%)***	***Recuperación (%)***
Retinol	105 ± 9	96 ± 3
Astaxantina	99 ± 7	113 ± 6
Luteína	99 ± 13	112 ± 9
Zeaxantina	101 ± 11	107 ± 5
trans-*β*-apo-8'-carotenal	98 ± 10	94 ± 3
Criptoxantina	103 ± 12	96 ± 3
15-*cis*-*β*-caroteno	90 ± 13	101 ± 2
13-*cis*-*β*-caroteno	105 ± 12	92 ± 5
α-caroteno	98 ± 12	89 ± 4
β-caroteno	97 ± 13	96 ± 2
9-*cis*-*β*-caroteno	112 ± 7	93 ± 3
trans-licopeno	100 ± 12	91 ± 3

Como se observa en la **Tabla 4**, los valores de recuperación obtenidos se encuentran entre 89 y 113%. Las recuperaciones más altas corresponden a la astaxantina y la luteína, mientras que las más bajas corresponden al *α*-caroteno y al *trans*-licopeno. No obstante, todos los analitos se encuentran dentro de los límites recomendados por la FDA y la AOAC International.

En esta misma tabla se presentan los resultados de la exactitud, que fue calculada inyectando plasma blanco al que se le añadió 3 concentraciones distintas de los analitos a analizar, en 5 repeticiones. El porcentaje de variabilidad que debe existir entre las distintas inyecciones y un estándar de referencia no debe exceder ± 15%. La exactitud de todos los analitos que se estudiaron se encuentra dentro del rango establecido, muy próximos a los valores de referencia de los estándares.

La resolución indica el nivel de separación de los compuestos y la simetría de los picos que se obtienen tras el análisis cromatográfico. Los valores superiores a 1,5 indican una buena separación y simetría. Como puede observarse en la **Tabla 5**, la resolución de la mayoría de compuestos analizados tiene valores superiores a 1,5, lo que indica una buena separación de los compuestos y una correcta definición de los picos cromatográficos.

Tabla 5. Resolución de los compuestos estudiados.

Analito	***TR (min)***	***Longitud de onda (nm)***	***Anchura (min)***	***Resolución (R)***
Retinol	8,54	330	0,60	N.d.
Astaxantina	34,07	450	0,38	2,3
Luteína	36,55	450	0,72	1,4
Zeaxantina	38,24	450	0,53	2,2
trans-β-apo-8'-carotenal	40,01	450	0,29	7,7
Criptoxantina	43,94	450	0,22	6,9
15-*cis-β*-caroteno	46,96	450	0,22	1,4
13-*cis-β*-caroteno	47,57	450	0,21	1,3
α-caroteno	48,12	450	0,20	2,9
β-caroteno	49,57	450	0,31	2,3
9-*cis-β*-caroteno	50,76	450	0,20	33,0
trans-licopeno	64,29	450	0,21	33,0

N.d.: No determinado; R= 2[(TR)B-(TR)A]/(Anch. A +Anch. B); TR= Tiempo de retención

La precisión intra- e inter-día fue calculada inyectando 3 niveles de concentración (baja, media y alta) de los analitos, ya sea en una misma tanda de inyecciones, o en

tres días diferentes, respectivamente. Se evaluó mediante el DER de la reproducibilidad del intra- e inter-día y los resultados no debían superar el 15% de la DER. Como puede observarse en la **Tabla 6**, la precisión de todos los analitos estudiados se encuentra dentro del rango recomendable por la FDA y la AOAC, con valores entre 1 y 15% para la variabilidad intra-día, y valores de entre 4,9 y 15% para la variabilidad inter-día.

Como se ha señalado, el LDD es la cantidad o concentración mínima de un analito que puede ser detectada con fiabilidad por un método analítico, pero no necesariamente cuantificada. Con el método desarrollado, se consiguieron LDD de entre 0,1 µg/mL y 1,3 µg/mL, correspondiendo los valores más bajos a la astaxantina y al *trans*-licopeno, mientras que el valor más elevado corresponde al 15-*cis*-*β*-caroteno. El LDC, por su parte representa la mínima cantidad de analito que puede cuantificarse en una muestra con exactitud y precisión. La tendencia del LDC es del orden de 0,7 µg/mL para la mayoría de los analitos; correspondiendo los valores más bajos a la astaxantina y al *trans*-licopeno y el valor más elevado al 15-*cis*-*β*-caroteno.
Por su parte, el rango de linealidad permite determinar los límites mínimo y máximo de concentración en que una medición será precisa. Tomando en cuenta el valor máximo encontrado en plasma de cada analito, se prepararon rectas de calibrado con plasma sintético al que se añadió distintas concentraciones de los analitos, desde el valor del LDC, hasta un máximo de 10 µg/mL. El procedimiento analítico demostró ser lineal en el intervalo de concentración determinado, con un coeficiente de correlación *(r)* de entre 0,9952 y 0,9984 para todos los compuestos en las muestras de plasma, lo que demuestra una buena linealidad de las curvas. En la **Tabla 7** se resumen los resultados.

Tabla 6. Precisión de los analitos estudiados

	Precisión											
	1,5 µg/mL (n=5)				6 µg/mL (n=5)				9 µg/mL (n=5)			
Analito	Día 1 (DER%)	Día 2 (DER%)	Día 3 (DER %)	DER Interday	Día 1 (DER%)	Día 2 (DER%)	Día 3 (DER %)	DER Interday	Día 1 (DER%)	Día 2 (DER%)	Día 3 (DER %)	DER Interday
Retinol	3	4	5	4,9	10	12	8	14,1	13	10	11	14,3
Astaxantina	7	3	3	14,5	11	13	12	14,6	15	12	12	14,7
Luteína	8	9	4	14,2	12	14	8	14,3	10	9	12	13,3
Zeaxantina	4	15	4	10,8	12	3	7	12,9	11	11	10	14,8
trans-β-apo-8'-carotenal	11	10	4	14,3	11	12	8	14,7	13	9	12	13,8
Cryptoxantina	5	13	3	12,1	11	6	9	14,3	11	10	8	13,9
15-*cis*-β-caroteno	5	13	1	9,4	11	5	9	15,1	10	7	11	13,4
13-*cis*-β-caroteno	6	14	3	14,9	12	15	12	14,0	11	10	10	14,9
α-caroteno	4	15	2	9,4	11	15	11	13,7	11	9	10	14,8
β-caroteno	11	10	8	11,2	11	15	14	14,9	12	6	10	12,8
9-*cis*-β-caroteno	6	10	7	10,4	12	12	4	13,9	11	11	11	14,8
trans-licopeno	5	5	2	11,6	10	13	9	13,8	10	15	8	14,8

DER: Desviación estándar relativa

Tabla 7. Límite de detección, límite de cuantificación, rango de concentración, rectas de calibrado y coeficiente de correlación de los analitos inyectados en plasma blanco.

Analito	***LDD (µg/mL)***	***LDC (µg/mL)***	***Rango de linealidad (µg/mL)***	***Recta de calibrado***	***Coeficiente de correlación (r)***
Retinol	0,2	0,7	0,7 - 10	y = 69,68x - 15,86	0,9952
Astaxantina	0,1	0,3	0,3 - 10	y = 49,14x + 3,02	0,9964
Luteína	0,4	1,3	1,3 - 10	y = 119,37x - 2,26	0,9954
Zeaxantina	0,2	0,7	0,7 - 10	y = 69,14x + 1,53	0,9955
trans-*β*-apo-8'-carotenal	0,2	0,7	0,7 - 10	y = 249,40x - 20,91	0,9957
Cryptoxantina	0,2	0,7	0,7 - 10	y = 200,48x - 15,76	0,9952
15-*cis*-*β*-caroteno	1,3	4,3	4,3 - 10	y = 91,23x - 10,85	0,9984
13-*cis*-*β*-caroteno	0,4	1,3	1,3 - 10	y = 90,59x + 8,74	0,9969
α-caroteno	0,5	1,6	1,6 - 10	y = 243,07x - 37,13	0,9966
β-caroteno	0,2	0,7	0,7 - 10	y = 105,80x - 2,94	0,9983
9-*cis*-*β*-caroteno	0,2	0,7	0,7 - 10	y = 75,68x - 8,42	0,9959
trans-licopeno	0,1	0,3	0,3 - 10	y = 34,53x - 4,29	0,9952

LDD: Límite de detección; LDC: Límite de cuantificación

Posteriormente, para corroborar la aplicabilidad del método a estudios epidemiológicos y/o ensayos clínicos, una vez validado, el método fue aplicado a un estudio piloto de intervención con 8 voluntarios adultos (69,9 ± 3,8 años; 32.3 ± 3.8 IMC) a los que se les suministró 250 mL de zumo de tomate durante 4 semanas. Las muestras de sangre fueron recogidas tanto al inicio como al final del estudio. Los analitos detectados en el plasma en estado basal, es decir antes de la intervención con el zumo de tomate, fueron retinol, astaxantina, luteína, *trans*-*β*-apo-8'-carotenal, criptoxantina, 13-cis-*β*-caroteno, *α*-caroteno, *β*-caroteno, licopeno y 5-*cis*-licopeno.

Tabla 8. Carotenoides y retinol en el plasma humano antes y después de una intervención dietética con 250 mL de zumo de tomate.

	Concentración			
	Basal	***Después de la intervención***	***Basal***	***Después de la intervención***
Analitos	***Media ± DE (µg/mL)***	***Media ± DE (µg/mL)***	***Media ± DE (µmol/L)***	***Media ± DE (µmol/L)***
Retinol	1,82 ± 0,37	1,90 ± 0,49	3,46 ± 0,70	3,62 ± 0,93
Astaxantina	0,76 ± 0,44	0,88 ± 0,37	1,27 ± 0,74	1,48 ± 0,63
Luteína	0,07 ± 0,02	0,06 ± 0,06	0,13 ± 0,03	0,11 ± 0,11
Zeaxantina	N.d.	N.d.	N.d.	N.d.
trans-β-apo-8'-carotenal	0,57 ± 0,03	0,57 ± 0,03	1,38 ± 0,06	1,38 ± 0,08
Criptoxantina	0,18 ± 0,12	0,20 ± 0,07	0,32 ± 0,22	0,37 ± 0,12
15-*cis*-*β*-caroteno	N.d.	N.d.	N.d.	N.d.
13-*cis*-*β*-caroteno	0,13 ± 0,00	0,14 ± 0,02	0,23 ± 0,00	0,26 ± 0,04
α-caroteno	0,22 ± 0,00	N.d.	0,41 ± 0,00	N.d.
β-caroteno	0,95 ± 0,50	1,09 ± 0,53	1,77 ± 0,93	2,03 ± 0,98
9-*cis*-*β*-caroteno	N.d.	N.d.	N.d.	N.d.
13-*cis*-licopeno	N.d.[a]	2,79 ± 1,44[a]	N.d.[a]	5,20 ± 2,69[a]
9-*cis*-licopeno	N.d.[a]	0,38 ± 1,42[a]	N.d.[a]	0,71 ± 2,64[a]
trans-licopeno	1,15 ± 0,83[a]	5,19 ± 2,35[a]	2,14 ± 1,54[a]	9.67 ± 4,38[a]
5-*cis*-licopeno	0,75 ± 1,10[a]	3,07 ± 1,43[a]	1,41 ± 2,06[a]	5,72 ± 2,67[a]

N.d.: No determinado

DE: Desviación estándar

[a]Los valores de la fila que tengan la misma letra son significativamente diferentes ($p<0,05$) Datos analizados mediante test de Wilcoxon para medidas repetidas.

En la **Tabla 8** se muestran los valores de carotenoides y vitamina A, en µg/mL y en µmol/L respectivamente, cuantificados en el plasma de los voluntarios a quienes se les suministró 250 mL de zumo de tomate durante 4 semanas. Como se observa, el método desarrollado permitió la identificación y cuantificación de 9 de los compuestos estudiados en estado basal, es decir sin antes haber consumido alimentos ricos en carotenoides, así como también tras la intervención. Con este método también se pudieron identificar y cuantificar 3 isómeros *cis* del licopeno, tanto antes como después de la intervención, observándose un incremento de la concentración tras el consumo del zumo.

2. El *trans*-licopeno del zumo de tomate atenúa los biomarcadores inmunoinflamatorios: estudio dosis-respuesta.

Un total de 283 participantes fueron evaluados para participar en el estudio; de los cuales 223 no fueron elegibles: 78 no cumplían los criterios de inclusión, 21 informaron de su voluntad de no cambiar su dieta, y 140 declinaron de participar, dejando un total de 44 pacientes para ser asignados al azar a los diferentes grupos de intervención. Veintiocho de ellos completaron el estudio debido a que 2 participantes decidieron abandonar el estudio por problemas después de la dieta, 3 por problemas de salud y otros 11 por cuestiones personales. En la **Figura 8** se presenta el diagrama de flujo de los voluntarios a lo largo del estudio.

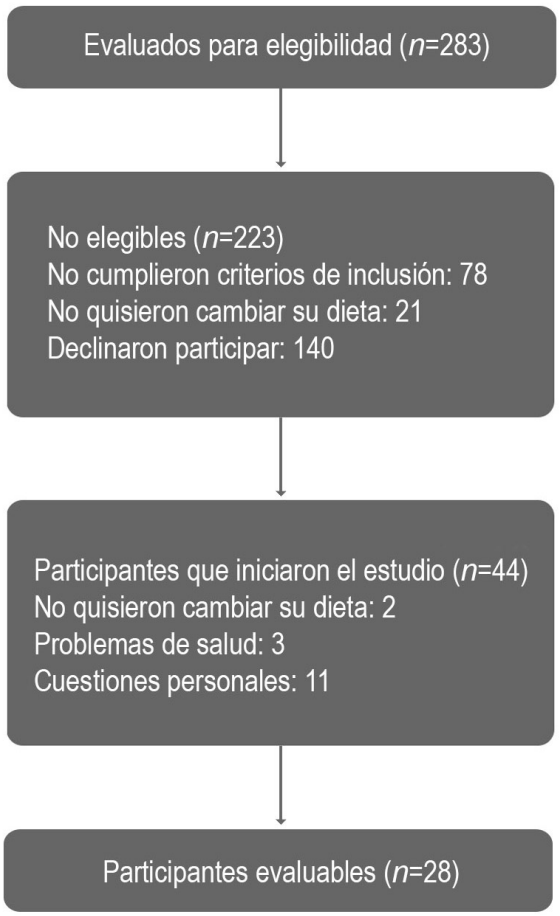

Figura 8. Diagrama de flujo de los participantes durante el estudio.

Para la realización del estudio se reclutaron voluntarios que no padecían enfermedad cardiovascular, pero con alto riesgo de desarrollarla. Los criterios de inclusión fueron: tener hipertensión arterial y uno o más de los siguientes factores de riesgo: a) ser fumador, b) presentar hipercolesterolemia (LDL-colesterol >160 mg/dL), c) tener bajas concentraciones de HDL-colesterol (<40 mg/dL), d) tener sobrepeso u obesidad (IMC >25 kg/m^2), e) tener diabetes tipo II, f) historia familiar de ECV temprana (parientes de primer orden, hombres <55 años; mujeres <65 años). Los voluntarios que hayan padecido o que presentasen una ECV diagnosticada, así como cualquier otra enfermedad crónica, alcoholismo u otras adicciones, alergia o intolerancia al tomate, fueron excluidos del estudio. En la **Tabla 9** se presentan las características demográficas y clínicas de los pacientes que participaron en el estudio. La media de edad de los voluntarios fue de 69,7 ± 3,1 años. El 61% de los voluntarios eran mujeres, mientras que el 39% eran hombres y todos presentaban sobrepeso u obesidad, con una media de IMC de 31,5 ± 3,6 kg/m^2.

Tabla 9. Características demográficas y clínicas de la población estudiada.

Variables	***Total (n=28)***
Sexo	
Masculino *(n)*	11
Femenino *(n)*	17
Edad (años)	69,7 ± 3,1[a]
Hábitos tabáquicos	
Nunca ha fumado *(n)*	15
Ex fumador *(n)*	8
Fumador habitual *(n)*	5
IMC (kg/m^2)	31,5 ± 3,6[a]
25-30 *(n)*	13
>30 *(n)*	15
Hipertensión *(n)*	28
Diabetes tipo II *(n)*	11
Dislipemia *(n)*	21
Historia familiar de ECV prematura *(n)*	4
Medicación utilizada	
Antihipertensivos *(n)*	8
Insulina *(n)*	4
Hiploglucemientas orales *(n)*	13
Antiagregantes plaquetarios *(n)*	8
Agentes hipolipemiantes *(n)*	22

[a]Valores expresados en media ± DE; DE: Desviación estándar

La **Tabla 10** resume el contenido de los principales carotenoides identificados en el zumo de tomate proporcionado a los participantes durante el ensayo. Los principales compuestos cuantificados fueron el *trans*-licopeno y *β*-caroteno, con una media de 193 ± 20 y 190 ± 15 µmol/L, respectivamente. La suma de los isómeros de licopeno representó el 52,2% de la ingesta total de carotenoides, siendo el *trans*-licopeno el principal compuesto con un 48,1%, mientras que el *β*-caroteno representó el 47,4% del total, por lo que la suma de *trans*-licopeno y *β*-caroteno representan el 95,5% de los carotenoides consumidos a través del zumo de tomate.

Tabla 10. Contenido de carotenoides del zumo de tomate administrado a los voluntarios durante el estudio.

	Contenido en el zumo (µmol/L)	**Dosis Baja µ*mol/dosis administrada*[a]**	**Dosis Alta µmol/*dosis administrada*[b]**
Luteína	0,46 ± 0,04	0,09 ± 0,01	0,19 ± 0,02
α-caroteno	0,88 ± 0,06	0,18 ± 0,01	0,35 ± 0,02
β-caroteno	190 ± 15	38,0 ± 3,00	75,9 ± 5,98
trans-licopeno	193 ± 20	38,6 ± 3,98	77,3 ± 7,95
5-*cis*-licopeno	2,76 ± 0,21	0,55 ± 0,04	1,10 ± 0,08
13-*cis*-licopeno	5,40 ± 0,57	1,08 ± 0,11	2,16 ± 0,22
9-*cis*-licopeno	8,30 ± 0,76	1,66 ± 0,15	3,32 ± 0,31
Carotenoides totales	401	80,10	160

Valores expresados como media ± DE; [a]Dosis administrada: 200 mL/ZT; [b]Dosis administrada: 400 mL/ZT
ZT: Zumo de tomate
DE: Desviación estándar

En la **Tabla 11** se presentan las concentraciones de carotenoides medidas en el plasma de los voluntarios, antes y después de la intervención con las distintas dosis de zumo de tomate. Después de la dieta de lavado, debido a la abstinencia en el consumo de tomate, productos a base de tomate y otros alimentos ricos en carotenoides, la concentración plasmática de carotenoides fue considerada la más baja. Los principales carotenoides cuantificados en el plasma después del período de lavado fueron el *trans*-licopeno, y los isómeros *cis*, 13-*cis*, 5-*cis*, y 9-*cis*-licopeno, respectivamente.

Tabla 11. Concentración plasmática de carotenoides antes y después de la intervención con zumo de tomate (*n*=28).

Analito	***Intervención***	***Concentración (µmol/L)***	***p*[1]**
Astaxantina	Basal	0,73±0,53	0,276
	Control	0,67±0,54	
	DB	0,51±0,27	
	DA	0,80±0,49	
Luteína	Basal	0,05±0,03	0,036
	Control	0,04±0,02	
	DB	0,02±0,02	
	DA	0,04±0,02	
trans-*β*-apo-8'-carotenal	Basal	0,53±0,03	0,025
	Control	0,52±0,03	
	DB	0,52±0,02	
	DA	0,54±0,03	
Criptoxantina	Basal	0,09±0,08	0,077
	Control	0,10±0,08	
	DB	0,10±0,06	
	DA	0,16±0,14	
15-*cis*-*β*-caroteno	Basal	n.d.	<0,001
	Control	n.d.	
	DB	n.d.	
	DA	0,18±0,21	
13-*cis*-*β*-caroteno	Basal	n.d.[a,b]	<0,001
	Control	0,09±0,00[a]	
	DB	0,10±0,02[b]	
	DA	0,11±0,03[a]	
α-caroteno	Basal	n.d.	< 0,001
	Control	n.d.	
	DB	n.d.	
	DA	0,51±0,43	
β-caroteno	Basal	0,69±0,41	0,006
	Control	0,65±0,41	
	DB	0,80±0,50	
	DA	1,19±0,98	
trans-licopeno	Basal	1,43±1,11[a]	<0,001
	Control	0,84±0,56[b]	
	DB	3,91±1,76[a,b]	
	DA	6,64±2,65[a,b]	

5-*cis*-licopeno	Basal	1,19±0,99[a]	<0,001
	Control	1,13±0,74[b]	
	DB	2,37±1,09[a,b]	
	DA	4,07±1,77[a,b]	
13-*cis*-licopeno	Basal	1,04±0,76[a]	<0,001
	Control	1,12±0,90[b]	
	DB	1,87±1,05[a]	
	DA	4,00±2,00[a,b]	
9-*cis*-licopeno	Basal	0,79±0,88	0,003
	Control	0,63±0,76	
	DB	0,89±0,56[a]	
	DA	1,94±1,26[a]	
Total isómeros *cis* del licopeno	Basal	1,74±1,60[a]	<0,001
	Control	1,71±1,46[b]	
	DB	4,77±2,60[a,b]	
	DA	9,57±4,78[a,b]	
Licopenos totales	Basal	3,00±2,66[a]	<0,001
	Control	2,55±1,87[b]	
	DB	8,68±4,22[a,b]	
	DA	16,22±7,26[a,b]	
Carotenoides totales	Basal	4,94±3,49[a]	<0,001
	Control	4,95±3,39[b]	
	DB	10,64±4,64[a,b]	
	DA	18,62±7,99[a,b]	

Valores expresados como media ± DE
[1]valor *p* de ANOVA para medidas repetidas de las diferencias entre intervenciones.
Valores con la misma letra son estadísticamente significativos entre intervenciones ($p < 0.05$).
n.d.; no determinado; DE: Desviación estándar; DB: Dosis baja; DA: Dosis alta

Tras las intervenciones, se observaron aumentos estadísticamente significativos de *trans*-licopeno, 5-*cis*-licopeno y 13-*cis*-licopeno entre todas las intervenciones, mientras que el 9-*cis*-licopeno solo aumentó significativamente después de la intervención DA. Por otra parte, el *β*-caroteno incrementó después de las distintas intervenciones, mientras que el *α*-caroteno lo hizo después de la intervención DA, pero estos cambios no fueron estadísticamente significativos. Las xantófilas, por su parte, no mostraron diferencias significativas tras las distintas intervenciones.

En la **Tabla 12** se presentan los valores de las concentraciones de moléculas de inflamación y de adhesión medidas en el plasma de los voluntarios, tanto antes como después de las distintas intervenciones.

Tabla 12. Marcadores inmunoinflamatorios medidos antes y después de la intervención con zumo de tomate (*n=28*).

	Intervención				
Biomarcador plasmático	***Basal***	***Control (C)***	***Dosis baja (DB)***	***Dosis alta (DA)***	***p[d]***
ICAM-1 (ng/mL)	3693±1377[a]	3609±1107[b]	318±116[a,b]	159±57[a,b]	<0,001
VCAM-1 (ng/mL)	3993±890[a]	3939±801[b]	400±101[a,b]	218±39[a,b]	<0,001
CRP (ng/mL)	1521±236[a,b,c]	539±200[a]	446±254[b]	532±158[c]	<0,001
IL-8 (pg/mL)	22±9[a]	40±17[a,b,c]	23±16[b]	24±15[c]	0,015
Eotaxina (pg/mL)	135±67	172±114	137±75	181±112	0,172
IFN-γ (pg/mL)	304±80	489±220	399±131	400±144	0,074
CXCL10 (pg/mL)	2908±1598	3397±1489	3366±1671	3606±2185	0,519

Valores expresados como media ± DE; valores con la misma letra son estadísticamente significativos (p<0,05); [d]valor *p* de ANOVA para medidas repetidas de las diferencias entre intervenciones.
DE: Desviación estándar

Las moléculas de adhesión ICAM-1 y VCAM-1 presentaron una disminución significativa entre las diferentes intervenciones, siendo esta disminución más relevante después de la intervención con la dosis más alta de zumo de tomate. Del mismo modo, la PCR ha presentado una disminución significativa después de las diferentes intervenciones, pero en este caso se observó una mejor respuesta después de intervención con la dosis más baja. La citoquina pro-inflamatoria y quimiotáctica IL-8 aumentó significativamente después de la intervención control, pero en contraste, presentó una disminución significativa después de la intervención DA, lo que sugiere que consumir zumo de tomate podría tener efectos protectores sobre el estado inflamatorio en esta población. Por su parte, la eotaxina, el IFN-γ, y la CXCL10 mostraron una tendencia a disminuir, principalmente con la dosis baja de zumo de tomate, en comparación con la intervención control, pero las disminuciones no fueron estadísticamente significativas para estas moléculas.

Con el fin de determinar con precisión si los carotenoides plasmáticos guardaban relación con la concentración plasmática de los biomarcadores inflamatorios, se aplicó la prueba de coeficiente de correlación de *Pearson* de dos colas, seguido de un análisis de regresión lineal. En primer lugar, se realizaron correlaciones entre las concentraciones de carotenoides del zumo de tomate, y las concentraciones que se cuantificaron en el plasma de los voluntarios tras las intervenciones. El *trans*-licopeno y el 5-*cis*-licopeno mostraron una correlación positiva significativa (r=0,798 y r=0,706, respectivamente, p <0,01) entre las concentraciones del zumo y las concentraciones plasmáticas, mientras que el β-caroteno, el α-caroteno, el 9-*cis*, y 13-*cis*-licopeno,

mostraron una correlación modesta, con valores de *r* de entre 0,320 y 0,655 (*p* <0,001). Posteriormente, se analizó la asociación existente entre los biomarcadores inflamatorios y aquellos carotenoides que mostraron una correlación positiva significativa entre las cantidades de carotenoides consumidas y las concentraciones plasmáticas, para poder determinar cuál de estos carotenoides guardaba correlación con las moléculas inflamatorias y de adhesión. Se observó que el 13-*cis*-*β*-caroteno presentó una correlación negativa considerable con la PCR (*r*=0,632; *p* <0,001), la ICAM-1 (*r*=0.650; *p* <0,001) y la VCAM-1 (*r*=0,658; *p* <0,001). En cuanto a las formas isoméricas de licopeno, el 5-*cis*-licopeno presentó una correlación negativa con la ICAM-1 (*r*=0,526; *p* <0,001) y la VCAM-1 (*r*=0,604; *p* <0,001), así como el 13-*cis*-licopeno, el cual mostró correlaciones negativas con la ICAM-1 (*r*=0,453; *p* <0,001) y la VCAM-1 (*r*=0,525; *p* <0,001), mientras que el *trans*-licopeno mostró una importante correlación negativa con la ICAM-1 (*r*=0,625; *p* <0,001) y la VCAM-1 (*r*=0,697; *p* <0,001), pero sin embargo, una correlación negativa menor con la PCR (*r*=0,227; *p*=0,042). Se observaron correlaciones más bajas entre el 15-*cis*-*β*-caroteno y la VCAM-1 (*r*=0,303; *p*=0,045); el *α*-caroteno y la VCAM-1 (*r*=0,245, *p*=0,045); el 9-*cis*-licopeno y la VCAM-1 (*r*=0,357; *p*=0,013); el *β*-caroteno y la VCAM-1 (*r*=0,204; *p*=0,037); y el *β*-caroteno y la ICAM-1 (*r*=0,219, *p*=0,035). Asimismo, se observó una asociación débil o ninguna asociación entre los carotenoides plasmáticos y las moléculas inflamatorias IFN-*γ*, IL-8 y CXCL10.

Como las pruebas de correlación no determinan causalidad, una vez establecidas las correlaciones, se realizó un análisis de regresión lineal para determinar con certeza si los cambios en las concentraciones de los biomarcadores que presentaron una correlación negativa significativa respecto a los carotenoides plasmáticos, fueron debidos a los carotenoides presentes en el plasma. Tras los análisis de regresión lineal se pudo determinar que los carotenoides tenían una influencia de entre el 13 y el 49% en la disminución de la VCAM-1, especialmente el *trans*-licopeno (r^2=0,489; *p* <0,01). En el caso de ICAM-1, los carotenoides pudieron explicar las variaciones entre un 5 y un 39%, siendo también el *trans*-licopeno el principal contribuyente (r^2=0,390; *p* <0,01). Sin embargo, para la PCR, los carotenoides pudieron explicar las disminuciones entre un 2,5 y un 40%, especialmente debido al 13-*cis*-*β*-caroteno.

Finalmente, para corroborar si los efectos positivos del *trans*-licopeno sobre estas moléculas podrían estar influenciados por la presencia de los isómeros *cis* en el plasma, que son reconocidos por su mejor biodisponibilidad, se realizaron correlaciones con ajustes por *cis* y por *trans*-licopeno para comparar los resultados. Después de ajustar por *cis*-licopeno, las correlaciones entre el *trans*-licopeno y la PCR disminuyeron, pero este cambio no fue estadísticamente significativo. En el caso del

trans-licopeno-ICAM-1, y el *trans*-licopeno-VCAM-1, los valores de *r* disminuyeron a -0,453 y -0,501, respectivamente, mostrando que, aunque en menor medida, el *trans*-licopeno todavía guardaba correlación con la disminución de estas moléculas. Sorprendentemente, después de ajustar por *trans*-licopeno, los isómeros *cis* del licopeno perdieron la correlación que tenían con las moléculas inflamatorias. En la **Tabla 13** se muestran los valores de correlación entre las moléculas inflamatorias y de adhesión que presentaron cambios estadísticamente significativos antes y después de ajustar por los isómeros *cis* y *trans*-licopeno.

Tabla 13. Correlaciones entre los isómeros *cis* y *trans* del licopeno y las moléculas inflamatorias y de adhesión, antes y después de ajustar por los mismos.

Ajustado por	***Biomarcador***		***Coeficiente de correlación de Pearson***	***valor p***
	PCR	*trans*-Licopeno	-0,227	0,042
		cis-Licopenos totales	-0,157	0,154
	VCAM-1	*trans*-Licopeno	-0,697	<0,001
		cis-Licopenos totales	-0,628	<0,001
	ICAM-1	*trans*-Licopeno	-0,625	<0,001
		cis-Licopenos totales	-0,551	<0,001
cis-Licopenos totales	PCR	*trans*-Licopeno	-0,414	<0,001
	VCAM-1	*trans*-Licopeno	-0,501	<0,001
	ICAM-1	*trans*-Licopeno	-0,453	<0,001
trans-Licopeno	PCR	*cis*-Licopenos totales	0,374	0,002
	VCAM-1	*cis*-Licopenos totales	0,356	0,003
	ICAM-1	*cis*-Licopenos totales	0,309	0,010

Discusión

VI. Discusión

Las ECV representan la principal causa de mortalidad en el mundo. Según la OMS, en el año 2012, catorce millones de personas fallecieron por enfermedades relacionadas con la aterosclerosis[127,128]. Entre los principales factores protectores está la alimentación y se ha observado que una alimentación equilibrada, que contemple el consumo regular de frutas y hortalizas es un importante factor protector contra las ECV[154,155]. Entre las frutas/hortalizas, una de las más consumidas en todo el mundo, ya sea de forma cruda, o en diferentes preparaciones, como salsas, sopas o zumos, es el tomate y numerosos estudios epidemiológicos y ensayos clínicos han coincidido en que su consumo regular está asociado con una menor incidencia de ECV[156-158]. Sus propiedades beneficiosas sobre la salud cardiovascular se han atribuido a la presencia de compuestos bioactivos en su composición, como lo son los carotenoides, sobre todo el licopeno, que es uno de sus principales pigmentos, responsable de su coloración roja.

Para la realización de ensayos clínicos y estudios epidemiológicos que buscan elucidar el vínculo entre dieta y salud, es muy importante contar con herramientas sensibles y específicas que permitan identificar y cuantificar de manera precisa los compuestos de interés, para que de esta forma, los resultados obtenidos puedan interpretarse correctamente.

Durante la realización de esta tesis doctoral, se ha desarrollado y validado un método cromatográfico que permite la identificación y cuantificación de 8 carotenoides, 3 isómeros del *β*-caroteno y retinol (vitamina A) en muestras de plasma humano, mediante HPLC-UV/DAD. Para la preparación de las muestras, es imprescindible la precipitación previa de las proteínas del plasma, por ello, es fundamental la elección de un solvente que permita eliminar los compuestos proteicos que pudieran interferir durante el análisis cromatográfico y, a su vez, proteger a los carotenoides para que no se produzcan pérdidas por oxidación y/o isomerización. Al mismo tiempo no debe dejar de considerarse la sostenibilidad y el impacto ambiental que produce la utilización de solventes químicos y se tiene que tratar de minimizar en lo posible la utilización de aquellas sustancias de difícil tratamiento y eliminación. Entre los solventes más utilizados para la desproteinización de matrices complejas como el plasma, están el metanol, el etanol y el acetonitrilo. Debido a su menor coste económico, así como por las buenas recuperaciones de carotenoides obtenidas tras distintas pruebas de extracción, se optó por la desproteinización con etanol y la extracción de carotenoides y retinol con hexano-BHT.

En cuanto a la identificación y cuantificación de carotenoides, diversos métodos cromatográficos han sido descritos y validados[97, 159-162]. La cromatografía líquida de alta resolución es una de las herramientas más versátiles para la identificación de múltiples compuestos, ya que permite el acoplamiento con diversos sistemas, como los espectrofotométricos, fluorimétricos, electroquímicos, o los de espectrometría de masas, que aumentan la sensibilidad y especificidad en la identificación. El HPLC acoplado a UV-DAD es una de las técnicas más comúnmente utilizadas para la identificación de los carotenoides tanto en alimentos como en muestras biológicas. Las ventajas en la utilización del HPLC-UV/DAD se relacionan con la eficiencia en la separación, la sensibilidad y alta resolución que tiene sobre distintos compuestos en muestras complejas[163,164].

Otro aspecto importante a tener en cuenta para los análisis cromatográficos es la elección de una columna adecuada para la separación de los compuestos seleccionados. Las columnas más utilizadas para la separación de carotenoides de distintas matrices son la C_{18} y la C_{30}; sin embargo, uno de los mayores inconvenientes que tiene la C_{18} es que no resuelve correctamente los isómeros geométricos de los carotenoides y resuelve de forma ineficiente los isómeros de posición, particularmente la luteína y la zeaxantina[165]. Utilizando una columna C_{30} se resuelve fácilmente este inconveniente, aunque en contrapartida se incrementan notablemente los tiempos de análisis.

Por otra parte, la separación de carotenoides puede llevarse a cabo utilizando tanto una fase normal como una fase reversa. Se ha observado que la separación de carotenoides en fase normal no es la más adecuada, debido a una mala separación de los carotenoides no polares (como el *β*-caroteno o el licopeno). En contraste, la separación en fase inversa permite un aumento significativo de la interacción entre el analito y la fase estacionaria no polar que lleva, permitiendo una mejor resolución de los carotenoides[166]. En la validación del método que se llevó a cabo en esta tesis, se pudo constatar que aunque los tiempos de análisis sean prolongados, la utilización de HPLC-UV/DAD con una columna C_{30} en fase reversa, permite la separación simultánea de una gran variedad de carotenoides y de sus isómeros geométricos, consiguiendo una buena identificación y resolución de todos los compuestos.

En cuanto a los parámetros de validación, el método desarrollado cumplió con los criterios de exactitud, precisión, recuperación, límites de detección y cuantificación y linealidad de acuerdo con la AOAC International y la FDA, organismos americanos mundialmente reconocidos, encargados de la publicación de estándares de validación de métodos científicos aplicados a la industria química y alimentaria, respectivamente.

Comparando los resultados con los obtenidos por otros autores, se puede observar que la recuperación del retinol (96%) fue similar al valor observado por Kandar *et. al*, 2013[167], quienes desarrollaron un método para retinol, *α*-tocoferol, licopeno y *β*-caroteno en plasma utilizando HPLC-UV. En lo que respecta a los carotenoides, las recuperaciones que se obtuvieron fueron de entre un 89 y un 113%, correspondiente al *α*-caroteno y a la astaxantina, respectivamente. Al comparar los resultados con los publicados por Talwar *et al*., 1998[168], quienes ensayaron un método para la cuantificación de retinol, *α*-tocoferol, *β*-caroteno, luteína, licopeno y criptoxantina, se obtuvo una recuperación 18% y 9% más alta para la luteína y para el *β*-caroteno, respectivamente, con el método desarrollado; mientras que si se compara con los datos presentados por Tzeng *et al*., 2004[159], que validaron un método para la identificación de la luteína, el *β*-caroteno y el licopeno en plasma, se observa una recuperación del 20% superior de la luteína. En cambio, en comparación a los resultados presentados por Rajendran *et al*., 2005[169], quienes lograron identificar y cuantificar 21 carotenoides en plasma humano, se obtuvieron una mejor recuperación para la luteína (19% superior), la zeaxantina (13% superior) y la criptoxantina (7% más). Karppi *et al*., 2008[170] desarrollaron un método para determinar retinol, *α*-tocoferol, luteína, zeaxantina, criptoxantina, licopeno, *α*-caroteno y *β*-caroteno, observando valores de recuperación similares a los obtenidos en esta tesis doctoral, a excepción de la luteína, la zeaxantina y el *β*-caroteno que fueron 10%, 17% y 16%, respectivamente, más bajas que las recuperaciones obtenidas con este método. En la **Tabla 14** se resumen los analitos estudiados y las recuperaciones obtenidas por los autores citados, así como las recuperaciones obtenidas en nuestro estudio de validación.

Tabla 14. Recuperaciones obtenidas por distintos autores

Autor	***Analitos estudiados***	***Recuperación obtenida***
Colmán-Martínez *et al*., 2015[153]	Retinol, astaxantina, luteína, zeaxantina, *trans*-ϐ-apo-8'-carotenal, cryptoxanthin, 15-*cis*-ϐ-caroteno, 13-*cis*-ϐ-caroteno, α-caroteno, ϐ-caroteno, 9-*cis*-ϐ-caroteno y licopeno	96% Retinol 113% Astaxantina 112% Luteína 107% Zeaxantina 94% *trans*-β-apo-8'-carotenal 96% Criptoxantina 101% 15-*cis*-β-caroteno 92% 13-*cis*-β-caroteno 89% α-caroteno 96% β-caroteno 93% 9-*cis*-β-caroteno 91% Licopeno
Kandar *et al*., 2013[167]	Retinol, α-tocoferol, licopeno y β-caroteno	97% Retinol 91,6% Licopeno 93,9% β-caroteno
Karppi *et al*., 2008[170]	Retinol, α-tocoferol, luteína, zeaxantina, criptoxantina, licopeno, α-caroteno y β-caroteno	90% Retinol 101,6% Luteína 90,1% Zeaxantina 97% Criptoxantina 92,5% Licopeno 85,8% α-caroteno 80,2% β-caroteno
Rajendran *et al*., 2005[169]	Luteína, zeaxantina, criptoxantina, α-caroteno, β-caroteno y licopeno	93% Luteína 94% Zeaxantina 89% Criptoxantina 92% α-caroteno 91% β-caroteno 94% Licopeno
Tzeng *et al*., 2004[159]	Luteína, β-caroteno y licopeno	92% Luteína 90% β-caroteno 87% Licopeno
Talwar *et al*., 1998[168]	Retinol, α-tocoferol, β-caroteno, luteína, licopeno y criptoxantina	94% Retinol 87% β-caroteno 94% Luteína 96% Criptoxantina

Con relación a los LDD y LDC, Mitrowska *et al*., 2012[171], quienes desarrollaron un método para la identificación de 15 carotenoides en plasma humano, obtuvieron valores muy similares a los de esta validación para el *trans*-*β*-apo-8'-carotenal, la astaxantina y el licopeno, mientras que comparando con los resultados obtenidos por Talwar *et al.,* 1998[168], solo el retinol tiene valores similares. Sin embargo, la ventaja de este método es la separación e identificación de 11 carotenoides y una vitamina liposoluble en un mismo análisis cromatográfico, mientras que Talwar *et al.* identificaron solamente 4 carotenoides y 2 vitaminas liposolubles.

Una vez validado el método, se analizaron muestras de un ensayo clínico preliminar con ocho voluntarios adultos, de alto riesgo cardiovascular, consiguiendo identificar y separar correctamente nueve de los 12 compuestos validados, obteniendo diferencias cualitativas entre el basal y después de la administración de distintas dosis de zumo de tomate durante 4 semanas de intervención. En las muestras basales se identificaron los siguientes compuestos: retinol, astaxantina, luteína, *trans*-*β*-apo-8'-carotenal, criptoxantina, 13-*cis*-*β*-caroteno, *α*-caroteno, *β*-caroteno, *trans*-licopeno, 5-*cis*-licopeno, mientras que los compuestos que fueron identificados después de la intervención fueron retinol, astaxantina, luteína, *trans*-*β*-apo-8'-carotenal, criptoxantina, 13-*cis*-*β*-caroteno, *β*-caroteno, *trans*-licopeno, 5-*cis*-licopeno, 9-*cis*-licopeno y 13-*cis*-licopeno, con un valor mínimo de 0,11 µmol/L correspondiente a la luteína y un valor máximo de 9,67 µmol/L correspondiente al *trans*-licopeno. Como puede observarse, hay diferencias cualitativas entre las muestras basales y tras la intervención; por ejemplo el *α*-caroteno pudo cuantificarse en las muestras basales, pero tras la intervención con el zumo no fue identificado, mientras que tras la intervención se pueden cuantificar las tres formas *cis* del licopeno, que son consideradas más biodisponibles. De hecho, en plasma humano, el licopeno total es una mezcla isomérica que contiene entre un 50% y un 60% de isómeros *cis*, mientras que las proporciones de isómeros *trans* alcanzan aproximadamente un 40%[172].

En un estudio llevado a cabo por Pellegrini *et al*., 2000[173], se describe una concentración de 0,58 µmol/L de licopeno y 0,32 µmol/L de *β*-caroteno en plasma después de una intervención con 25 g de puré crudo de tomate (aproximadamente 7 mg de licopeno y 0,25 mg de *β*-caroteno) con 5 g de aceite de oliva a 11 voluntarios sanos durante 14 días. Las diferencias observadas entre las concentraciones plasmáticas de estos compuestos en los voluntarios que participaron de dicho estudio y las concentraciones plasmáticas observadas en los voluntarios de nuestro estudio pueden deberse a que, aunque consumieron el puré de tomate conjuntamente con una matriz oleosa para asegurar la absorción de los carotenoides, las cantidades de licopeno y *β*-caroteno administradas a estos voluntarios fueron mucho menores que

las cantidades que se administraron a los voluntarios de nuestro estudio de intervención (7 mg *vs.* 21 mg de licopeno y 0,25 mg vs. 20 mg de *β*-caroteno, respectivamente).

En otro estudio realizado por Porrini *et al*., 1998[174] se obtuvieron valores del orden de 0,34 y 0,43 µmol/L para el licopeno y el *β*-caroteno, respectivamente, después de una suplementación diaria a 10 mujeres sanas durante 7 días con puré de tomate (60 g) o tomates frescos (300 g), equivalentes a aproximadamente 16,5 mg de licopeno cada uno, enriquecido con 10 g de aceite de oliva. Comparando sus resultados con los nuestros, las diferencias pueden atribuirse a la menor cantidad de licopeno que recibieron por dosis, así como al tiempo de duración del estudio (7 días *vs.* 4 semanas). Así también Gärtner *et al.,* 1997[175] describieron valores de 0,04 y 0,002 µmol/L para el licopeno, y *β*-caroteno, respectivamente, después de que 5 voluntarios sanos consumieran diariamente pasta de tomate (23 mg de licopeno) con 15 g de aceite durante 2 días. Aunque en este estudio la cantidad de licopeno administrada a los voluntarios fue similar a la nuestra y acompañaron la ingesta con aceite, el tiempo de intervención ha sido mucho menor que el nuestro. En cuanto al retinol, los resultados obtenidos con el método validado son más altos que los publicados por Liu *et al*., 2011[165], quienes cuantificaron una media de 1,92 µmol/L en plasma de voluntarios participantes del *Toronto Nutrigenomic Health Study*, mientras que la media cuantificada en nuestras muestras fue de 6,35 µmol/L. Con ello se pudo comprobar que el método desarrollado y validado es apto para ser aplicado en muestras de ensayos clínicos o bien en estudios epidemiológicos nutricionales.

Una vez que ya se tuvo un método adecuado para la identificación y cuantificación de los carotenoides en plasma, para la consecución de los objetivos 2 al 4 de esta tesis doctoral, se llevó a cabo un estudio de intervención en 28 voluntarios adultos de elevado riesgo cardiovascular, con una edad media de 69,7 ± 3,1 años y un IMC promedio de 31,5 ± 3,6 kg/m^2. Los voluntarios no debían tener ninguna ECV diagnosticada en el momento de ingresar en el estudio, pero sí debían tener hipertensión diagnosticada y uno o más de los siguientes factores de riesgo: a) ser fumador, b) tener hipercolesterolemia (LDL-colesterol >160 mg/dL), c) tener bajas concentraciones de HDL-colesterol (<40 mg/dL), d) tener sobrepeso u obesidad (IMC >25 kg/m^2), e) tener diabetes tipo II, y/o f) historia familiar de ECV temprana (parientes de primer orden, hombres <55 años; mujeres <65 años). Se ha demostrado que todos estos factores, de manera individual, incrementan el riesgo de desarrollar ECV[176], por lo que todos estos pacientes fueron considerados de alto riesgo cardiovascular.

Al estudiar la relación dosis-respuesta entre los niveles de carotenoides plasmáticos tras la ingesta durante 3 meses de dos distintas dosis de zumo de tomate, se observó

que los carotenoides que aumentaron de manera significativa tras la ingesta fueron el *trans*-licopeno y los isómeros *cis,* como el 5-*cis*, el 13-*cis* y el 9-*cis*-licopeno. Así también, se vio que el *β*-caroteno incrementaba de manera significativa, mientras que las xantófilas, aunque estaban presentes en el zumo de tomate, no aumentaron significativamente en el plasma. Estos resultados concuerdan con los obtenidos previamente en el estudio piloto.

Para la consecución del tercer objetivo de esta tesis doctoral, que fue el de determinar la asociación existente entre los carotenoides del tomate y las moléculas inflamatorias, como marcadores de riesgo cardiovascular en pacientes sin ECV, pero con alto riesgo de desarrollarla, se utilizó la bibliografía para la elección de los biomarcadores relacionados con la aterosclerosis que se determinarían, como la PCR, el IFN-*ɣ,* la IL-8, o las moléculas de adhesión ICAM-1 y VCAM-1.

Estas moléculas están relacionadas tanto con el inicio, como con la progresión de lesiones ateroscleróticas. Por ejemplo, la PCR es un reconocido reactante de fase aguda que actúa sobre las células endoteliales, con efectos agudos como la inducción de trombosis, y efectos crónicos que podrían contribuir con la formación y maduración de las placas de ateroma. La PCR se ha convertido en el marcador inflamatorio de elección en el ámbito clínico debido a esta asociación consistente con eventos cardiovasculares, su larga vida media y la estabilidad cuando se almacena congelada durante períodos prolongados de tiempo[177]. Por su parte, el IFN-*ɣ* es una citoquina proinflamatoria, considerada a menudo como un regulador clave del desarrollo de la aterosclerosis, ya que es capaz de influir en varios pasos fundamentales durante el desarrollo de la misma, incluyendo la expresión de genes proinflamatorios, el reclutamiento de monocitos circulantes hacia el endotelio activado y también se lo ha relacionado con el crecimiento y la estabilidad de la placa aterosclerótica[178]. La IL-8, a su vez, es una citoquina pro inflamatoria implicada en la migración de monocitos al espacio subendotelial, el cual es un paso clave en los estadios iniciales de la aterosclerosis, y también ha demostrado ser un potente quimiotáctico para los neutrófilos, dirigiéndolos a los sitios de lesión tisular[179]. Concentraciones elevadas de IL-8 se asociaron con un aumento del riesgo de enfermedad arterial coronaria futura en pacientes aparentemente sanos[180], como lo son los voluntarios que han participado en el estudio, y además se ha visto que es una quimoquina capaz de predecir eventos cardiovasculares de manera independiente a otras citoquinas[179].

Como ya se ha descrito, las moléculas de adhesión ICAM-1 y VCAM-1 facilitan el reclutamiento de leucocitos al espacio subendotelial. Además, las concentraciones plasmáticas elevadas de moléculas de adhesión se han correlacionado con varios factores de riesgo cardiovascular, tales como fumar[181,182], hipertensión[183,184], bajas

concentraciones de HDL-colesterol, hipercolesterolemia y/o hipertrigliceridemia[185,186]; y dado que todos estos factores de riesgo se corresponden con las características de los voluntarios que participaron en el estudio de intervención, se consideró fundamental determinar las concentraciones de estas moléculas en el plasma de los voluntarios.

Asimismo, se quiso observar el comportamiento de otras moléculas menos estudiadas con relación al riesgo cardiovascular, tras el consumo crónico de carotenoides del tomate, como por ejemplo la eotaxina y la CXCL10, y de esta forma poder elucidar el papel de los carotenoides con relación a estas moléculas. Por ejemplo, se ha observado que la CXCL10 es un potente mitogénico y quimiotáctico para el factor estimulante de colonia de macrófagos, importantes para la diferenciación de los monocitos en macrófagos, y además puede modular el equilibrio local del sistema inmune a través de la inducción de células T reguladoras. En animales de experimentación se ha observado que aquellos deficientes en CXCL10 ($ApoE^{-/-}$/$CXCL10^{-/-}$) presentaban lesiones ateroscleróticas más moderadas que los ratones de control que sí expresaban CXCL10 ($ApoE^{-/-}$/$CXCL10^{+/+}$), tras la ingesta de una dieta alta en grasas saturadas y colesterol durante un período de 6-12 semanas[187].

Por su parte, en cultivos de células a partir de células de músculo liso aórtico humano, la eotaxina se relacionó con la modulación de la función de los macrófagos, y probablemente en la activación y/o reclutamiento de mastocitos[147], y más recientemente, en un ensayo clínico realizado en pacientes obesos, la eotaxina se asoció con el grosor de la carótida íntima-media, como un factor de predicción temprana del proceso aterosclerótico[148].

Diversos estudios de intervención se llevaron a cabo con el fin de determinar los efectos del tomate, productos derivados del tomate, o incluso de los suplementos de licopeno sobre moléculas inmunoinflamatorias relacionadas sobre el desarrollo de la aterosclerosis. Una de las más estudiadas es la PCR y aunque autores como Biddle *et al*., 2015[188] observaron una correlación negativa entre las concentraciones elevadas de licopeno en plasma y las concentraciones de PCR, otros autores como Abete *et al*., 2013[189], Thies *et al*., 2012[190] y Blum *et al*., 2007[191] no encontraron ninguna asociación entre la suplementación con tomate o productos derivados del tomate y la atenuación de las concentraciones plasmáticas de esta molécula. Una posible explicación sobre los resultados negativos que se observaron en estos estudios podría ser que los productos del tomate que fueron administrados a los voluntarios, no tenían, o tenían muy poca cantidad de aceite y se ha demostrado que el consumo conjunto de una matriz lipídica junto con los alimentos ricos en carotenoides, mejora la biodisponibilidad de los mismos[192-194], siendo 3-5 g, la cantidad óptima de grasas para una mejor absorción[192].

Dado que el incremento de la ICAM-1 y la VCAM-1 está relacionado con estímulos pro-inflamatorios, tales como un aumento de las concentraciones de PCR en el plasma[137] y, como se observó una reducción de sus concentraciones tras la intervención con zumo de tomate, al mismo tiempo se esperaba una reducción de las concentraciones de ICAM-1 y VCAM-1. Sin embargo, en la literatura se observan resultados contradictorios; Thies *et al*., 2012[190] tras realizar un estudio de intervención durante 12 semanas para evaluar los efectos del consumo de una dieta rica en tomate y productos derivados del mismo sobre marcadores de riesgo cardiovascular, como la PCR, IL-6 o ICAM-1 frente al consumo de una dieta baja en tomate o una dieta suplementada con cápsulas de licopeno. La media de consumo de licopeno fue de 226-351 mg/semana en el grupo de voluntarios que llevaron la dieta rica en tomate, mientras que la media del grupo suplementado con licopeno y del grupo de voluntarios control (dieta baja en tomate), fue tan solo de 0-2 mg/semana. Al finalizar el estudio, no observaron cambios en los niveles de ICAM-1, aún después de haber incrementado las concentraciones de licopeno plasmático en un 267% en el grupo de intervención con la dieta rica en tomate. En nuestro estudio, la media de consumo de licopeno en la intervención con la dosis alta de zumo de tomate fue de 280 mg/semana, pero sin embargo el promedio de incremento del licopeno plasmático en este grupo de intervención fue de 410%. Estas diferencias podrían deberse principalmente a que las raciones de tomate o productos derivados del tomate que debían consumir los voluntarios de este estudio, no iban acompañadas de aceite. Por otra parte, Blum *et al*., 2007[191], no solo no encontraron diferencias entre las concentraciones basales de PCR e ICAM-1 y las concentraciones observadas tras una intervención con tomate fresco durante 1 mes, sino que además notaron un incremento de las concentraciones de ICAM-1 en el grupo control (dieta sin tomate); mientras que García-Alonso *et al*., 2012[195] observaron una reducción en las concentraciones de VCAM-1 tras una intervención con zumo de tomate durante 2 semanas, pero las concentraciones de ICAM-1 solamente disminuyeron si el zumo de tomate estaba enriquecido con ácidos grasos poliinsaturados ω-3. Resumiendo, las diferencias observadas respecto a nuestros resultados, podrían ser tanto por la dosis administrada como por la adición de aceite.

Con relación a la IL-8, algunos estudios que se llevaron a cabo para determinar la capacidad del licopeno sobre la inhibición de esta molécula, sobre todo en cultivos celulares[196] y en modelos animales[197], se observó una disminución de los niveles de IL-8 tras intervenciones/suplementaciones con licopeno. No obstante, no hay muchos ensayos clínicos en humanos publicados en los cuales se estudie el efecto del licopeno sobre esta quimoquina. Ghavipour *et al*., 2013[198] llevaron a cabo un estudio

para investigar si el consumo diario de 330 mL de zumo de tomate (37 mg/día de licopeno) durante 20 días podía reducir el estado inflamatorio en personas obesas. Los biomarcadores analizados fueron IL-6, IL-8, PCR y TNF-*α*. En concordancia con los resultados de este estudio, observaron una disminución significativa de la IL-8, pero sin embargo, no encontraron diferencias en las concentraciones de PCR plasmático tras la intervención. Estos autores hipotetizaron que los efectos antiinflamatorios atribuidos al licopeno no fueron suficientemente potentes para mermar el estado inflamatorio exacerbado observado en los pacientes obesos.

Uno de los posibles mecanismos mediante el cual tanto el *cis* como el *trans*-licopeno podría atenuar las concentraciones de la quimoquina IL-8, así como las moléculas de adhesión ICAM-1 y VCAM-1 y, posiblemente también modular las concentraciones plasmáticas de la PCR, es mediante la inhibición de la translocación del factor nuclear kappa B, implicado en la activación de la cascada inflamatoria que puede estimular la expresión de estas moléculas[191].

Por otra parte, en un ensayo clínico llevado a cabo por Tarantino *et al*., 2014[148], la eotaxina se asoció con el grosor de la íntima de la carótida media, como un predictor temprano de aterosclerosis en personas obesas. Mientras que la CXCL10, a su vez, se detectó en placas ateromatosas proclives a la ruptura[199]. Aunque en este estudio no se pudieron observar un descenso significativo en las concentraciones de estas moléculas tras la intervención con el zumo de tomate, sí se observó una tendencia a disminuir, sugiriendo futuras investigaciones con una población más numerosa para evaluar el efecto del consumo de los carotenoides del tomate, principalmente de los isómeros *trans*, sobre estas moléculas y su relación con el desarrollo de la aterosclerosis. En la **Tabla 15** se presentan los estudios citados junto con los resultados obtenidos tras las distintas intervenciones.

Tabla 15. Resultados obtenidos por los autores citados.

Autor	*Tipo de estudio*	*Duración*	*Intervención*	*Moléculas estudiadas*	*Resultados observados*
Biddle *et al.*, 2015[188]	Ensayo clínico randomizado con pacientes con insuficiencia cardíaca	30 días	326 g de zumo comercial de vegetales rico en licopeno (29,4 mg de licopeno) *vs.* agua como control	PCR	Disminución de la PCR en el grupo intervención
Tarantino *et al.*, 2014[148]	Estudio transversal de una cohorte de pacientes obesos con esteatosis hepática no alcohólica	11 meses	Medición del grosor de la íntima de la carótida y 27 moléculas inflamatorias	IL-1b, IL-1ra, IL-2, IL-4, IL- 5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, eotaxina (CCL11), basic FGF, G-CSF, GMCSF, IFN-g, IP-10 (CXCL10), MCP-1 (CCL2), MIP-1a (CCL3), MIP-1b (CCL4), PDGF-bb, RANTES (CCL5), TNF-α y VEGF	La eotaxina se correlacionó de manera negativa con el grosor de la íntima de la carótida
Abete *et al*., 2013[189]	Ensayo clínico randomizado con voluntarios sanos	28 días	160 g de salsa de tomate rica en licopeno (27, 2 mg licopeno/día) *vs*. 160 g de salsa de tomate convencional (12,3 mg licopeno/día)	PCR	Aumento de la PCR en el grupo que consumió salsa de tomate rica en licopeno
Ghavipour *et al*., 2013[198]	Ensayo clínico randomizado con mujeres obesas	20 días	330 mL de zumo de tomate *vs.* agua como control	IL-6, IL-8, PCR, TNF-*α*	Disminución de la IL-8 y TNF-*α* en el grupo intervención
Thies *et al.*, 2012[182]	Ensayo clínico randomizado con pacientes obesos	12 semanas	Dieta alta en tomate (35-50 mg licopeno/día) o dieta suplementada con cápsulas de licopeno (10 mg/día) vs. Dieta baja en tomate como control	PCR, ICAM-1, IL-6	No observaron diferencias significativas entre las concentraciones basales y las concentraciones tras las intervenciones en estas moléculas
García-Alonso *et al.,* 2012[195]	Ensayo clínico randomizado con mujeres sanas	2 semanas	500 mL de zumo de tomate enriquecido con Ω-3 *vs.* 500 mL de zumo de tomate solo (26,5 mg/licopeno)	ICAM-1, VCAM-1	Disminución de las concentraciones de VCAM-1 en ambos grupos de intervención. Disminución de las concentraciones de ICAM-1 en el grupo de intervención de zumo de tomate enriquecido con Ω-3
Blum *et al.*, 2007[191]	Ensayo clínico randomizado con voluntarios sanos	30 días	Dieta enriquecida con 300 g de tomate fresco *vs.* Dieta regular sin tomate	ICAM-1, PCR	No observaron diferencias significativas entre las concentraciones basales y las concentraciones tras las intervenciones de la PCR. Aumento de la ICAM-1 al finalizar el estudio en grupo de dieta control

Respecto al *β*-caroteno, coincidiendo con los resultados obtenidos en esta tesis doctoral, Jhonson *et al*., 1997[200] tampoco observaron cambios significativos en las concentraciones plasmáticas de este compuesto tras la administración de suplementos de 60 mg de *β*-caroteno (100%) solo, o en combinación con licopeno (97% *trans*-licopeno, 1% 13-*cis*-licopeno), ambos enriquecidos con aceite de maíz, mientras que las concentraciones plasmáticas de licopeno incrementaron tras la suplementación. Los autores señalaron que el consumo conjunto de *β*-caroteno y licopeno tiene mínimos efectos en la absorción del *β*-caroteno, pero sin embargo, la presencia del caroteno aumenta la absorción del licopeno, probablemente porque algunos componentes de la molécula del *β*-caroteno, aumentan la solubilización del licopeno cristalino y, por ende, mejorarían su respuesta plasmática[200]. Esta hipótesis concuerda con los resultados que se obtuvieron tras nuestro estudio de intervención y podría explicar la falta de correlación entre el *β*-caroteno y las moléculas estudiadas.

En cuanto al papel del licopeno en la nutrición humana, puede llegar a ser de difícil interpretación debido a que el isómero predominante en los alimentos, como por ejemplo en el tomate, es el *trans*, mientras que en plasma y en tejidos se encuentra principalmente en la configuración *cis*[201]; de hecho, el contenido de isómero *trans* en el tomate crudo o en productos derivados del tomate puede llegar a ser de entre un 60 y un 96%[196], mientras que en el plasma se ha observado una concentración de tan solo un 30-40%[172,202] y en tejidos un 20-65%[196,203,204], predominando en estos últimos el isómero *cis* (50-90%)[205]. Estas observaciones han llevado a la hipótesis de que los cambios de configuración isomérica de *trans* a *cis* podrían tener alguna influencia biológica con relación a la salud. Aunque se reconoce que los isómeros *cis* son mejor absorbidos y tienen una mayor biodisponibilidad, relacionándolos con los efectos beneficiosos del licopeno sobre la salud, el papel que desempeña la forma *trans* sobre la salud no está bien esclarecido. No obstante, en esta tesis se pudo observar que tras realizar ajustes por la forma *trans*, el efecto modulador del *cis*-licopeno se perdía, mientras que los ajustes por *cis*-licopeno disminuyeron los efectos protectores del *trans*-licopeno, sin llegar a ser una disminución significativa. De esta forma, se llegó a la conclusión de que la presencia de isómeros *trans* del licopeno, tanto en plasma como en los tejidos, cumple una función biológica importante, pudiendo llegar a ser clave en la salud cardiovascular, mediante la modulación de la producción de moléculas inflamatorias relacionadas con la aterosclerosis.

Fortalezas y debilidades de esta tesis

Las fortalezas observadas en esta tesis doctoral son las siguientes:

- El desarrollo y la validación de un método cromatográfico sensible, específico, que permite la separación, identificación y cuantificación simultánea de un elevado número de carotenoides en el plasma, además de una vitamina liposoluble, que ha demostrado ser eficiente en su aplicación a ensayos clínicos.
- El realizar la cuantificación en plasma de no sólo el *trans* licopeno, sino también de los tres isómeros *cis*: el 5,9 y 13 *cis* licopeno.
- En cuanto a los ensayos clínicos realizados, una fortaleza importante ha sido la elucidación del efecto beneficioso que tienen los isómeros *trans* del licopeno sobre la regulación de las citoquinas inflamatorias, quimoquinas y moléculas de adhesión tras el consumo crónico de zumo de tomate, a pesar de la mejor biodisponibilidad y mayor proporción de isómeros *cis* en el plasma humano.
- El análisis de dos biomarcadores: la eotaxina y la CXCL10 capaces de predecir futuros eventos cardiovasculares, sobre los que no había ningún estudio previo correlacionándolos con el consumo de carotenoides.

Algunas de las limitaciones que tiene esta tesis son:

- Debido a que el estudio de intervención con zumo de tomate se llevó a cabo con voluntarios adultos de alto riesgo cardiovascular, los resultados observados no pueden ser extrapolados a la población en general.
- Aunque el estudio era cruzado y aleatorizado, el número de pacientes incluidos era pequeño (n=28), por lo que podría existir una confusión residual, a pesar de los ajustes por potenciales factores de confusión que hemos realizado durante los análisis de correlación.
- Los posibles efectos sinérgicos entre distintos componentes del tomate y otros componentes de la dieta de los voluntarios no fueron evaluados.
- Las limitaciones intrínsecas de los biomarcadores estudiados, como pueden ser, errores en las mediciones durante los análisis o la variabilidad interindividual de los voluntarios.

Conclusiones

VII. Conclusiones

Conclusiones generales

El consumo regular de zumo de tomate tiene un efecto protector sobre parámetros de riesgo cardiovascular, al incrementar las concentraciones plasmáticas de carotenoides, lo cual a su vez se relaciona con la modulación de las concentraciones plasmáticas de moléculas inflamatorias y moléculas de adhesión relacionadas con el inicio y la progresión de la aterosclerosis.

Conclusiones específicas

- Es fundamental contar con una herramienta sensible y específica que permita la cuantificación de los carotenoides en el plasma. El método desarrollado por HPLC-UV/DAD ha sido validado y permite la separación, identificación y cuantificación de once carotenoides y una vitamina liposoluble en plasma humano: luteína, zeaxantina, astaxantina, criptoxantina, *trans*-*β*-apo-8'-carotenal, 15-*cis*-*β*-caroteno, 13-*cis*-*β*-caroteno, *α*-caroteno, *β*-caroteno, 9-cis-*β*-caroteno, *trans*-licopeno y retinol, además de la identificación de 3 isómeros del licopeno (5-*cis*, 9-*cis* y 13-*cis*-licopeno), previamente descritos por nuestro grupo de investigación.
- El consumo regular de zumo de tomate durante 4 semanas se relaciona con un incremento significativo de las concentraciones plasmáticas de determinados carotenoides, especialmente del licopeno y sus isómeros, tanto el *trans*-licopeno, como el 5-*cis*, el 9-cis y el 13-cis-licopeno, así como del *β*-caroteno.
- Se observa una relación dosis-respuesta en el nivel de carotenoides. El incremento de las concentraciones plasmáticas de carotenoides es proporcional a la cantidad consumida de zumo de tomate, siendo por lo tanto más elevadas después del consumo de dos raciones (400 mL), que de una ración (200 mL).
- El aumento de carotenoides plasmáticos se correlaciona con una menor concentración plasmática de moléculas de inflamación tales como la PCR, la citocina pro-inflamatoria y quimiotáctica IL-8 y las moléculas de adhesión ICAM-1 y VCAM-1.

- Los carotenoides plasmáticos también podrían modular otros biomarcadores cardiovasculares relacionados con la aterosclerosis, como la eotaxina, el IFN-ɣ y la CXCL10.
- A pesar de la mayor biodisponibilidad y la mayor proporción de los isómeros *cis* del licopeno en el plasma humano, el isómero *trans*-licopeno puede explicar la menor concentración plasmática de moléculas de adhesión celular.
- Los isómeros *cis* del licopeno no se correlacionan con la disminución de las concentraciones de moléculas inflamatorias y de adhesión celular.

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VIII. Referencias Bibliográficas

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Anexos

IX. Anexos

A. Publicaciones

En los anexos se incluyen dos publicaciones (Publicación 1 y 2) donde se muestran los resultados obtenidos en esta tesis doctoral y otras dos publicaciones (Publicación 3 y 4) que no forman parte propiamente del trabajo de la tesis.

Publicación 1. Un nuevo método para la cuantificación simultánea de antioxidantes: carotenos, xantófilas 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, Ramón Estruch, and Rosa M. Lamuela-Raventós. *Oxidative Medicine and Cellular Longevity.* 2016; 2016: 9268531. doi: 10.1155/2016/9268531

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Research Article

A New Method to Simultaneously Quantify the Antioxidants: Carotenes, Xanthophylls, and Vitamin A in Human Plasma

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A simple and accurate reversed phase high-performance liquid chromatography coupled with diode array detector (HPLC-DAD) method for simultaneously determining and quantifying the antioxidants carotenes, xanthophylls, and retinol in human plasma is presented in this paper. Compounds were extracted with hexane, a C30 column, and a mobile phase of methanol, methyl *tert*-butyl ether, and water were used for the separation of the compounds. A total of 8 carotenoids, 3 *Z*-*β*-carotene isomers, and 1 fat-soluble vitamin (retinol) were resolved within 72 min at a flow rate of 0.6 mL/min. Detection was achieved at 450 nm for carotenoids and 330 nm for retinol. To evaluate the effectiveness of themethod, it has been applied to an intervention study conducted on eight volunteers. *Results.* Limits of detection were between 0.1 *μ*g/mL for lycopene and astaxanthin and 1.3 *μ*g/mL for 15-*Z*-*β*-carotene. Recoveries were ranged between 89% and 113% for *α*-carotene and astaxanthin, respectively. Accuracy was between 90.7% and 112.2% and precision was between 1% and 15% RSD. In human plasma samples compounds studied were identified besides three lycopene isomers, demonstrated to be suitable for application in dietary intervention studies. *Conclusions.* Due to its accuracy, precision, selectivity, and reproducibility, this method is suitable to dietary habits and/or antioxidants status studies.

1. Introduction

Several epidemiologic studies have shown that oxidative stress plays an essential role in the pathogenesis of many degenerative diseases, such as cancer, diabetes, age-related eye diseases, and cardiovascular diseases [1–6] and it has been suggested that antioxidants may exert a protective role against these chronic diseases by defending against oxidative damage [7, 8]. There is an increasing interest in the analysis of carotenoids and some fat-soluble vitamins such as retinol (vitamin A) due to their antioxidant properties and their relationship with the prevention of chronic diseases [9–12]. The characterization and quantification of carotenoids and retinol in human plasma are essential for best interpretation of epidemiologic studies linking oxidative stress, diet, and health.

Carotenoids comprise a group of fat soluble phytochemicals widely distributed in nature, responsible for the colours of many fruits and vegetables, as well as certain animal tissues and leaf coloration after the degradation of chlorophyll [13]. Considering the number of double bonds in the molecules, carotenoids can be found with *cis* or *trans* configuration (or *E*/*Z* isomers). In general the all-*E* form is predominant in nature but numerous researches show that more than 50% of some carotenoids, as lycopene, present in human plasma and tissues are *Z*-isomers [14] and it is believed that geometrical configuration of carotenoids could have implications in the solubility, absorption, and transport in humans [15, 16] or even geometrical isomers of provitamin A carotenoids have different vitamin A activities [17]. The enhanced absorption of lycopene *Z*-isomers is hypothesised to result from higher

solubility in mixed micelles, the shorter length of the Z-isomers, and/or a lower tendency to aggregate [14, 18]. This hypothesis was supported by studies in both animals and humans [18, 19].

Among fat-soluble vitamins, retinol exerts an important antioxidant action via the inhibition of lipid peroxidation and has free-radical-scavenging properties [11, 20]; meanwhile, carotenoids are known to be effective quenchers of singlet oxygen, as well as strong scavengers of different reactive oxygen species (ROS) [21].

High-performance liquid chromatography (HPLC) is one of the most used techniques for the identification of carotenoids [22, 23] as well as vitamin A in human plasma [24–26]. The advantages of the use of this technique are speed, sensitivity, and accuracy for the determination of the compounds in addition to the economy of the solvents required and the simple coupling with other techniques.

In light of the importance of these compounds for health maintenance, an accurate determination and quantification in plasma is necessary. The aim of this study is to develop a simple HPLC method to identify the main carotenoids and their geometrical isomers as well as retinol, one of the major antioxidant fat-soluble vitamins, in human plasma.

2. Experimental Procedures

2.1. Materials and Methods

2.1.1. Reagents and Standards. Carotenoids and vitamins standards: retinol, astaxanthin, lutein, zeaxanthin, *E*-β-apo-8′-carotenal, cryptoxanthin, 15-*Z*-β-carotene, 13-*Z*-β-carotene, α-carotene, β-carotene, 9-*Z*-β-carotene, and lycopene were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Methanol (MeOH) and methyl-*tert*-butyl ether (MTBE) of HPLC grade were obtained from Panreac Quimica SA (Barcelona, Spain). Ultrapure water (Milli-Q) was generated by a Millipore System (Bedford, MA, USA). Human plasma and butylated hydroxytoluene (BHT) were acquired from Sigma-Aldrich.

2.1.2. Preparation of Standard and Stock Solutions. Individual working standards of retinol, astaxanthin, lutein, zeaxanthin, *E*-β-apo-8′-carotenal, cryptoxanthin, 15-*Z*-β-carotene, 13-*Z*-β-carotene, α-carotene, β-carotene, 9-*Z*-β-carotene, and lycopene were prepared at a concentration of 1 mg/mL in MTBE. All working standards were manipulated under protection of light to minimise light-induced isomerisation, stored in eppendorf tubes, and kept at −20°C until use.

The stock solution used to spike plasma samples was prepared by mixing individual working standards at a concentration of 50 mg/mL in MTBE.

2.1.3. Extraction and Isolation of Carotenoids and Retinol. Plasma was subjected to a liquid-liquid extraction procedure previously described by our working group [27]. Briefly, 800 μL of plasma was mixed with 800 μL of ethanol and 2 mL of hexane/BHT (100 mg/L). Then, 300 μL of stock solution was added followed by a vortex-mixing for 1 minute and centrifuged at 2062 gr for 5 minutes at 4°C. The upper nonpolar layer was removed and the remaining aqueous plasma mixture was reextracted as described above. The two nonpolar extracts were combined in a glass vial and dried under nitrogen gas at <25°C followed by a reconstitution with 300 μL of MTBE. Then, the samples were stored into insert-amber vials for HPLC at −20°C until the day of analysis.

2.1.4. Instrumentation. Chromatographic analysis was carried out in an HP 1100 HPLC system (Hewlett-Packard, Waldbronn, Germany), consisting of a quaternary pump and an autosampler coupled to a diode array detector DAD G1315B. Chromatographic separation was performed on a reversed-phase column YMC Carotenoid S-5 μm, 250 mm × 4.6 mm (Waters, Milford, MA), maintained at 25°C, and connected to a precolumn YMC Guard Cartridge Carotenoid 20 × 4.0 mm i.d, S-5 μm. The guard column was replaced every 500 injections. The integration was performed with Agilent ChemStation Software. The DAD detector was adjusted at 450 nm for the carotenoids and 330 nm for retinol, respectively. All compounds were identified by retention time compared with pure standards as well as by the UV-Vis spectra of each compound.

2.1.5. Chromatographic Conditions. The chromatographic separation was performed using the following solvents: Milli-Q water (A), methanol (B), and MTBE (C). Solvent A was used isocratically at 4% while the following linear gradient was used for B (*t* (min), %B): (0.0, 90); (40.0, 40); (60.0, 6); (62.0, 90); (72.0, 90). Twenty microliter aliquots of the samples were injected in the HPLC-DAD system. Total run time was 72 minutes at a flow rate of 0.6 mL/min.

2.1.6. Quality Parameters. The method was fully validated based on the criteria of the AOAC International and the U.S. Department of Health and Human Services Food and Drug Administration (FDA) [28, 29]. The quality parameters established for the validation of the method were accuracy, intra- and interday precision, recovery, limit of detection (LoD), limit of quantification (LoQ), and linearity.

Three dilutions were prepared from the stock solution in MTBE to the final concentrations of low (1.5 μg/mL), medium (4 μg/mL), and high (9 μg/mL) for all analytes for precision and accuracy assays.

Accuracy consisted in the closeness of agreement between the measured value and the reference value and was established by repetitively spiking blank plasma with three known concentrations of analyte standards: low, medium, and high with respect to the calibration curves, in five replicates. The results were determined as the percentage of the ratio of the mean observed concentration and the known spiked concentration in the biological matrices. The mean value should be within ±15% of the nominal value.

Intraday precision and interday precision were considered using five determinations per three concentration levels: low, medium, and high in a single analytical run or in three different days, respectively. The precision of the method was assessed on the % RSD (percentage of relative standard deviation) of intra- and interday repeatability and the values determined at each concentration level should not exceed

15% of RSD, according to the regulation of the AOAC and FDA.

Recovery was accomplished by preparing seven-point calibration curves and seven-point external curves, spiked before and after extraction, respectively. The detector response obtained from the amount of analyte added to and extracted from the biological matrix was compared to the detector response obtained for the same concentration of the pure authentic standard. A linear regression between the ratio analyte concentration against the calculated concentration has been applied, and the slope multiplied by 100 corresponded to the analyte recovery.

Limit of detection (LoD) and limit of quantification (LoQ) were determined by comparing measured signals from samples with the low concentration of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected or quantified. A signal-to-noise ratio in the order of 3 : 1 and 10 : 1, for LoD and LoQ, respectively, was considered acceptable.

Linearity was tested by assessing signal responses of target analytes from plasma samples spiked in duplicate at seven different concentrations and by calculation of linear regression.

2.2. Method Application: Pilot Dietary Intervention Study

2.2.1. Biological Material. To assess the efficiency in the identification of carotenoids and retinol in human plasma, the present method was applied to a small-scale prospective, open and controlled, single-arm intervention study conducted in eight volunteers free of cardiovascular disease but with high risk of developing it, aged 69.9 ± 3.8 years with a mean body mass index of 32.3 ± 3.8 kg/m^2.

The volunteers were instructed to avoid the consumption of tomato and tomato-based products 3 days before the study. On the experimental day, after 12 hours of fasting, blood samples were collected early in the morning to quantify carotenoids and retinol as baseline. After that, all volunteers have followed a similar diet developed by a trained dietitian, which took into account their preferences and tastes, as well as the consideration that participants were diabetic, obese, hypertensive, and/or dyslipidemic. Each day during 4 weeks, participants consumed 250 mL of tomato juice before dinner and at the end of the study blood samples were taken for comparing with baseline. All samples were 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). Written informed consent was obtained from all participants.

2.3. Statistical Analysis. Statistical analysis was performed using the SPSS Statistical Analysis System (version 22.0; SPSS Inc, Chicago, IL). Data are presented as means and standard deviation (SD). Statistical differences between the two interventions were analysed by the nonparametric statistical Wilcoxon test for paired comparisons. Statistical significance was set at $p < 0.05$.

3. Results and Discussion

3.1. HPLC Method Development

3.1.1. Extraction. For the extraction of carotenoids and retinol, precipitation of proteins from plasma as a prior step is required. To achieve this issue, most methods use organic solvents such as methanol, ethanol, or acetonitrile [30]. Some studies use ethanol-BHT at different concentrations [31, 32] while others use ethanol-ascorbic acid [33] or ethanol-saline [34] for protection of carotenoids besides deproteinization. Different solvents and solvent combinations have been described in the literature for removing lipophilic analytes from biosamples. Hexane alone [35, 36] or combined with other solvents, such as hexane/acetone [37], hexane/ether [34], or hexane/ethanol/acetone/toluene [38], seems to be the most used solvents for the extraction of carotenoids and lipophilic compounds from plasma. There are few studies where hexane-BHT is used for protection of carotenoids during the extraction [39, 40] while other authors prefer to use hexane-saline [34]. Both mixtures, hexane-BHT and hexane-saline, were tested for extraction. Recoveries obtained with hexane-saline were between 57.4% and 86.9% corresponding to *E*-lycopene and 13-*Z*-β-carotene, respectively, versus recoveries range between 73.4% and 90.4% corresponding to *E*-lycopene and β-carotene, respectively, using hexane-BHT as extraction solvent. Due to its simplicity, speed, and good recoveries achieved, ethanol for deproteinization and repeated extraction with hexane-BHT were chosen for performing this study.

3.1.2. Separation and Identification. Numerous HPLC methods are reported for separation and identification of carotenoids and fat-soluble vitamins from diverse complex matrices such as food, food products, or plasma. The use of isocratic or gradient systems coupled to different types of columns and/or coupled to different detectors or even the use of different temperatures for the stability of the analytes, depending of the compounds studied is the best described technique for these purposes.

Reversed-phase HPLC with C_{18} columns and isocratic or gradient elution seems to be the modality most commonly used for identification and quantification of carotenoids and fat-soluble vitamins [41–44]. One of the major problems in the identification of carotenoids and lipophilic compounds using C_{18} columns seems to be the separation of geometrical isomers of carotenoids [41–47]. Tzeng et al. [33] have developed an isocratic method for the identification of carotenoids using a C_{18} column, but they could only separate three carotenoids: lutein, lycopene, and β-carotene. Olmedilla et al. [40] also have described a gradient method with a C_{18} column but could not identify isomers of the carotenoids. To solve this issue, polymeric C_{30} columns were developed for separation of *Z*-*E* isomers [22, 48, 49]. The use of this kind of column has allowed us the separation of the twelve compounds studied, including 3 *Z*-isomers of β-carotene and another 3 *Z*-isomers

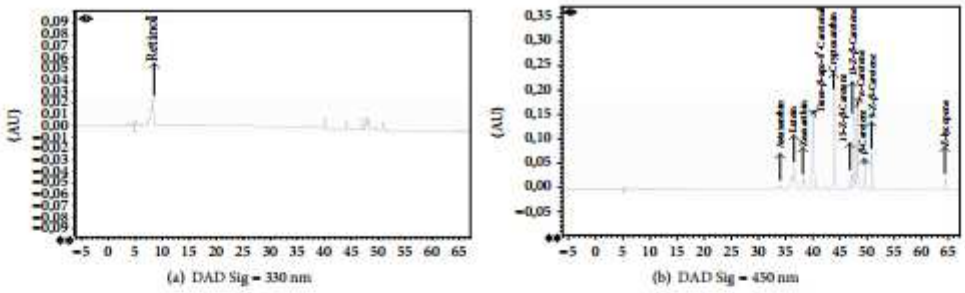

Figure 1: Representative HPLC chromatogram of carotenoids and retinol standards in MTBE.

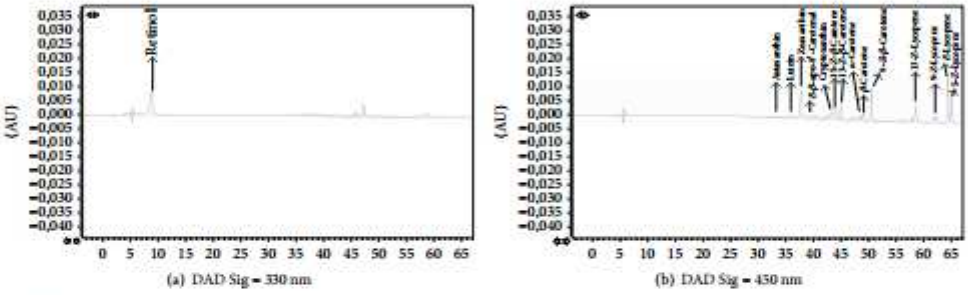

Figure 2: Representative HPLC chromatogram of carotenoids and retinol in human plasma corresponding to a volunteer after tomato juice intervention.

of lycopene. Figures 1 and 2 show a representative HPLC-DAD chromatogram of carotenoids and retinol in a standard mixture and a human plasma sample, respectively.

Likewise lutein and zeaxanthin are reported as two difficult compounds to separate on monomeric C_{18} columns [42, 43]. Gueguen et al. [44] found an inadequate resolution of lutein and zeaxanthin with the method proposed using a C_{18} column and an isocratic elution system; Thibeault et al. [31] were not able to differentiate lutein and zeaxanthin with these same conditions. A high resolution can be achieved by using polymeric C_{30} columns with gradient elution [22, 49, 50]. With the present method, all analytes, including lutein and zeaxanthin, have reached a resolution higher than 1.5, indicating a good separation of the compounds and a good symmetry of all peaks. Results are shown in Table 1.

Coupling of photodiode array detector (DAD) and fluorescence detector (FLD) is the technique chosen by Epler et al. [51] and Liu et al. [32] for identification of carotenoids and retinols from human serum and foods. Using more complex techniques, such as photoisomerization of some analytes as a prior step, Ferruzzi et al. [49] identified 13 lycopene isomers (*Z*/*E*) by using an electrochemical detector (ECD). Lyan et al. [46] and Lee et al. [47, 52] have identified various carotenoids by the coupling of two different detectors or columns. Other authors like Gleize et al. [53] have set up a gradient method for the identification of eleven carotenoids and fat-soluble vitamins from complex matrixes such as food samples, human plasma, and human adipose tissue using a single C_{30} column kept at 35°C. All these techniques allow the separation and identification of lipid compounds such as carotenoids and fat soluble vitamins from different matrices but are complex and require coupling of columns and use of temperature or combination of detectors, which is not always possible in all laboratories.

In this study, carotenoids and retinol were separated in a single run on a reversed-phase column using a gradient system of water, methanol, and MTBE. The mobile phase was optimized in order to obtain the best separation of the compounds in the shortest time possible and to achieve this, several gradients were assessed. The best results obtained for the conditions were described in the chromatographic conditions section.

3.2. Validation Parameters

3.2.1. Linearity. According to the maximal reported value in plasma for each analyte, plasma samples were spiked in duplicate at seven different concentrations ranged from LoQ of each compound to 10 μg/mL. The analytical procedure was linear over the concentration range tested with the correlation coefficient from 0.9952 to 0.9984 for all compounds in plasma samples, demonstrating a good linearity of the curves. Table 4

Table 1: Resolution of the analytes studied.

Analyte	Rt (min)	Wavelength (nm)	Width (min)	Resolution
Retinol	8.54	330	0.6	N.d.
Astaxanthin	34.07	450	0.38	2.3
Lutein	36.55	450	0.72	1.4
Zeaxanthin	38.24	450	0.53	2.2
E-β-apo-8′-carotenal	40.01	450	0.29	7.7
Cryptoxanthin	43.94	450	0.22	6.9
15-*Z*-β-carotene	46.96	450	0.22	1.4
13-*Z*-β-carotene	47.57	450	0.21	1.3
α-carotene	48.12	450	0.2	2.9
β-carotene	49.57	450	0.31	2.3
9-*Z*-β-carotene	50.76	450	0.2	33.0
E-lycopene	64.29	450	0.21	33.0

N.d.: not determined.
$R = 2[(\mathrm{Rt})B - (\mathrm{Rt})A]/(WA + WB)$.
R = resolution.
Rt = retention time.
W = width.

Table 2: Accuracy and recovery of the compounds studied.

Analyte	Accuracy (%)	Recoveries (%)
Retinol	105 ± 9	96 ± 3
Astaxanthin	99 ± 7	113 ± 6
Lutein	99 ± 13	112 ± 9
Zeaxanthin	101 ± 11	107 ± 5
E-β-apo-8′-carotenal	98 ± 10	94 ± 3
Cryptoxanthin	103 ± 12	96 ± 3
15-*Z*-β-carotene	90 ± 13	101 ± 2
13-*Z*-β-carotene	105 ± 12	92 ± 5
α-carotene	98 ± 12	89 ± 4
β-carotene	97 ± 13	96 ± 2
9-*Z*-β-carotene	112 ± 7	93 ± 3
E-lycopene	100 ± 12	91 ± 3

summarizes the correlation coefficients of the curves of all compounds.

3.2.2. Accuracy and Precision. Accuracy and intra and interday precision were studied. All compounds analysed met the acceptance criteria to not overcome 15% RSD in both intra- and interday precision and in the three concentration levels. The highest values were 15% belonging to astaxanthin, zeaxanthin, α-carotene, β-carotene, and 13-*Z*-β-carotene. Accuracy results obtained were between 90.7% and 112.2% being within limits of accuracy, 85–115%. The method proposed demonstrated good accuracy and precision in plasma samples, asserting that was feasible for the determination of carotenoids and retinol in human plasma. Results are expressed in Tables 2 and 3.

3.2.3. Recovery. Recovery for retinol was 96%, similar to the value achieved by Kand'ár et al. [54]. For carotenoids, the recoveries were between 89% and 113%, corresponding to α-carotene and astaxanthin, respectively. Comparing our results with those reported by Talwar et al. [43] we achieved a recovery 18% higher for lutein and 9% higher for β-carotene with the described method; comparing with the data presented by Tzeng et al. [33], we achieved a better recovery for lutein (20% higher), and comparing with Rajendran et al. [22] we obtained a better recovery for lutein (19% higher), zeaxanthin (13% higher), and cryptoxanthin (7% higher). Karppi et al. [39] have reported similar values of recovery except for lutein, zeaxanthin, and β-carotene that were 10%, 17%, and 16%, respectively, lower than in the present study.

The extraction procedure was really effective, since high recoveries can be observed in Table 2.

3.2.4. Limit of Detection (LoD) and Limit of Quantification (LoQ). The LoD found was 0.1 μg/mL for astaxanthin and lycopene; 0.2 μg/mL for retinol, zeaxanthin, *E*-β-apo-8′-carotenal, cryptoxanthin, β-carotene, and 9-*Z*-β-carotene; 0.4 μg/mL for lutein and 13-*Z*-β-carotene; 0.5 μg/mL for α-carotene, and 1.3 μg/mL for 15-*Z*-β-carotene. The LoQ was from 0.3 μg/mL to 4.4 μg/mL for astaxanthin and 15-*Z*-β-carotene, respectively. All LoD and LoQ values obtained are expressed in Table 4. In a study carried out by Mitrowska et al. [55] similar values were obtained for *E*-β-apo-8′-carotenal, astaxanthin, and lycopene. In another study developed by Talwar et al. [43] a similar value for retinol but not for the other compounds studied can be observed; nonetheless, with the presented method we have achieved the separation and identification of 11 carotenoids and 1 fat-soluble vitamin in a single run.

3.2.5. Plasma Levels of Carotenoids and Retinol. The method was used for measuring concentrations of carotenoids and retinol in human plasma samples. All compounds were determined following the procedure described above and the results are expressed in Table 5.

Nine of the 12 validated analytes were identified in plasma at baseline: retinol, astaxanthin, lutein, *E*-β-apo-8′-carotenal, cryptoxanthin, 13-*Z*-β-carotene, α-carotene, β-carotene, and lycopene. It is important to highlight that, besides the identification of the validated compounds, we also have searched for *cis* isomers of lycopene which have been suggested to be more bioavailable than *E*-lycopene, typically found in raw food [18]. In fact, in human plasma, total lycopene is an isomeric mixture containing 40% to 50% as *Z*-isomers [5] and according to the antioxidant properties of lycopene, from greatest to least are 5-*Z*, 9-*Z*, 7-*Z*, 13-*Z*, 15-*Z*, 11-*Z*, and all-*E* lycopene [56]. We have identified 5-*Z*, 9-*Z*, and 13-*Z* lycopene, all present in human plasma according to the study carried out by Arranz et al. [27]

After tomato juice intervention, an increase in retinol, astaxanthin, cryptoxanthin, 13-*Z*-β-carotene, β-carotene, 13-*Z*-lycopene, 9-*Z*-lycopene, *E*-lycopene, and 5-*Z*-lycopene was observed, with values between 5.194 and 0.140 μg/mL corresponding to *E*-lycopene and 13-*Z*-β-carotene, respectively.

Table 3: Intra- and interday precision.

Analyte	Precision											
	1.5 μg/mL ($n = 5$)				6 μg/mL ($n = 5$)				9 μg/mL ($n = 5$)			
	Day 1 (RSD %)	Day 2 (RSD %)	Day 3 (RSD %)	RSD Interday	Day 1 (RSD %)	Day 2 (RSD %)	Day 3 (RSD %)	RSD Interday	Day 1 (RSD %)	Day 2 (RSD %)	Day 3 (RSD %)	RSD Interday
Retinol	3	4	5	4.9	10	12	8	14.1	13	10	11	14.3
Astaxanthin	7	3	3	14.5	11	13	12	14.6	15	12	12	14.7
Lutein	8	9	4	14.2	12	14	8	14.3	10	9	12	13.3
Zeaxanthin	4	15	4	10.8	12	3	7	12.9	11	11	10	14.8
E-β-apo-8′-carotenal	11	10	4	14.3	11	12	8	14.7	13	9	12	13.8
Cryptoxanthin	5	13	3	12.1	11	6	9	14.3	11	10	8	13.9
15-*Z*-β-carotene	5	13	1	9.4	11	5	9	15.1	10	7	11	13.4
13-*Z*-β-carotene	6	14	3	14.9	12	15	12	14.0	11	10	10	14.9
α-carotene	4	15	2	9.4	11	15	11	13.7	11	9	10	14.8
β-carotene	11	10	8	11.2	11	15	14	14.9	12	6	10	12.8
9-*Z*-β-carotene	6	10	7	10.4	12	12	4	13.9	11	11	11	14.8
E-lycopene	5	5	2	11.6	10	13	9	13.8	10	15	8	14.8

Nd.: not determined.
RSD: relative standard deviation.

Table 4: Limit of detection (LoD), limit of quantification (LoQ), range of concentration, calibration curve, and correlation coefficient of the analytes in blank plasma spiked with standard solution.

Analytes	LoD (μg/mL)	LoQ (μg/mL)	Linearity range (μg/mL)	Calibration curve	Correlation coefficient (r)
Retinol	0.2	0.7	0.7–10	$y = 69.684x - 15.857$	0.9952
Astaxanthin	0.1	0.3	0.3–10	$y = 49.138x + 3.0197$	0.9964
Lutein	0.4	1.3	1.3–10	$y = 119.37x - 2.263$	0.9954
Zeaxanthin	0.2	0.7	0.7–10	$y = 69.14x + 1.53$	0.9955
Apo-8′-carotenal	0.2	0.7	0.7–10	$y = 249.4x - 20.908$	0.9957
Cryptoxanthin	0.2	0.7	0.7–10	$y = 200.48x - 15.759$	0.9952
15-Z-β-carotene	1.3	4.3	4.3–10	$y = 91.235x - 10.848$	0.9984
13-Z-β-carotene	0.4	1.3	1.3–10	$y = 90.591x + 8.7376$	0.9969
α-carotene	0.5	1.6	1.6–10	$y = 243.07x - 37.135$	0.9966
β-carotene	0.2	0.7	0.7–10	$y = 105.8x - 2.9437$	0.9983
9-Z-β-carotene	0.2	0.7	0.7–10	$y = 75.685x - 8.4189$	0.9959
E-lycopene	0.1	0.3	0.3–10	$y = 34.532x - 4.2861$	0.9952

Table 5: Carotenoids and retinol in human plasma before (baseline) and after the dietary intervention.

Analytes	Concentration			
	Baseline Mean ± SD (μg/mL)	After intervention Mean ± SD (μg/mL)	Baseline Mean ± SD (μmol/L)	After intervention Mean ± SD (μmol/L)
Retinol	1.82 ± 0.37	1.90 ± 0.49	3.46 ± 0.70	3.62 ± 0.93
Astaxanthin	0.76 ± 0.44	0.88 ± 0.37	1.27 ± 0.74	1.48 ± 0.63
Lutein	0.07 ± 0.02	0.06 ± 0.06	0.13 ± 0.03	0.11 ± 0.11
Zeaxanthin	N.d.	N.d.	N.d.	N.d.
E-β-apo-8′-carotenal	0.57 ± 0.03	0.57 ± 0.03	1.38 ± 0.06	1.38 ± 0.08
Cryptoxanthin	0.18 ± 0.12	0.20 ± 0.07	0.32 ± 0.22	0.37 ± 0.12
15-Z-β-carotene	N.d.	N.d.	N.d.	N.d.
13-Z-β-carotene	0.13 ± 0.00	0.14 ± 0.02	0.23 ± 0.00	0.26 ± 0.04
α-carotene	0.22 ± 0.00	N.d.	0.41 ± 0.00	N.d.
β-carotene	0.95 ± 0.50	1.09 ± 0.53	1.77 ± 0.93	2.03 ± 0.98
9-Z-β-carotene	N.d.	N.d.	N.d.	N.d.
13-Z-lycopene	N.d.[a]	2.79 ± 1.44[a]	N.d.[a]	5.20 ± 2.69[a]
9-Z-lycopene	N.d.[a]	0.38 ± 1.42[a]	N.d.[a]	0.71 ± 2.64[a]
E-lycopene	1.15 ± 0.83[a]	5.19 ± 2.35[a]	2.14 ± 1.54[a]	9.67 ± 4.38[a]
5-Z-lycopene	0.75 ± 1.10[a]	3.07 ± 1.43[a]	1.41 ± 2.06[a]	5.72 ± 2.67[a]

N.d.: not determined.
SD: standard deviation.
[a]Values in a row with the same letter are significantly different ($p < 0.05$). Data analyzed by Wilcoxon test for repeated measures.

Among these compounds, lycopene and its isomers have presented a significant increase after tomato juice consumption ($p < 0.05$). Results are shown in Table 5.

In a study carried out by Pellegrini et al. [57], they found a concentration of 0.31 μg/mL of lycopene and 0.17 μg/mL of β-carotene in human plasma after a consumption of tomato purée. In another study performed by Porrini et al. [58], they have seen values in the order of 0.18, 0.21, 0.02, 0.13, 0.03, and 0.23 μg/mL for lycopene, lutein, zeaxanthin, β-cryptoxanthin, α-carotene, and β-carotene, respectively, after a daily supplementation of tomato purée. Gärtner et al. [59] have determined values of 0.02, 0.0002, and 0.001 μg/mL for lycopene, α-carotene, and β-carotene, respectively, after a consumption of tomato paste. Regarding retinol, the results obtained with the present method are higher than those reported by Liu et al. [32] who have found a mean of 0.55 μg/mL and 0.56 μg/mL in plasma of women and men, respectively, participants of the Toronto Nutrigenomics and Health Study.

4. Conclusions

Based on previous reported work, the present method is simple, accurate, reliable, sensitive, and selective for the determination of the antioxidants carotenoids and retinol in human plasma samples. With this method, all analytes of interest were successfully resolved, including lutein, zeaxanthin, and Z-isomers of β-carotene which have previously

been reported as critical compounds to identify and separate. The sample preparation procedure in this method provides excellent recoveries for all analytes.

A total of 8 carotenoids, 3 *Z*-isomers of the *β*-carotene, 1 fat-soluble vitamin, and also 3 *Z*-isomers of the lycopene were simultaneously separated and identified in human plasma by the use of polymeric C_{30} chromatography column with a gradient elution.

Future approaches to enhance the analysis of the isomers should focus on the conclusive identification of the compounds that remain tentatively identified. For this purpose, the isolation of standards of most *cis*-isomers and the determination of their absorption coefficients are needed for their accurate quantification.

The HPLC method was completely validated and due to the good results obtained after the intake of tomato juice, this method may be applied to evaluate the liposoluble antioxidants carotenoids and vitamin A in clinical intervention antioxidants trials and epidemiological studies status investigations.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Publicación 2. El *trans* licopeno del zumo de tomate atenúa los biomarcadores inmuno-inflamatorios: estudio de dosis-respuesta.

trans-Lycopene from tomato juice attenuates immune-inflammatory biomarkers: dose-response intervention trial. **Mariel Colmán-Martínez**, Miriam Martínez-Huélamo, Palmira Valderas-Martínez, Sara Arranz-Martínez, Enrique Almanza-Aguilera, Dolores Corella-Piquer, Ramón Estruch, and Rosa M. Lamuela-Raventós. *Molecular Nutrition & Food Research* (Enviado).

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Keywords:	Atherosclerosis, Cellular adhesion molecules, Inflammation, trans/cis-Lycopene isomers, Tomato juice

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Research Article

trans-Lycopene from tomato juice attenuates immune-inflammatory biomarkers: a dose-response intervention trial

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Abbreviations: AHT, arterial hypertension; **B**, baseline; **C**, control intervention; **CRP**, C-reactive protein; **CVD,** cardiovascular disease; **CXCL10**, CXC motif chemokine 10;

DM, diabetes mellitus; **HD**, high-dose intervention; **ICAM-1**; inter-cellular adhesion molecule 1; **IFNγ**, interferon gamma; **IL-8**, interleukin 8; **LD**, low-dose intervention; **MD**, Mediterranean diet; **NO**, nitric oxide; **TJ**, tomato juice; **VCAM-1**, vascular-cell adhesion molecule 1

Keywords: Atherosclerosis, Cellular adhesion molecules, Inflammation, *trans*/*cis*-Lycopene isomers, Tomato juice

Abstract

Scope: To evaluate the effects of carotenoids from tomato juice (TJ) on inflammatory biomarkers, by performing a 4-week dose-response nutritional trial in a population at high cardiovascular risk.

Methods and results: An open, prospective, randomized, cross-over, and controlled clinical trial was carried out with 28 volunteers (mean age 69.7 ± 3.1 years; mean BMI 31.5 ± 3.6 kg/m^2) at high cardiovascular risk, which were assigned to consume daily for 4 weeks in random order: 200 mL or 400 mL of TJ, or water as a control (C). Blood samples were collected at baseline (B) and after each intervention. Endpoints included changes in plasmatic carotenoids, C-reactive protein (CRP), IFN-γ, interleukin-8 (IL-8), eotaxin, CXCL motif chemokine 10 (CXCL10), inter-cellular adhesion molecule 1 (ICAM-1), and vascular-cell adhesion molecule 1 (VCAM-1). Compared to C, both TJ interventions induced significant decreases in ICAM-1, VCAM-1, CRP, and IL-8 (p <0.05), in a dose dependent manner. Decreases were significantly correlated mainly with the *trans*-lycopene, while the other carotenoids present in TJ were not associated with any significant changes in these molecules.

Conclusion: *trans*-Lycopene from TJ may attenuate the risk of CVD in a dose-response manner by reducing the concentration of inflammatory molecules related to atherosclerosis.

INTRODUCTION

Tomato (*Solanum Lycopersicum* L.) is one of the most widely consumed fruits in the Western world and is also a key food item in healthy dietary patterns such as the Mediterranean diet (MD). Diets rich in tomato or tomato-based products have been associated with decreased levels of cardiovascular risk factors [1–3], and these effects are attributed mainly to lycopene [4, 5]. In fruits and vegetables, lycopene is found in the all-*trans* configuration, which is a more thermodynamically stable form, whilst in human tissues and plasma the *cis* form is predominant. Numerous studies have suggested that isomerization from *trans* to *cis* forms increases the bioavailability of lycopene, and hence its biological benefits [6–8].

Atherosclerosis is a low-grade chronic inflammation disease of the wall of large- and medium–sized arteries, involving many components of the vascular, metabolic, and immune systems. Accumulation of oxidized lipids in the sub endothelium space of the arteries triggers the recruitment of circulating leukocytes, mainly monocytes, to remove these particles, which differentiate into macrophages [9–11]. At the same time, endothelium activation causes expression of adhesion molecules on its surface, such as ICAM-1, VCAM-1, and chemoattractant agents, such as E-selectin, and P-selectin, which work synergistically with the chemokines interleukin 8 (IL-8), CCL-2, CCL-5, and CXCL-10. These molecules facilitate the roll, adherence, and transmigration of leukocytes to the subendothelial space, creating a feedback mechanism for the recruitment cycle, and leading to the development of atheromatous plaque [11, 12].

The protective effects of lycopene on cardiovascular health have been attributed to its capacity to prevent atherogenesis by its antioxidant activity, which inhibit LDL

oxidation [1, 13], as well as by its immune-modulatory and anti-inflammatory properties [14].

Despite the predominance of dietary all-*trans* lycopene, *cis*-isomers account for 58-73% of total lycopene in human serum, and a surprisingly high 79-88% in benign or malignant prostate tissue [15]. However, which of the isoforms is the more bioactive in reducing inflammatory biomarkers has still to be elucidated. Hence, we embarked on an open, prospective, randomized, cross-over, dose-response, and controlled feeding intervention trial in order to evaluate the effects of the consumption of different doses of tomato juice (TJ), as a rich source of lycopene, on markers of inflammation in a population without overt CVD, but at high risk of developing it. Moreover, we wanted to evaluate which of the carotenoids present in plasma was more effective in modulating the response of these molecules.

MATERIALS AND METHODS

2.1. *Tomato juice samples*

The TJ was kindly supplied by Gallina Blanca Foods (GB Foods, Spain) and consisted of a mixture of crushed tomatoes and common olive oil (5%). TJ was packaged in 200 mL Tetra Brik packs.

2.2. *Study population*

Twenty-eight patients with a mean age of 69.7 ± 3.1 years, and mean BMI of 31.5 ± 3.6 kg/m^2 participated in the study. Inclusion criteria were: age between 55 and 80 years, diagnosed AHT, no reported clinical features of CVD (ischemic heart disease -angina pectoris or myocardial infarction recent or old-, stroke, peripheral vascular disease) but at high risk of developing CVD, namely, diagnosed DM and two or more of the following risk factors: i) smoking, ii) hypercholesterolemia (LDL-cholesterol >160 mg/dL), iii) lower concentrations of HDL-cholesterol (values <40 mg/dL), iv) obesity or overweight (BMI >25 kg/m^2), and/or family history of early CVD (first-order relatives, men <55 years old or women <65 years old). Patients with a history of CVD, chronic illnesses, alcoholism or another addictions, tomato allergy or intolerance were excluded. Demographic and clinical characteristics of the studied population are presented in **Supporting Information Table S1.**

2.3. *Study design*

The study was an open, prospective, randomized, cross-over, and controlled clinical trial aimed to assess the effects of different daily doses of TJ consumption on inflammatory biomarkers related to atherosclerosis in patients at high cardiovascular risk. After a run-in period of 3 weeks, all participants followed-up three 4-week intervention periods in a random sequence. During the run-in period, participants were asked to avoid the consumption of tomato or tomato based-products as well as carotenoid-rich products. At baseline (B), all volunteers followed-up a similar MD designed by a trained dietician for 3 weeks in which they limited tomato and tomato based-product consumption, while maintaining all other components of diet and physical activity. Before and after each intervention, volunteers were asked to fill out a 24-h food recall to assess their compliance with the prescribed diet. Total energy, macronutrient, and micronutrient intake were calculated using the Food Processor Nutrition and Fitness Software (esha Research, Salem, OR, USA). The participants randomly received: 1) one portion (LD) of 200 mL TJ/day before dinner; 2) two portions (HD) of 400 mL TJ/day divided into 2 doses of 200 mL each, before lunch and dinner; or 3) 200 mL water as control intervention (C), according to a computer-generated random-number table. Between the different interventions there was a 21-day wash-out period, when the participants followed their regular diet, but avoiding the consumption of tomato or tomato-based products.

Ethic statements

The study protocol was approved by the Ethics Committee of Clinical Investigation of the University of Barcelona (Barcelona, Spain), and the clinical trial was registered at the International Standard Randomized Controlled Trial Number (ISRCTN99660610). Written informed consent was obtained from all participants.

2.3.2 *Recruitment and clinic visits*

Participants were recruited at the Department of Internal Medicine of the Hospital Clínic of Barcelona. After reviewing medical records, potential participants were invited to a screening interview, and those who met the inclusion criteria and signed the informed consent were randomly allocated into the corresponding sequence of interventions, that was determined by computer-generated random table. Afterwards, participants were called for an initial evaluation and to fill in a food consumption questionnaire. Likewise, at the end of each intervention, participants came for the corresponding follow-up visits at the Hospital.

2.3.3 *Participant flow*

A total of 283 participants were assessed for eligibility; 223 of them were not eligible: 78 did not meet inclusion criteria, 21 reported their willingness to not change their diet, and 140 declined to participate in the study, leaving a total of 44 participants randomly assigned to the different groups. Twenty-eight of these completed the study; 2 participants decided to drop put during the trial due to problems following the diet, 3 for

medical illnesses and 11 for personal issues. The flow chart of participants throughout the trial is summarized in **Supporting Information Figure S1.**

2.3.4 *Collection of blood samples*

Fasting blood samples (10 mL) were drawn via venipuncture from the arm into evacuated plasma tubes containing EDTA at B and after each intervention, and immediately centrifuged at 1500 g for 15 min at 4 °C to separate the plasma. Plasma was aliquoted and stored at -80 °C until the day of analysis.

2.4 Inflammatory biomarkers

Inflammatory molecules were determined at B and after each intervention using Human ProcartaPlex® Multiplex Immunoassays (Affymetrix, eBioscience Inc., CA, USA), according to the manufacturer's instructions. The molecules determined were: ICAM-1, VCAM-1, and C-reactive protein (CRP). Plates were analyzed on a Luminex MAGPIX® instrument (Luminex, Co., Austin, TX, USA) using ProcartaPlex® Analyst 1.0 Software (eBioscience, San Diego, CA, USA). For IL-8, eotaxin, IFN-γ, and CXCL10, Bio-Plex Pro™ Assay (Bio-Rad Laboratories, Inc., CA, USA) was used. Analysis was carried out on a Luminex Bio-Plex® 2200 System (Luminex Co., TX, USA) using the Bio-Plex Manager™ Software. Concentrations were obtained by extrapolation of the results with standard calibration curves. Results were shown in ng/mL for ICAM-1, VCAM-1, and CRP; for IL-8, eotaxin, IFN- γ, and CXCL10 results were presented in pg/mL.

2.5 Extraction of carotenoid compounds from TJ

The extraction of carotenoids was carried out as quickly as possible, avoiding exposure to light, oxygen, high temperatures and pro-oxidant metals such as iron or copper, in order to minimize autoxidation and *cis/trans* isomerization. TJ samples (0.5 g) were homogenized with 5 mL of ethanol/hexane (4:3 v/v), following a procedure described elsewhere by Vallverdú-Queralt *et al.* [16]. The homogenate was sonicated for 5 min and centrifuged at 2140 g for 15 min at 4 °C. The supernatant was transferred into a flask and the extraction was repeated. Both supernatants were mixed and dried under a nitrogen flow at <25 °C. Finally, the residue was dissolved in methyl *tert*-butyl ether (MTBE) up to 1 mL and filtered through a 13 mm, 0.45 µm polytetrafluoroethylene (PTFE) filter (Waters Co., Milford, MA, USA) into an amber vial for HPLC analysis. Samples were stored at -20 °C until the analysis. Extractions were performed in triplicate.

2.6 Extraction of carotenoid compounds from human plasma

Plasma was subjected to a liquid-liquid extraction procedure previously described by our working group [17]. Briefly, 800 µL of ethanol was added to 800 µL of plasma. After vortex-mixing for 45 s, plasma was extracted twice with hexane (2 mL), stabilized with 0.1 g/L of butylated hydroxytoluene, and the extracts were vortex-mixed for 1 min and centrifuged at 2062 g for 5 min at 4 °C. The nonpolar layers were removed, pooled, and evaporated under a gentle stream of nitrogen at <25 °C. Finally, the samples were reconstituted with 300 µL of a solution of MTBE, filtered through a 13 mm, 0.22 µm PTFE filter (Waters Co., Milford, MA, USA) into an insert-amber vial for HPLC and stored at -80 °C until the day of analysis.

2.7 HPLC separation and identification of carotenoids in TJ and human plasma

Chromatographic analysis was performed using an HP 1100 HPLC system (Hewlett-105 Packard, Waldbronn, DE), equipped with a quaternary pump and an auto sampler, coupled to a DAD G1315B. The separation was carried out on a RP column YMC Carotenoid S-5 µm, 250 mm x 4.6 mm (Waters, Milford, MA, US) and connected to a precolumn YMC Guard Cartridge Carotenoid 20 x 4.0 mm i.d., S-5µm, following the procedure previously described by our working group [17]. The HPLC–UV chromatograms were acquired at 450 nm wavelength and the integration was performed with Agilent ChemStation™ software. The tentative identification of carotenoids and their geometrical isomers (*cis*-isomers) was done on the basis of the retention times and absorption spectrum characteristics previously described by our group [16] and also by comparing with other references in the literature [18, 19]. Results were expressed as µmol/L.

2.8 Statistical analysis

Values are expressed as means ± SD. Continuous variables were tested for normal distribution using the Kolmogorov-Smirnov test. In order to determine statistically significant differences between interventions for both plasmatic carotenoids and inflammatory molecules, the ANOVA test for repeated measures was performed. Associations between plasmatic carotenoids and inflammatory molecules were calculated by Pearson's correlation test for parametric variables. After that, linear regression models were used to determine the relationship between them. Intervention-associated differences were evaluated in terms of changes relative to B and, at common time points, between interventions. Statistical significance was set at $p < 0.05$. Analyses

were performed using the Statistical Package for Social Sciences (SPSS, version 19.0 for Windows; SPSS Inc., Chicago, IL, USA).

3. RESULTS

3.1. Carotenoid content in TJ

The carotenoid content in TJ was analyzed at 3 different times: before starting, in the middle, and at the end of the trial, in order to ensure that the amount of carotenoids ingested did not vary throughout. No statistically significant differences were found across time (data not shown). **Table 1** summarizes the content of the major carotenoids identified in the TJ given to the participants during the trial. The main compounds quantified were *trans*-lycopene and *β*-carotene with a mean of 193 ± 20 and 190 ± 15 µmol/L, respectively, *trans*-lycopene being the major contributor with 48.1 %, whilst *β*-carotene represented 47.4 % of the total. Thus, *trans*-lycopene and *β*-carotene accounted for 95.5% of the total carotenoids quantified. The sum of lycopene isomers represented 52.2 % of the total carotenoid intake.

3.2. Carotenoid profile of human plasma

The lowest carotenoid plasma concentrations were after the wash-out period, due to the no consumption of tomato, tomato-based products, and other carotenoid-rich foods. The major carotenoids quantified in plasma after the wash-out period were *trans*-lycopene, and the *cis*-isomers 13-*cis*, 5-*cis*, and 9-*cis*-lycopene.

After the TJ interventions, statistically significant increases were observed for *trans*-lycopene, 5-*cis*-lycopene, and 13-*cis*-lycopene, whilst the increase in 9-*cis*-lycopene was significant only after HD interventions. On the other hand, β-carotene significantly increased after the HD intervention, whilst α-carotene increased after the HD intervention, but this change was not statistically significant. Xanthophylls showed no significant differences throughout the interventions. These data are summarized in **Table 2.**

3.3. Inflammatory biomarkers

Compared to baseline, adhesion molecules ICAM-1 and VCAM-1 showed a significant decrease after the different TJ interventions, in a dose dependent manner (p <0.001). Similarly, CRP decreased significantly (p <0.001) after the different interventions, particularly LD. The pro-inflammatory cytokine IL-8 increased significantly after the control intervention, but in contrast, showed a significant decrease after the LD and HD intervention, suggesting that TJ consumption had anti-inflammatory effects in this population. Eotaxin, IFN-γ, and CXCL10 showed a tendency to decrease after both, LD and HD, interventions compared to control intervention, but the reduction did not attain statistical significance. The results are set out in **Table 3.**

3.4. Relationship between carotenoid intake and plasmatic inflammatory biomarkers

In order to accurately determine whether plasmatic carotenoids were related to the concentration of inflammatory biomarkers, a two-tail Pearson's coefficient test,

followed by a linear regression analysis, was applied. Regarding TJ and plasmatic carotenoid concentrations, *trans*-lycopene and 5-*cis*-lycopene showed a substantial positive correlation (r=0.798 and 0.706; p <0.001, respectively) while β-carotene, α-carotene, 9-*cis*, and 13-*cis*-lycopene presented a modest correlation, with r values between 0.320 and 0.655 (p <0.001).

The association between the inflammatory biomarkers and plasmatic carotenoids was also explored, and 13-*cis*-β-carotene showed a considerable negative correlation with CRP (r=0.632; p <0.001), ICAM-1 (r=0.650; p <0.001), and VCAM-1 (r=0.658; p <0.001). Regarding isomeric forms of lycopene, the 5-*cis*-lycopene presented a negative correlation with ICAM-1 (r=0.526; p <0.001) and VCAM-1 (r=0.604; p <0.001), as 13-*cis*-lycopene, with negative correlations for ICAM-1 (r=0.453; p <0.001), and VCAM-1 (r=0.525; p <0.001), whilst *trans*-lycopene showed an important negative correlation with ICAM-1 (r=0.625; p <0.001) and VCAM-1 (r=0.697; p <0.001), but a lower negative correlation with CRP (r=0.227; p =0.042). Lower correlations were observed between 15-*cis*-β-carotene -VCAM-1 (r=0.303; p =0.045); α-carotene-VCAM-1 (r=0.245; p =0.045); 9-*cis*-lycopene-VCAM-1 (r=0.357; p =0.013); β-carotene-VCAM-1 (r=0.204; p =0.037); and β-carotene-ICAM-1 (r=0.219; p =0.035). A weak association or no association was observed between plasmatic carotenoids and inflammatory molecules IFN-γ, IL-8 and CXCL10.

Subsequently, a linear regression analysis was applied to establish the relationship between the carotenoids and molecules with a significant negative correlation. It was found that carotenoids could have an influence of between 13 and 49% on the decrease of VCAM-1, especially *trans*-lycopene (r^2=0.486; p<0.001). In the case of ICAM-1, carotenoids could explain between 5 and 39% of the variations, *trans*-lycopene also

being the major contributor (r^2=0.390; p<0.001). In the case of CRP, carotenoids could explain between 2.5 and 40% of the decrease, especially due to 13-*cis*-β-carotene.

In order to corroborate whether positive effects of *trans*-lycopene on these molecules could be influenced by the *cis*-lycopene isomers, which are recognized for their better bioavailability, correlations adjusting for total *cis*- and *trans*-lycopenes were made. After adjusting for total *cis*-lycopene, correlations between *trans*-lycopene and CRP decreased, although not significantly. In the case of *trans*-lycopene-ICAM-1, and *trans*-lycopene-VCAM-1, *r* values changed to -0.453 and -0.501, respectively, showing that *trans*-lycopene was still correlated with the decrease in the expression of these molecules, although to a lesser extent. Surprisingly, after adjusting for *trans*-lycopene, the *cis*-isomers lost the correlation with the inflammatory molecules. **Table 4** shows the differences in correlations between total *cis*- and *trans*-lycopene and the inflammatory biomarkers adjusted for each one.

4. DISCUSSION

Atherosclerosis is an inflammatory disease of the arteries, involving several components of the vascular, metabolic, and immune systems [20–22]. In this study, we measured the expression of several chemokines, cell adhesion molecules, as well as inflammatory cytokines, responsible for the initiation and progression of atherosclerosis, after a 4-week intervention with different amounts of TJ. A downregulation of serum concentrations of ICAM-1, VCAM-1, CRP, and IL-8 was observed in a dose-dependent manner after TJ consumption and these effects were attributable mainly to the intake of *trans*-lycopene.

Several epidemiologic studies have focused on elucidating the effects of tomato, tomato-based products, or even lycopene supplementation on different immune-inflammatory biomarkers at diverse stages of the atherosclerosis process. One of the most studied molecules is the acute phase protein CRP, which is a sensitive biomarker of inflammatory processes. In this regard, lycopene has been negatively correlated with plasmatic concentration of CRP in a TJ intervention carried out by Biddle *et al.*, 2015 [24]. In contrast, Abete *et al.*, 2013 [25], Thies *et al.*, 2012 [26], and Blum *et al.*, 2007 [27] did not find any association between the ingestion of tomato or tomato-based products and the attenuation of CRP plasmatic concentration in human feeding interventions. A possible explanation for these negative results could be the absence, or very low amount, of an oil matrix in the tomato-based products provided to volunteers, since it has been shown that dietary fats enhance carotenoid bioavailability [28–30]. 3-5 grams being the optimal amount for efficient absorption [28]. On the other hand, Jacob *et al.*, 2008 [31] observed an inverse association between the consumption of TJ (20 mg/day of lycopene) and CRP plasmatic concentration, but they could not attribute this result only to lycopene, because their TJ was enriched with vitamin C (435 mg/day).

Other important molecules involved in the development of atherosclerosis are the adhesion molecules ICAM-1 and VCAM-1, whose expression is related to pro-inflammatory stimuli such as oxidized LDL, TNF-α, or CRP [32]. Similarly to the reduction of the CRP concentration by the chronic consumption of TJ, we also expected a reduction of ICAM-1 and VCAM-1 molecules. Nonetheless, the above-cited Thies *et al.*, 2012 [26] did not observe any changes in ICAM-1 after a 12-week dietary intervention with different doses of tomato, tomato by-products or lycopene supplements, even after a significant increase in plasmatic lycopene within the different intervention groups. It is noteworthy that the discrepancies between their results and

ours could be due to the characteristics of the studied population; in our trial, all subjects included were at high cardiovascular risk. In a study by Blum *et al.*, 2007 [27], there was no difference from the baseline concentration of CRP after a tomato intervention, but an increase of ICAM-1 was observed in the control group. García-Alonso *et al.*, 2012 [33] also observed a reduction of VCAM-1 after TJ supplementation for 2 weeks, whereas ICAM-1 concentration only decreased when the TJ was enriched with n-3 polyunsaturated fatty acids.

The ability of lycopene to inhibit the expression of IL-8, a remarkable chemokine involved in the monocyte recruitment from the circulation to inflammation sites, has been observed, mainly in cell cultures [34] and animal models [35]. However, human feeding trials that evaluate the effects of lycopene on this inflammatory biomarker are scarce. Ghavipour *et al.*, 2013 [36] investigated if daily consumption of 330 mL of TJ (37 mg/day of lycopene) could reduce inflammation in overweight and obese people, using IL-6, IL-8, CRP, and TNF-α as biomarkers. In agreement with our results, they found a significant decrease in IL-8. Nevertheless, they could not find differences in the CRP plasmatic concentration in this population, although the dose of TJ they administered to the volunteers was similar to our higher dose. They hypothesized that the anti-inflammatory actions of lycopene were not potent enough to diminish the exacerbated inflammation in the obese group. One of the possible mechanisms by which *cis*- and *trans*-lycopene may reduce the expression of the chemokine IL-8, as well as the adhesion molecules ICAM-1 and VCAM-1, and possibly CRP, is by inhibiting the translocation of the nuclear factor kappa B, involved in the activation of the inflammatory cascade that could stimulate their expression [37]. The importance of these inflammatory molecules is well established, not only in early stages of

atherosclerosis, but also to predict future vascular events, even in apparently healthy individuals.

Regarding β-carotene, it was notable that its plasmatic concentration decreased after the interventions, despite its high levels in TJ. This is in agreement with the results of Johnson *et al*., 1997 [38], who observe that a combined consumption of β-carotene and lycopene has minimal effects on the absorption of β-carotene, while the absorption of lycopene improves, probably because some components in the β-carotene suspension enhance the solubilization of crystalline lycopene and thus provide a better serum response [38]. This explanation supports our results and could account for the lack of influence of β-carotene on the modulation of the studied immune-inflammatory molecules.

One of the strong points of the present study was the elucidation of the beneficial effects of *trans*-lycopene on the downregulation of chemokines and adhesion molecules after TJ consumption, which was observed by correlation and regression analyses. Although *cis*-lycopene isomers are well recognized for their better bioavailability and greater proportion in human plasma and tissues compared with the *trans* isoform [6, 15, 19, 39–41], after adjusting for *trans*-lycopene, negative correlations between *cis*-lycopene and these molecules were lost, suggesting an important effect of *trans*-lycopene on inflammatory molecules. To the best of our knowledge, no previous studies have reported the positive impact of *trans*-lycopene on immune and inflammatory biomarkers.

Another important aspect was the analysis of two biomarkers, the chemokines eotaxin and CXCL10, which are capable of predicting future cardiovascular events, but have not been previously explored in depth in relation to carotenoid consumption. In cell cultures

derived from human aortic smooth muscle cells, eotaxin was related to the modulation of macrophage function, and probably participates in mast cell activation and/or recruitment [42]. In a recent clinical trial carried out by Tarantino *et al.*, 2014 [43], eotaxin was associated with the carotid intima-media thickness, as an early predictor of the atherosclerotic process in obese people. The chemokine CXCL10 participates in heart damage initiation and progression by the mediation of inflammatory cell infiltration [44], and has been detected in low shear stress regions of atherosclerotic plaques, where the fibrous caps are thinner and necrotic cores are larger [45], suggesting a relationship with the development of plaques prone to rupture. In animal models, it has been seen that CXCL10 deficiency modulates early atherosclerotic lesions in ApoE$^{-/-}$ mice [46, 47], whilst in humans, an elevated concentration of CXCL10 has been associated with coronary atherosclerosis [48]. Although in this study we did not observe a significant decrease of these biomarkers after TJ interventions *vs.* the control, the data revealed a decreasing tendency. These results warrant further studies with a larger population to investigate the effects of carotenoid consumption, mainly lycopene *trans*-isomers, on these molecules in the establishment and progression of atherosclerosis.

R.L.R., R.E., and D.C. designed the research; P.V.M., S.A.M. carried out the study and collected plasma samples; M.M.H., M.C.M. conducted the research and analyzed the data; E.A.A. provided statistical support; M.C.M. wrote the paper. M.M.H., P.V.M., S.A.M., R.E., and D.C. critically reviewed the manuscript. R.L.R. has the responsibility for the final content. All authors read and approved the final manuscript.

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Conflict of interest:

Dr. Estruch reports serving on the board of and receiving lecture fees from the Research Foundation on Wine and Nutrition (FIVIN); serving on the boards of the Beer and Health Foundation and the European Foundation for Alcohol Research (ERAB); receiving lecture fees from Cerveceros de España and Sanofi-Aventis; and receiving grant support through his institution from Novartis. Dr. Lamuela-Raventós reports serving on the board of and receiving lecture fees from FIVIN; receiving lecture fees from Cerveceros de España; and receiving lecture fees and travel support from PepsiCo. Nevertheless, these foundations had no involvement in the study design, the collection, analysis and interpretation of data, the writing of the manuscript or the

decision to submit the manuscript for publication. The other authors declare no conflict of interest.

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TABLES

Table 1. Carotenoid content of the TJ administered to the volunteers during the trial.

	Concentration (*μmol/L*)	LD: 200 mL TJ *μmol/dose administered*	HD: 400 mLTJ μmol/*dose administered*
Lutein	0.46 ± 0.04	0.09 ± 0.01	0.19 ± 0.02
α-carotene	0.88 ± 0.06	0.18 ± 0.01	0.35 ± 0.02
β-carotene	190 ± 15	38.0 ± 3.0	75.9 ± 5.98
trans-lycopene	193 ± 20	38.6 ± 3.98	77.3 ± 7.95
5-*cis*-lycopene	2.76 ± 0.21	0.55 ± 0.04	1.10 ± 0.08
13-*cis*-lycopene	5.40 ± 0.57	1.08 ± 0.11	2.16 ± 0.22
9-*cis*-lycopene	8.30 ± 0.76	1.66 ± 0.15	3.32 ± 0.31
Total carotenoids	401	80.1	160

Values are expressed as mean ± SD
TJ: Tomato juice

576 **Table 2.** Plasma carotenoid concentration at baseline and after interventions (n=28).

Analyte	*Time*	*Concentration (μmol/L)*	*p'*
Astaxanthin	Baseline	0.73±0.53	0.276
	Z0	0.67±0.54	
	Z1	0.51±0.27	
	Z2	0.80±0.49	
Lutein	Baseline	0.05±0.03	0.036
	Z0	0.04±0.02	
	Z1	0.02±0.02	
	Z2	0.04±0.02	
trans-β-apo-8'-carotenal	Baseline	0.53±0.03	0.025
	Z0	0.52±0.03	
	Z1	0.52±0.02	
	Z2	0.54±0.03	
Cryptoxanthin	Baseline	0.09±0.08	0.077
	Z0	0.10±0.08	
	Z1	0.10±0.06	
	Z2	0.16±0.14	
15-*cis*-β-carotene	Baseline	n.d.	<0.001
	Z0	n.d.	
	Z1	n.d.	
	Z2	0.18±0.21	
13-*cis*-β-carotene	Baseline	n.d.^{a,b}	<0.001
	Z0	0.09±0.00^{a}	
	Z1	0.10±0.02^{b}	
	Z2	0.11±0.03^{a}	
α-carotene	Baseline	n.d.	<0.001
	Z0	n.d.	
	Z1	n.d.	
	Z2	0.51±0.43	
β-carotene	Baseline	0.69±0.41	0.006
	Z0	0.65±0.41	
	Z1	0.80±0.50	
	Z2	1.19±0.98	
trans-lycopene	Baseline	1.43±1.11^{a}	< 0.001
	Z0	0.84±0.56^{b}	
	Z1	3.91±1.76^{a,b}	
	Z2	6.64±2.65^{a,b}	
5-*cis*-lycopene	Baseline	1.19±0.99^{a}	< 0.001

	Z0	1.13±0.74[b]	
	Z1	2.37±1.09[a,b]	
	Z2	4.07±1.77[a,b]	
13-*cis*-lycopene	Baseline	1.04±0.76[a]	< 0.001
	Z0	1.12±0.90[b]	
	Z1	1.87±1.05[a]	
	Z2	4.00±2.00[a,b]	
9-*cis*-lycopene	Baseline	0.79±0.88	0.003
	Z0	0.63±0.76	
	Z1	0.89±0.56[a]	
	Z2	1.94±1.26[a]	
Total *cis* lycopene isomers	Baseline	1.74±1.60[a]	< 0.001
	Z0	1.71±1.46[b]	
	Z1	4.77±2.60[a,b]	
	Z2	9.57±4.78[a,b]	
Total lycopenes	Baseline	3.00±2.66[a]	< 0.001
	Z0	2.55±1.87[b]	
	Z1	8.68±4.22[a,b]	
	Z2	16.22±7.26[a,b]	
Total carotenoids	Baseline	4.94±3.49[a]	< 0.001
	Z0	4.95±3.39[b]	
	Z1	10.64±4.64[a,b]	
	Z2	18.62±7.99[a,b]	

Values are expressed as mean ± SD.
[1]*p* value of the ANOVA for repeated measures from the differences between interventions.
Values with the same letter are statistically significant between interventions ($p < 0.05$).

577

578 **Table 3.** Immune and inflammatory biomarkers measured at baseline and after

579 interventions (n=28).

		Intervention			
Plasmatic biomarker	*Baseline*	*Control (Z0)*	*Low-dose (LD)*	*High-dose (HD)*	p^d
ICAM-1 (ng/mL)	3693±1377[a]	3609±1107[b]	318±116[a,b]	159±57[a,b]	<0.001
VCAM-1 (ng/mL)	3993±890[a]	3939±801[b]	400±101[a,b]	218±39[a,b]	<0.001
CRP (ng/mL)	1521±236[a,b,c]	539±200[a]	446±254[b]	532±158[c]	<0.001
IL-8 (pg/mL)	22±9[a]	40±17[a,b,c]	23±16[b]	24±15[c]	0.015
Eotaxin (pg/mL)	135±67	172±114	137±75	181±112	0.172
IFN-γ (pg/mL)	304±80	489±220	399±131	400±144	0.074
CXCL10 (pg/mL)	2908±1598	3397±1489	3366±1671	3606±2185	0.519

Values are expressed as mean ± SD. Values with the same letters are significantly different (p <0.05).

[d] p value of the ANOVA for repeated measures from the differences between interventions.

580

581 **Table 4.** Correlations between *cis* and *trans*-lycopene and inflammatory molecules after

582 and before adjusting.

Adjusting for	*Biomarker*		*Pearson's coefficient test*	*p value*
	CRP	*trans*-Lycopene	-0.227	0.042
		Total *cis*-Lycopenes	-0.157	0.154
	VCAM-1	*trans*-Lycopene	-0.697	<0.001
		Total *cis*-Lycopenes	-0.628	<0.001
	ICAM-1	*trans*-Lycopene	-0.625	<0.001
		Total *cis*-Lycopenes	-0.551	<0.001
Total *cis*-Lycopenes	CRP	*trans*-Lycopene	-0.414	<0.001
	VCAM-1	*trans*-Lycopene	-0.501	<0.001
	ICAM-1	*trans*-Lycopene	-0.453	<0.001
trans-Lycopene	CRP	Total *cis*-Lycopenes	0.374	0.002
	VCAM-1	Total *cis*-Lycopenes	0.356	0.003
	ICAM-1	Total *cis*-Lycopenes	0.309	0.010

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Publicación 3. Desarrollo de un método avanzado de HPLC-MS/MS para la determinación de carotenoides y vitaminas liposolubles en plasma humano.

Development of an advanced HPLC-MS/MS method for the determination of carotenoids and fat soluble vitamins in human plasma. Barbora Hrvolová, Miriam Martínez-Huélamo, **Mariel Colmán-Martínez**, Sara Hurtado-Barroso, Rosa M. Lamuela-Raventós and Jiří Kalina. *International Journal of Molecular Sciences.* 2016; 2016: Oct; 17(10): 1719. doi: 10.3390/ijms17101719

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Article

Development of an Advanced HPLC–MS/MS Method for the Determination of Carotenoids and Fat-Soluble Vitamins in Human Plasma

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Abstract: The concentration of carotenoids and fat-soluble vitamins in human plasma may play a significant role in numerous chronic diseases such as age-related macular degeneration and some types of cancer. Although these compounds are of utmost interest for human health, methods for their simultaneous determination are scarce. A new high pressure liquid chromatography (HPLC)-tandem mass spectrometry (MS/MS) method for the quantification of selected carotenoids and fat-soluble vitamins in human plasma was developed, validated, and then applied in a pilot dietary intervention study with healthy volunteers. In 50 min, 16 analytes were separated with an excellent resolution and suitable MS signal intensity. The proposed HPLC–MS/MS method led to improvements in the limits of detection (LOD) and quantification (LOQ) for all analyzed compounds compared to the most often used HPLC–DAD methods, in some cases being more than 100-fold lower. LOD values were between 0.001 and 0.422 µg/mL and LOQ values ranged from 0.003 to 1.406 µg/mL, according to the analyte. The accuracy, precision, and stability met with the acceptance criteria of the AOAC (Association of Official Analytical Chemists) International. According to these results, the described HPLC-MS/MS method is adequately sensitive, repeatable and suitable for the large-scale analysis of compounds in biological fluids.

Keywords: tandem mass spectrometry; carotenoids; fat-soluble vitamins; human plasma; pilot human study; high antioxidant diet

1. Introduction

A wide range of significant evidence has associated the human diet with chronic diseases in the last decades [1–5]. Based on this evidence, many dietary recommendations and guidelines have been formulated to prevent conditions such as age-related macular degeneration, cardiovascular diseases, some types of cancer, osteoporosis, diabetes, and others [6,7]. Owing to their content of bioactive compounds, notably carotenoids and fat-soluble vitamins, an increment of fruit and vegetable consumption is recommended [8]. Carotenoids, xanthophylls and carotenes are natural fat-soluble, red, yellow and orange pigments characterized by a wide distribution, structural diversity and numerous physio-chemical and biological properties [9]. Particularly due to their antioxidant activity, carotenoids have been the subject of many studies [10–14]. To date, over 700 carotenoid compounds have been identified in various natural sources, and 40 to 50 of these are usually consumed in the human

diet [15,16]. Since these antioxidants are synthesized only by plants, algae and fungi [9], their levels in human blood are directly dependent on food intake.

Another group of interesting and useful compounds are fat-soluble vitamins and their metabolites such as retinol, retinol acetate, cholecalciferol, and α-tocotrienol. These compounds also have free radical scavenging properties that allow them to function as antioxidants [17,18]. The presence of fat-soluble vitamins in human tissues and fluids is of vital importance for human health because of their catalytic functions in anabolic and catabolic pathways. Both carotenoids and fat-soluble vitamins can be labeled as fat-soluble micronutrients, and their absorption from the gastrointestinal tract depends on processes responsible for fat absorption or metabolism [19]. An excess or lack of these fat-soluble micronutrients has been associated with the expression of certain diseases. Consequently, there is an increasing demand for the analysis of these antioxidants.

Available methods can determine only a few representatives of the aforementioned fat-soluble micronutrients [20–26], and few of them can be applied for the simultaneous analysis of compounds in biological samples. Most use high pressure liquid chromatography (HPLC) separation coupled to UV–Vis or diode array detection (DAD) [23–25], but with these methods it is extremely challenging to obtain the sensitivity required for the analysis of human fluids, in which the concentration of fat-soluble micronutrients is very low. The problem of sensitivity can be solved by usage of tandem mass spectrometry (MS/MS) detection, although finding general ionization conditions suitable for all targeted analytes is very difficult. A few HPLC–MS/MS methods have been described for the simultaneous analysis of carotenoids and fat-soluble vitamins but usually for no more than 10 analytes [22,25–27]. Reported here is a unique HPLC–MS/MS method for the simultaneous determination of 16 carotenoids and fat-soluble vitamins in human plasma.

2. Results

2.1. HPLC-MS/MS Method Development

2.1.1. Extraction of Carotenoids and Fat-Soluble Vitamins

For the determination of carotenoids and fat-soluble vitamins in a complex matrix such as human plasma, it was necessary to develop an efficient extraction procedure. For our purpose, a double liquid-liquid extraction was designed. The biggest advantage of this extraction procedure is the small amount of human plasma (200 µL) and chemicals required to obtain good quality results. A detailed description is provided in Materials and Methods.

2.1.2. Optimization of Chromatographic and MS/MS Conditions

During the development of the HPLC–MS/MS method, different variations of mobile phase additives were compared: 0.4 g/L ammonium acetate (AMAC), 0.7 g/L AMAC, 1 g/L AMAC, 0.4 g/L AMAC + 0.1% acetic acid (AA), 0.7 g/L AMAC + 0.1% AA, 1 g/L AMAC + 0.1% AA. Table 1 shows the dependency of the MS signal intensity for product ions using multiple reaction monitoring (MRM) in APCI positive mode on the use of LC solvent additives. In general, a combination of AMAC and AA provided better results than the addition of only AMAC to the mobile phases. 0.4 g/L AMAC + 0.1% AA provided the best MS signal intensity for 25-hydroxycholecalciferol, retinol acetate, and cholecalciferol; meanwhile 0.7 g/L AMAC + 0.1% AA provided the best MS signal intensity for most of the analyzed analytes: retinol, cantaxanthin, cryptoxanthin, 13-*Z*-β-carotene, α-carotene, β-carotene, and 9-*Z*-β-carotene. For lutein, the same mass signal intensities were obtained with 0.7 g/L AMAC, 0.4 g/L AMAC + 0.1% AA, 0.7 g/L AMAC + 0.1% AA, and 1 g/L AMAC + 0.1% AA. In the case of *E*-β-apo-8′-carotenal, the MS signals obtained by 1 g/L AMAC, 0.4 g/L AMAC + 0.1% AA, 0.7 g/L AMAC + 0.1% AA, and 1 g/L AMAC + 0.1% AA were also comparable. For α-tocotrienol and astaxanthin, 1 g/L AMAC provided the best MS signal, which was 0.7 g/L AMAC for zeaxanthin, and 0.4 g/L AMAC for 5-*Z*-lycopene. The combination of 0.7 g/L AMAC + 0.1% AA added to both mobile

phases was chosen for the final data acquisition because they provided the highest MS signal intensity for product ions of most of the analytes, including the highly problematic carotenes.

Table 1. Influence of LC solvent additives (0.4 g/L ammonium acetate (AMAC), 0.7 g/L AMAC, 1 g/L AMAC, 0.4 g/L AMAC + 0.1% AA, 0.7 g/L AMAC + 0.1% AA, 1 g/L AMAC + 0.1% AA) on MS signal intensity for product ions using MRM in APCI positive mode. The fair blue represents lower intensities of MS signal than the highest obtained intensity under described conditions. The dark blue represents the highest MS signal intensity.

Analyte	0.4 g/L AMAC	0.7 g/L AMAC	1 g/L AMAC	0.4 g/L AMAC + 0.1% AA	0.7 g/L AMAC + 0.1% AA	1 g/L AMAC + 0.1% AA
retinol	2,200,000	1,400,000	1,700,000	1,600,000	2,400,000	1,800,000
25-hydroxycholecalciferol	1,700,000	1,600,000	1,700,000	1,900,000	1,800,000	1,800,000
retinol acetate	660,000	640,000	600,000	720,000	630,000	700,000
α-tocotrienol	88,000	96,000	110,000	100,000	90,000	98,000
cholecalciferol	730,000	790,000	740,000	860,000	800,000	840,000
astaxanthin	400,000	480,000	530,000	370,000	500,000	500,000
lutein	100,000	140,000	130,000	140,000	140,000	140,000
zeaxanthin	1150	1883	1200	1800	1772	1925
cantaxanthin	86,000	190,000	150,000	170,000	200,000	180,000
E-β-apo-8′-carotenal	1,190,000	1,900,000	2,000,000	2,000,000	2,000,000	2,000,000
cryptoxanthin	6000	7950	6483	7500	8000	7317
13-*Z*-β-carotene	108,000	100,000	89,000	107,000	110,000	100,000
α-carotene	40,000	40,000	32,000	40,000	43,000	37,000
β-carotene	5350	5000	4467	5000	5367	5242
9-*Z*-β-carotene	1440	1400	1360	1100	1500	1500
5-*Z*-lycopene	4000	3000	2942	3000	2800	2800

Values represent intensities of MS signal obtained for each analyte under specific conditions.

An excellent separation of 16 analytes in 50 min was accomplished (Figure 1). Flow rate, which allowed good quality resolution, was 0.6 mL/min, and the most suitable injection volume was 20 µL. The resolution and shape of individual peaks were greatly affected by the solvent used for sample reconstitution. During preliminary experiments, methyl *tert*-butyl ether (MTBE) and methanol (MeOH) were used for reconstitution. The effects of the solvents used for reconstitution are shown in Figure 2. Reconstitution by MeOH provided better separation and resolution of all analytes.

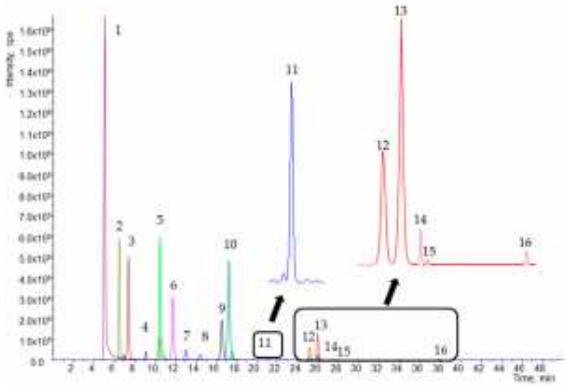

Figure 1. Chromatogram of working standard solutions obtained by high pressure liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis. Peaks: **(1)** retinol; **(2)** 25-hydroxycholecalciferol; **(3)** retinol acetate; **(4)** α-tocotrienol; **(5)** cholecalciferol; **(6)** astaxanthin; **(7)** lutein; **(8)** zeaxanthin; **(9)** cantaxanthin; **(10)** *E*-β-apo-8′-carotenal; **(11)** cryptoxanthin; **(12)** 13-*Z*-β-carotene; **(13)** α-carotene; **(14)** β-carotene; **(15)** 9-*Z*-β-carotene; and **(16)** 5-*Z*-lycopene.

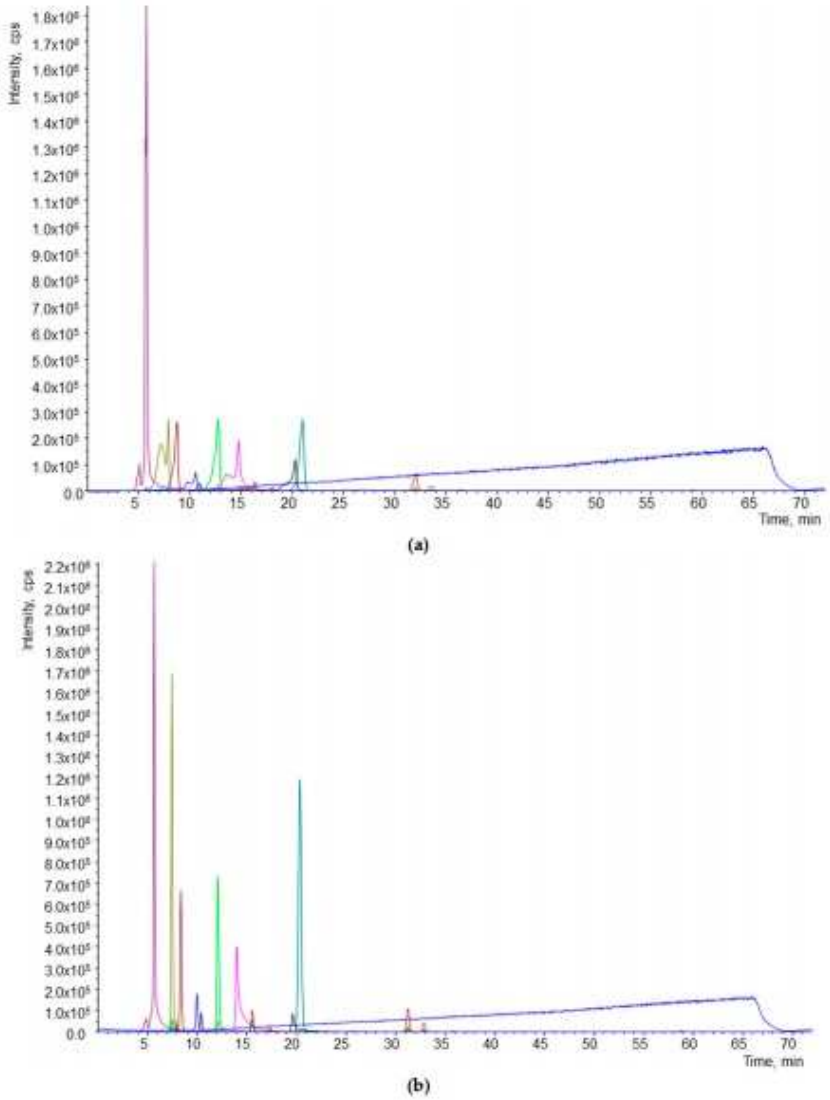

Figure 2. Effect of solvent used for reconstitution of samples. (**a**) Chromatogram of sample reconstituted in methyl *tert*-butyl ether (MTBE); (**b**) chromatogram of sample reconstituted in MeOH.

Retention time (Rt) and optimal value for mass detection of declustering potential (DP), focusing potential (FP), entrance potential (EP), cell exit potential (CXP), and quantification transitions, with their corresponding collision energy (CE) are shown in Table 2.

Table 2. Optimized values of declustering potential (DP), entrance potential (EP), cell exit potential (CXP) and retention time (Rt). Quantification transitions of the carotenoids and fat-soluble vitamins with the optimal collision energy (eV).

Analyte	Rt (min)	DP (V)	EP (V)	CXP (V)	Quantification Transition	CE (eV)
retinol	5.00	35	10	15	269 → 181	14
25-hydroxycholecalciferol	6.45	58	10	15	383 → 365	17
retinol acetate	7.34	41	10	15	329 → 269	18
α-tocotrienol	9.03	181	10	15	411 → 165	57
cholecalciferol	10.45	60	10	15	385 → 367	24
astaxanthin	11.69	84	10	15	597 → 147	40
lutein	12.98	102	10	15	551 → 429	26
zeaxanthin	14.40	85	10	15	568 → 476	25
cantaxanthin	16.47	70	10	15	565 → 363	15
E-β-apo-8′-carotenal	17.16	70	10	15	417 → 325	14
cryptoxanthin	20.71	94	10	15	553 → 535	20
13-Z-β-carotene	25.03	48	10	15	536 → 444	24
α-carotene	25.85	120	10	15	536 → 444	24
β-carotene	27.42	85	10	15	537 → 413	28
9-Z-β-carotene	28.13	75	10	15	537 → 413	30
5-Z-lycopene	37.65	87	10	15	537 → 413	23

2.2. *Validation of the Method*

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

LOD and LOQ were determined for individual analytes (Table 3). The sensitivity of the developed HPLC-MS/MS method was evaluated by these two parameters. LOD values were between 0.001 and 0.422 µg/mL, and LOQ values ranged from 0.003 to 1.406 µg/mL according to the analyte. The lowest LOD and LOQ were established for astaxanthin, and the highest for zeaxanthin. The LOD and LOQ values obtained demonstrate that the present HPLC–MS/MS method is suitable for the analysis of carotenoids and fat-soluble vitamins in human plasma.

Table 3. Limit of detection (LOD), limit of quantification (LOQ), recovery, matrix effect, linearity range and correlation coefficient obtained in samples by the HPLC-MS/MS method.

Analyte	LOD [a] (µg/mL)	LOQ [b] (µg /mL)	Rec. [c] (%)	ME [d] (%)	Conc. Range [e] (µg /mL)	(r^2) [f]
retinol	0.002	0.005	102.6 ± 8.9	108.5 ± 9.1	0.005–10	0.990
25-hydroxycholecalciferol	0.003	0.011	92.0 ± 3.2	87.3 ± 1.4	0.011–5	0.995
retinol acetate	0.002	0.008	103.4 ± 1.5	95.1 ± 1.7	0.008–10	0.995
α-tocotrienol	0.113	0.376	99.0 ± 5.3	89.9 ± 3.4	0.376–5	0.992
cholecalciferol	0.005	0.018	102.9 ± 2.9	89.6 ± 1.3	0.018–10	0.998
astaxanthin	0.001	0.003	102.3 ± 2.6	100.1 ± 3.3	0.003–1	0.995
lutein	0.008	0.028	86.1 ± 1.4	91.0 ± 2.0	0.028–10	0.992
zeaxanthin	0.422	1.406	86.2 ± 2.2	86.9 ± 3.2	1.406–10	0.992
cantaxanthin	0.002	0.006	100.1 ± 2.5	103.5 ± 8.0	0.006–1	0.992
E-β-apo-8′-carotenal	0.003	0.010	103.6 ± 2.1	100.4 ± 1.4	0.010–10	0.994
cryptoxanthin	0.244	0.812	104.8 ± 3.2	94.0 ± 2.3	0.812–10	0.993
13-Z-β-carotene	0.056	0.187	100.6 ± 2.0	87.8 ± 1.0	0.187–10	0.992
α-carotene	0.022	0.073	104.2 ± 5.4	102.8 ± 3.9	0.073–5	0.994
β-carotene	0.041	0.138	101.1 ± 2.2	95.7 ± 5.3	0.138–5	0.999
9-Z-β-carotene	0.293	0.975	97.4 ± 6.7	91.5 ± 2.4	0.975–10	0.996
5-Z-lycopene	0.189	0.631	104.6 ± 8.2	96.5 ± 3.3	0.631–10	0.998

Values are expressed as means ± standard deviation; LOD [a]: limit of detection; LOQ [b]: limit of quantification; Rec. [c]: recovery; ME [d]: matrix effect; Conc. range [e]: concentration range; (r^2) [f]: correlation coefficient.

2.2.2. Linearity

Linearity was evaluated according to signal responses of target analytes from spiked plasma samples at seven different concentrations and by calculating the linear regression. Calibration curves showed linear responses ranging from 0.003 to 10 μg/mL in plasma according to the target analyte. To obtain an adequate accuracy it was necessary to use a weighting factor for most of the analyzed compounds. Sufficient linearity was demonstrated by the coefficient of determination (r^2) values, ranging from 0.990 to 0.999. Linear concentration ranges of individual compounds and associated coefficients of determination are shown in Table 3.

2.2.3. Recovery

The recoveries of analyzed carotenoids and fat-soluble vitamins were between 86.1% and 104.8%. The lowest recovery was achieved in the case of lutein and zeaxanthin. Individual recoveries established during the method validation are presented in Table 3.

2.2.4. Accuracy and Precision

Intra- and inter-day accuracy and precision in three concentration levels with respect to calibration curves were studied. All the analytes met the acceptance criteria by not exceeding 15% RSD. The highest values of RSD% were 14% for 9-*Z*-β-carotene and cryptoxanthin. Obtained accuracy ranged from 85.9% to 114.0%. Values of intra- and inter-day precision are summarized in Table 4 and values of intra- and inter-day accuracy are shown in Table 5.

2.2.5. Matrix Effect

The final quality parameter of the validation procedure was the matrix effect. Matrix effect values ranged from 86.9% to 108.5% (Table 3). MS signal suppression was achieved for 13-*Z*-β-carotene, cryptoxanthin, lutein, zeaxanthin, β-carotene, 9-*Z*-β-carotene, retinol acetate, 25-hydroxycholecalciferol, cholecalciferol, α-tocotrienol, and 5-*Z*-lycopene. MS signal enhancement was obtained for *E*-β-apo-8′-carotenal, α-carotene, astaxanthin, cantaxanthin, and retinol.

2.3. *Quantification of Carotenoids and Fat-Soluble Vitamins in Human Plasma*

Concentrations of carotenoids and fat-soluble vitamins found in the human plasma after a high antioxidant intervention are shown in Table 6. In plasma samples from eight volunteers the following carotenoids and fat-soluble vitamins were found at quantifiable concentrations: retinol, 25-hydroxycholecalciferol, cantaxanthin, β-carotene, and α-carotene. The highest concentration was obtained in the case of β-carotene (2634.1 ± 1870.3 nM). Retinol acetate, astaxanthin, *E*-β-apo-8′-carotenal, and cryptoxanthin were also detected in plasma but at concentration levels lower than their LOQ. The other targeted analytes were not detected.

Table 4. Intra- and inter-day precision obtained in samples by the HPLC-MS/MS method.

Concentration	LOW (n = 5)				MEDIUM (n = 5)				HIGH (n = 5)			
Analyte	Day 1 (RSD%)	Day 2 (RSD%)	Day 3 (RSD%)	Inter-Day (RSD%)	Day 1 (RSD%)	Day 2 (RSD%)	Day 3 (RSD%)	Inter-Day (RSD%)	Day 1 (RSD%)	Day 2 (RSD%)	Day 3 (RSD%)	Inter-Day (RSD%)
retinol	11.5	8.4	3.6	3.8	6.0	6.0	6.7	6.7	9.5	9.2	6.8	3.4
25-hydroxycholecalciferol	10.2	10.0	8.0	1.8	4.4	6.0	11.5	3.3	7.2	8.5	2.5	1.7
retinol acetate	8.6	7.0	5.7	0.7	9.2	6.4	6.8	1.3	8.3	11.1	3.6	0.7
α-tocotrienol	4.7	2.9	3.0	3.5	3.5	8.0	8.8	10.4	9.5	3.8	7.9	3.5
cholecalciferol	12.7	10.0	4.6	1.1	7.4	2.8	9.5	2.0	8.0	9.1	5.7	1.0
astaxanthin	9.9	1.0	7.5	2.7	9.5	4.7	8.8	8.8	6.2	7.0	10.3	4.0
lutein	12.1	9.7	4.5	4.1	5.8	5.2	9.6	2.5	6.2	11.8	5.9	2.0
zeaxanthin	7.3	1.9	2.2	5.5	9.0	9.3	7.5	4.8	8.5	10.8	5.1	0.7
cantaxanthin	11.5	3.3	7.0	6.2	11.1	2.9	9.7	6.2	8.5	11.0	9.9	2.9
E-β-apo-8′-carotenal	11.5	5.3	5.1	10.6	8.6	8.0	9.7	13.1	5.3	5.8	2.9	9.4
cryptoxanthin	5.2	7.6	8.2	4.4	10.4	10.1	8.3	1.5	6.9	14.0	4.2	0.3
13-Z-β-carotene	10.8	3.1	4.6	3.1	4.4	10.6	10.0	9.1	12.3	13.6	13.1	4.2
α-carotene	5.5	9.8	9.4	5.9	0.7	5.3	10.2	5.0	7.1	12.1	8.1	5.7
β-carotene	8.9	12.2	5.7	2.4	8.1	3.7	2.5	3.4	10.5	2.9	7.8	1.8
9-Z-β-carotene	7.0	10.0	5.1	1.3	3.3	4.0	14.0	4.9	8.0	9.7	3.8	4.9
5-Z-lycopene	2.8	7.3	4.9	5.2	5.1	0.1	6.5	2.8	13.4	9.2	9.5	5.1

RSD: relative standard deviation; n: replicates; n = 5.

Table 5. Intra- and inter-day accuracy obtained in samples by the HPLC-MS/MS method.

Concentration	LOW (n = 5)				MEDIUM (n = 5)				HIGH (n = 5)			
Analyte	Day 1 (RSD%)	Day 2 (RSD%)	Day 3 (RSD%)	Inter-Day (RSD%)	Day 1 (RSD%)	Day 2 (RSD%)	Day 3 (RSD%)	Inter-Day (RSD%)	Day 1 (RSD%)	Day 2 (RSD%)	Day 3 (RSD%)	Inter-Day (RSD%)
retinol	100.6 ± 11.4	97.7 ± 8.6	105.3 ± 3.6	101.2 ± 3.7	98.8 ± 6.0	104.4 ± 6.0	91.4 ± 6.7	98.2 ± 6.6	100.6 ± 9.5	97.8 ± 9.2	104.6 ± 6.8	101.0 ± 3.4
25-hydroxycholecalciferol	99.2 ± 10.3	97.3 ± 10.1	100.7 ± 8.0	99.1 ± 1.7	101.6 ± 4.5	105.4 ± 5.9	98.8 ± 11.5	101.9 ± 3.3	99.2 ± 7.2	97.3 ± 8.5	100.7 ± 2.5	99.1 ± 1.7
retinol acetate	98.3 ± 8.5	97.1 ± 7.0	97.3 ± 5.6	97.5 ± 0.7	103.3 ± 9.2	106.0 ± 6.5	104.4 ± 7.0	104.6 ± 1.3	98.3 ± 8.3	97.0 ± 11.1	97.9 ± 3.6	97.7 ± 0.7
α-tocotrienol	100.8 ± 4.6	108.0 ± 2.8	104.0 ± 3.0	104.3 ± 3.5	96.4 ± 3.5	86.8 ± 1.7	87.7 ± 3.6	90.3 ± 5.9	100.6 ± 9.5	106.5 ± 3.8	104.0 ± 5.8	103.7 ± 2.9
cholecalciferol	97.9 ± 12.5	95.8 ± 9.8	96.8 ± 4.5	96.8 ± 1.0	103.9 ± 7.3	103.5 ± 11.0	101.6 ± 7.4	103.0 ± 1.2	97.9 ± 8.0	96.0 ± 9.1	97.6 ± 5.7	97.1 ± 1.0
astaxanthin	102.6 ± 9.8	105.3 ± 1.2	99.9 ± 7.5	102.6 ± 2.6	94.9 ± 9.7	92.1 ± 4.7	104.7 ± 5.6	97.2 ± 6.8	102.5 ± 6.2	103.7 ± 7.0	96.3 ± 10.3	100.8 ± 4.0
lutein	94.5 ± 8.2	97.0 ± 9.8	97.7 ± 4.5	96.4 ± 1.7	108.6 ± 5.8	105.2 ± 5.3	103.6 ± 9.5	105.8 ± 2.4	94.7 ± 6.2	97.5 ± 11.8	98.4 ± 5.9	96.9 ± 2.0
zeaxanthin	103.0 ± 7.2	103.8 ± 2.1	112.8 ± 1.7	106.5 ± 5.1	98.2 ± 9.0	97.2 ± 9.3	89.9 ± 7.4	95.1 ± 4.8	100.4 ± 8.5	100.5 ± 10.8	101.7 ± 5.1	100.9 ± 0.7
cantaxanthin	108.9 ± 11.4	104.8 ± 3.2	96.7 ± 7.1	103.5 ± 6.0	94.4 ± 10.9	92.6 ± 2.9	104.0 ± 9.7	97.0 ± 6.3	102.6 ± 8.5	108.0 ± 2.7	98.0 ± 9.9	102.9 ± 4.9
E-β-apo-8′-carotenal	111.3 ± 1.3	100.0 ± 5.1	96.6 ± 5.0	102.6 ± 7.5	95.2 ± 4.0	85.9 ± 4.1	103.3 ± 9.4	94.8 ± 9.2	109.0 ± 5.3	114.0 ± 2.3	97.0 ± 2.9	106.7 ± 8.2
cryptoxanthin	101.1 ± 5.2	94.7 ± 7.6	93.0 ± 8.2	96.2 ± 4.4	99.8 ± 10.6	101.6 ± 10.1	102.6 ± 8.4	101.3 ± 1.4	100.1 ± 6.9	99.8 ± 14.0	99.5 ± 4.2	99.8 ± 0.3
13-Z-β-carotene	96.5 ± 10.8	102.3 ± 2.9	97.9 ± 4.8	98.9 ± 3.0	108.7 ± 4.3	91.0 ± 10.6	103.5 ± 10.0	101.1 ± 9.0	93.9 ± 12.3	109.0 ± 1.6	97.8 ± 13.1	100.2 ± 7.8
α-carotene	111.5 ± 0.6	105.0 ± 9.5	103.3 ± 9.2	106.6 ± 4.1	86.1 ± 0.7	93.9 ± 5.4	94.2 ± 10.3	91.4 ± 5.0	109.5 ± 2.6	104.1 ± 12.2	102.9 ± 8.5	105.3 ± 3.1
β-carotene	100.3 ± 9.0	96.8 ± 12.0	101.8 ± 5.7	99.6 ± 2.6	99.8 ± 7.9	102.6 ± 3.5	95.9 ± 2.5	99.4 ± 3.4	100.1 ± 10.5	97.6 ± 2.9	101.1 ± 7.7	99.6 ± 1.8
9-Z-β-carotene	101.7 ± 7.0	99.2 ± 10.0	100.6 ± 5.1	100.5 ± 1.3	98.9 ± 3.0	107.2 ± 3.9	98.1 ± 14.3	101.4 ± 5.0	100.4 ± 8.0	92.1 ± 9.7	100.6 ± 3.8	97.7 ± 4.9
5-Z-lycopene	97.3 ± 2.8	100.7 ± 7.3	90.7 ± 4.9	96.2 ± 5.2	103.5 ± 5.3	98.6 ± 0.1	103.5 ± 6.4	101.8 ± 2.8	96.8 ± 13.4	106.8 ± 9.2	99.5 ± 9.5	101.0 ± 5.1

Values are expressed as means ± relative standard deviation; n: replicates; n = 5.

Table 6. Carotenoids and fat-soluble vitamins quantified in plasma by the HPLC-MS/MS method.

Analyte	Quantification Transition	In Plasma (nM)
retinol	269 → 181	115.2 ± 10.5
25-hydroxycholecalciferol	383 → 365	189.7 ± 32.5
retinol acetate	329 → 269	<LOQ [a]
α-tocotrienol	411 → 165	n.d. [b]
cholecalciferol	385 → 367	n.d. [b]
astaxanthin	597 → 147	<LOQ [a]
lutein	551 → 429	260.2 ± 138.9
zeaxanthin	568 → 476	n.d. [b]
cantaxanthin	565 → 363	28.32 ± 12.4
E-β-apo-8′-carotenal	417 → 325	<LOQ [a]
cryptoxanthin	553 → 535	<LOQ [a]
13-*Z*-β-carotene	536 → 444	n.d. [b]
α-carotene	536 → 444	100.6 ± 18.6
β-carotene	537 → 413	2634.1 ± 1870.3
9-*Z*-β-carotene	537 → 413	n.d. [b]
5-*Z*-lycopene	537 → 413	n.d. [b]

Values are expressed as means ± standard deviation (n = 8); <LOQ [a]: under limit of quantification; n.d. [b]: not detected.

3. Discussion

3.1. HPLC-MS/MS Method Development

3.1.1. Extraction of Carotenoids and Fat-Soluble Vitamins

To keep costs of analytical procedures down, it is necessary to minimize the quantity of plasma used, which entails a lower consumption of chemicals. Two hundred µL of human plasma was used in our experiments. In previously published work, quantities such as 200 µL [28,29], 100 µL [23], 800 µL [30], 1 mL [31], and 300 µL [32] of plasma have been required. Isolation of fat-soluble micronutrients from plasma generally consists of two steps. Deproteinization by the addition of methanol, acetonitrile [31,33] or ethanol [23,29,30,33] to plasma is required as a prior step. Deproteinization is usually followed by extraction with hexane [29], heptane [21] or a combination of hexane with another solvent [34]. Solvents used for extraction are very often enriched with an antioxidant, such as tert-butylated hydroxytoluene or ascorbic acid, to protect carotenoids and fat-soluble vitamins [29,32]. In our study, ethanol for deproteinization and n-hexane enriched with BHT at a concentration of 100 mg/L were chosen. The designed extraction technique is simple, repeatable, non-time consuming and provides good recoveries (Table 3).

3.1.2. Chromatographic and MS/MS Conditions

Numerous HPLC-DAD methods have been designed to determine carotenoids and fat-soluble vitamins [23–25]. However, chromatographic techniques coupled to mass detection used for the analysis of a wide range of analytes are scarce. The available HPLC–MS or HPLC–MS/MS methods analyze fewer analytes in comparison with the method described here [25,26,29,32,35,36]. During the development of this method, different combinations of solvents for the preparation of mobile phases and gradients were tested: A = 80:20 MeOH/W (v/v), B = 78:20:2 MTBE/MeOH/W (v/v/v); A = MeOH, B = MTBE and A = MeOH, B = 80:20 MTBE/MeOH (v/v). At the beginning of the study, the total run time for an adequate separation of our analytes was 72 min with the following linear gradient used for B (t (min), %B): (0.0, 90); (40.0, 40); (60.0, 6); (62.0, 90); (72.0, 90) [24]. Before the validation of the method, the resulting total run time was 50 min, with the linear gradient as described in Materials and Methods.

LC solvent additives such as formic acid, acetic acid, propionic acid, ammonium acetate, ammonium formate, and others are usually used in HPLC–MS and HPLC–MS/MS analyses to enhance the mass signal [22,25,26,35–38]. For example, Kopec et al. [25], who analyzed carotenoids, retinyl esters, α-tocopherol and phylloquinone in chylomicron-rich fractions of human plasma, compared the effects of adding water (no additive), formic acid, and ammonium acetate to the mobile phase, obtaining the best results with the addition of ammonium acetate. Ammonium acetate for mass signal enhancement was also used for the analysis of carotenoids by Meulebroek et al. [26] and Arathi et al. [22]. Another preferred LC solvent additive for MS analysis of carotenoids is ammonium formate [35,36]. In our preliminary experiments, all tested mobile phases were enriched with AMAC at a concentration of 0.4 g/L. Then, the effects of the following additives and their combinations were compared: 0.4 g/L AMAC, 0.7 g/L AMAC, 1 g/L AMAC, 0.4 g/L AMAC + 0.1% AA, 0.7 g/L AMAC + 0.1% AA, 1 g/L AMAC + 0.1% AA (Table 1). Due to providing the best MS signal enhancement for the most problematic analytes, the combination of 0.7 g/L AMAC + 0.1% AA was chosen for the final data acquisition.

These conditions allowed us to efficiently separate 16 analytes in an acceptable time, with very good resolution and suitable sensitivity for the analysis of biological fluids. To the best of our knowledge, a similar HPLC-MS/MS method for the determination of a large number of the afore mentioned analytes has not been previously published.

3.2. Method Validation

The proposed method was fully validated based on the criteria of the AOAC International [39]. Since few methods for the simultaneous determination of a wide range of carotenoids and fat-soluble vitamins using HPLC-MS/MS have been published, an effective comparison of our results was limited. As in Kopec et al. [25], lutein, cryptoxanthin, α-carotene, and β-carotene were analyzed and as in Meulebroek et al. [26], lutein, zeaxanthin, α-carotene, and β-carotene. In comparison with Kopec et al. [25], lower LOD and LOQ values were obtained for lutein, but the values were higher for the other analytes. In comparison with Meulebroek et al. [26], comparable LOD and LOQ values for lutein and α-carotene were obtained, but our values for zeaxanthin and β-carotene were higher. The most often used method for the analysis of carotenoids and fat-soluble vitamins is HPLC coupled to DAD [23–25]. In comparison with a previous study of our group [24], LOD and LOQ values achieved by the present HPLC-MS/MS method were lower for retinol, astaxanthin, lutein, E-β-Apo-8′-carotenal, 13-Z-β-carotene, α-carotene, and β-carotene, in some cases, more than 100-fold lower. For cryptoxantin, zeaxantin, and 9-Z-β-carotene we obtained comparable LOD and LOQ values. Thus, our method achieved a considerable improvement in LOD and LOQ values in relation to HPLC–DAD methods.

Another very important quality parameter is recovery. Achieved recoveries were in approximate agreement with those published by Lee et al. [23], Colmán-Martínez et al. [24], and Karppi et al. [29]. Recoveries obtained during our experiments were between 86.1% and 104.8%, in comparison with 87% to 105% reported by Lee et al. [23] for α-tocotrienol, retinol, α-carotene, β-carotene, lutein, zeaxanthin, and canthaxanthin. Karppi et al. [29] published recoveries between 86.8% and 103% for retinol, lutein, zeaxanthin, E-β-apo-8′-carotenal, cryptoxanthin, α-carotene, and β-carotene. Recoveries achieved by Colmán-Martínez et al. [24] ranged from 89% to 107% for retinol, astaxanthin, lutein, E-β-apo-8′-carotenal, 13-Z-β-carotene, α-carotene, β-carotene, cryptoxantin, zeaxantin, and 9-Z-β-carotene. The biggest differences in recovery were for lutein and zeaxanthin, which in our case were 86.1% and 86.2%, respectively. The average recovery for these compounds published in the above-mentioned studies is approximately 100%.

For the quality evaluation of our method, we also calculated the matrix effect. It is essential to calculate this parameter for HPLC-MS/MS methods designed to determine compounds in complex matrices such as human plasma, because the matrix can enhance or suppress the acquired MS signal. The matrix effect calculated for each analyte is shown in Table 3. Data to compare the matrix effect was not found.

Perhaps the most valuable parameters for assessing the potential application of the developed method to real samples were intra- and inter-day precision and accuracy, which were determined by injection of plasma extracts spiked at three different concentrations (low, medium, and high) in 5 replications. Intra-day precision ranged from 0.1 to 14.0 RSD% and inter-day from 0.7 to 13.1 RSD%. Values of intra-day accuracy were between 85.9% and 114%. Inter-day accuracy was between 90.3% and 106.6%. These results are summarized in Tables 4 and 5. Kopec et al. [25] report an accuracy ranging from 93% to 102%, and inter-day precision from 4.5 RSD % to 15 RSD % for lutein, cryptoxanthin, α-carotene and β-carotene. The highest conformity with our analytes (11 common analytes) is found in the study by Colmán-Martínez et al. [24], whose values ranged from 1 to 15 RSD% for intra-day precision; 4.9 to 15.1 RSD% for inter-day precision; and an accuracy of 90.7%–112.2%. In comparison with Colmán-Martínez et al. [24], the method presented here is more precise and the accuracy is comparable. In any case, based on the intra- and inter-day precision and accuracy, our method is repeatable and reproducible and therefore highly suitable for application to real biological samples such as human plasma.

3.3. *Quantification of Carotenoids and Fat-Soluble Vitamins in Human Plasma*

The plasma samples collected after a high antioxidant intervention were analyzed. As the content of carotenoids and fat-soluble vitamins depends on alimentary intake, we could not compare our results (concentrations in plasma before the intervention) with other results such as those published by Colmán-Martínez et al. [24].

4. Materials and Methods

4.1. *Standards, Solvents and Reagents*

Carotenoids and fat-soluble vitamin standards: *E*-β-apo-8′-carotenal, α-carotene, 13-*Z*-β-carotene, cryptoxanthin, lutein, zeaxanthin, astaxanthin, cantaxanthin, β-carotene, 9-*Z*-β-carotene, retinol, retinol acetate, 25-hydroxycholecalciferol, cholecalciferol and α-tocotrienol were purchased from Sigma-Aldrich (St. Louis, MO, USA). The standard of 5-*Z*-lycopene was supplied by CaroteNature GmbH (Ostermundigen, Switzerland).

MeOH of LC-MS grade, *n*-hexane, ethanol and MTBE of HPLC grade, synthetic plasma and butylated hydroxytoluene (BHT) were obtained from Sigma-Aldrich. AMAC and AA of HPLC grade were purchased from Panreac Quimica SA (Barcelona, Spain). Ultrapure water (Milli-Q) was generated by a Millipore system (Millipore, Bedford, MA, USA).

Preparation of Standard Solutions

All carotenoid and fat-soluble vitamin standards were prepared at a concentration of 1 mg/mL of MTBE by dissolving 1 mg of each standard in 1 mL of MTBE. All standards were processed and manipulated under dim light to protect against light-induced isomerization or possible degradation. Individual working standards were stored in eppendorf tubes and kept in a freezer at −80 °C until analysis. The stock solution used to spike synthetic plasma was prepared by mixing individual working standards at a concentration of 10 µg/mL in MeOH.

4.2. *UHPLC-MS/MS Method Development*

4.2.1. Instrumentation

HPLC-MS/MS was carried out in an Agilent 1100 HPLC system (Agilent Technologies, Hewlett-Packard, Waldbronn, Germany), consisting of a binary pump and an autosampler coupled to a QTRAP4000 (AB Sciex, Foster City, CA, USA) triple quadrupole mass spectrometer with a DAD detector, which was operated in multiple reaction monitoring (MRM) mode. Chromatographic

separation was performed on a reversed phase column YMC Carotenoid S-5 µm, 250 × 4.6 mm (Waters, Milford, MA, USA), maintained at 40 °C.

4.2.2. Chromatographic Conditions

For chromatographic separation, the following combination of mobile phases was used. Mobile phase A consisted of MeOH, AMAC at a concentration of 0.7 g/L and 0.1% of acetic acid. Mobile phase B contained MTBE and MeOH (80:20, *v*/*v*), AMAC at a concentration of 0.7 g/L and 0.1% of acetic acid. The following linear gradient of A was used (*t* (min), %A): (0.0, 90); (10.0, 75); (20.0, 50); (25.0, 30); (35.0, 10); (37.0, 6); (39.0, 90); (50.0, 90). Total run time of analysis was 50 min. The mobile phase flow rate was 600 µL/min, and 20 µL of the sample was injected into the UHPLC system.

4.2.3. MS Conditions

A triple quadrupole mass spectrometer QTRAP4000 (Sciex, Foster City, CA, USA) equipped with APCI ionization source and controlled by Analyst v.1.6.2 software (Sciex) was used for direct infusion experiments. During the infusion experiments, the equivalent mixture (50:50, *v*/*v*) of mobile phase containing MeOH:W (80:20, *v*/*v*) + 0.4 g/L AMAC and mobile phase containing MTBE:MeOH:W (78:20:2, *v*/*v*/*v*) + 0.4 g/L AMAC was used. All individual standards were injected at a concentration of 1 µL. An atmospheric-pressure chemical ionization source was working in positive ionization mode. After various optimization experiments, the following parameters were chosen to analyze the final samples: curtain gas, 20 psi; source temperature, 400 °C; ion source gas 1 (GS1), 45 psi; ion source gas 2 (GS2), 0 psi; entrance potential (EP), 10 V; collision cell exit potential (CXP), 15 V. Values of declustering potential (DP) were individual for each standard. During the preliminary experiments, the various combinations of precursor-product ions were obtained. The most selective combination for the analyte with the highest MS signal intensity was chosen for final acquisition data. The resulting data acquisition was performed via MRM mode with a dwell time of 120 ms, with 1412 cycles, and between 10 and 14 data points on the peaks. The product ions were generated by collision-activated dissociation (CAD) applied to selected precursor ions in the mass spectrometer collision cell. The chosen MRM transitions and optimal values of DP and collision energy (CE) for all 17 standards are shown in Table 2.

4.2.4. Quality Parameters

The present method was validated according to the criteria of AOAC International [39]. The quality parameters established for the validation of the method were accuracy, intra- and inter-day precision, recovery, limit of detection (LOD), limit of quantification (LOQ), and linearity. LOD is the lowest quantity of a substance that can be distinguished from the absence of that substance (a blank value) within a stated confidence limit. The LOD is estimated from the mean of the blank, the standard deviation of the blank and the confidence factor. The LOD was estimated from the chromatograms of spiked blank plasma at the lowest analyte concentration tested for a signal-to-noise ratio of 3. Similarly, the LOQ is the lowest concentration not only at which the analyte can be reliably detected but at which predefined goals for precision and accuracy are met. The LOQ is at a higher concentration than LOD. The LOQ was determined for a signal-to-noise ratio of 10.

Another quality parameter, recovery, was established by preparing internal calibration curves (synthetic plasma was spiked before the extraction procedure) and external calibration curves (samples were spiked after extraction procedure). Firstly, the concentration had to be calculated by interpolation of individual analyte areas obtained from the samples spiked after the extraction procedure by samples spiked before the extraction procedure. Further, the dependency of ratio analyte concentration to internal standard concentration on calculated concentration was plotted. Then, a linear regression was applied. The slope multiplied by 100 corresponded to the analyte recovery.

To determine the presence or non-presence of a plasma matrix effect, two calibration curves were prepared. MS/MS areas of known amounts of standards (calibration curve prepared by dissolving

of working mixture of standards in MeOH–(A)) were compared with those measured in a blank plasma extract spiked, after extraction, with the same amount of the working mixture of standards (B). The ratio (B/A × 100) was defined as the matrix effect (ME). A value of 100% indicates that there was no matrix effect. There was signal enhancement if the value was higher than 100% and signal suppression if the value was lower than 100%.

Linearity was tested by evaluating signal responses of target analytes from spiked plasma samples at seven different concentrations and by calculating linear regression.

Accuracy expresses the closeness of mean test results obtained by the developed method to the actual concentration of the analyte, and was determined by spiking blank plasma with three known concentrations (low, medium, and high with respect to the calibration curve). The accuracy was expressed as the percentage of the ratio of the mean observed concentration and the known spiked concentration in the plasma matrix. The mean accuracy should be within ±15% of the nominal value. Precision of a method is the closeness of agreement between independent test results obtained from homogenous test material under specified conditions of use. Intra-day precision and inter-day precision were studied. It was decided to use five replicates per three concentration levels: low, medium, and high in a single run or in three different days. The precision of the developed method was evaluated by the %RSD (percentage of relative standard deviation of intra- and inter-day repeatability). The values determined at each concentration level should not exceed 15% of RSD.

4.3. Method Application to Real Samples: Human Dietary Intervention Study

The human plasma samples were obtained from healthy, non-smoking male volunteers (8 volunteers), aged between 18 and 32 years. The plasma samples in the pilot human study were collected after two weeks of a high antioxidant dietary intervention consisting of an increased consumption of fruits and vegetables. Blood collection was performed in the morning at 08:00 after fasting. Collected blood samples were immediately centrifuged at 3500 rpm for 15 min at 6 °C. Plasma was separated and samples were stored at −80 °C until analysis. For the determination of carotenoids and fat-soluble vitamins, 200 µL of human plasma was used. The study protocol was approved by the Ethics Committee of Clinical Investigation of the University of Barcelona (Spain) (Institutional Review Board IRB00003099; Approval date: 12 April 2016).

Extraction of Carotenoids and Fat-Soluble Vitamins

In preliminary experiments, liquid-liquid extraction as previously described by our group in [30] was used for the isolation of selected carotenoids and fat soluble vitamins. For our purpose, the following double liquid-liquid extraction method was designed. Two hundred microliters of ethanol causing deproteinization and 0.5 mL of *n*-hexane/BHT (100 mg/L) were added to 200 µL of human plasma in an eppendorf tube. This step was followed by a vortexing for 1 min and centrifugation at 2070× *g* for 5 min at 4 °C. The upper nonpolar hexane layer was removed from the two-phase liquid system to another eppendorf tube. The remaining aqueous plasma phase was re-extracted by the second addition of 0.5 mL of *n*-hexane/BHT (100 mg/L) followed by vortexing for 1 min and centrifugation at 2070× *g* for 5 min at 4 °C. The upper nonpolar hexane layer was again removed. Both nonpolar layers were combined in an eppendorf tube and evaporated to dryness by a sample concentrator under nitrogen gas at 25 °C followed by a reconstitution with 100 µL of MeOH. Finally, the samples were stored in glass amber vials with inserts in an ultra-freezer at −80 °C until HPLC-MS/MS analysis. In comparison to a previously published extraction method, we have only proportionally changed volumes of the used solvents and we have also exchanged the solvent for reconstitution of a sample. The same design was used to prepare internal and external calibration curves with purified human plasma. Stock solution of synthetic plasma was prepared by dissolving the purified human plasma in 50 mL of Mili-Q water. For this purpose, mixtures of the above-mentioned individual working standards in methanol at 10, 5, 2.5, 1, 0.5, 0.1, 0.05, 0.01 and 0 µg/mL concentrations have been prepared. For preparation of the internal calibration curve, the following extraction procedure was

performed on each concentration level. Two hundred microliters of ethanol was added to 200 µL of human plasma in an eppendorf tube. Then, the plasma sample was spiked by 100 µL of stock solution of individual working standards. This step was followed by the addition of 0.5 mL of *n*-hexane/BHT (100 mg/L) and by a vortexing for 1 min and centrifugation at 2070× *g* for 5 min at 4 °C. The upper nonpolar hexane layer was removed from the two-phase liquid system to another eppendorf tube. The remaining aqueous plasma phase was re-extracted as described above. Both nonpolar layers were combined in an eppendorf tube and evaporated to dryness by a sample concentrator under nitrogen gas at 25 °C, followed by a reconstitution with 100 µL of MeOH. The processed samples were also stored into glass amber vials with inserts in an ultra-freezer at −80 °C until HPLC-MS/MS analysis. For preparation of external calibration curve the exact same extraction procedure was performed on each concentration level, but in this case, removed combined nonpolar phases were spiked by 100 µL of stock solution of individual working standards before evaporating to dryness and reconstitution. All samples were manipulated under dim light during the all procedure steps in order to avoid oxidation and/or isomerization of the bioactive compounds.

5. Conclusions

A unique HPLC-MS/MS method for the simultaneous quantification of 16 carotenoids and fat-soluble vitamins in human plasma was designed and fully validated. Good quality values of LOD, LOQ, recovery, linearity, matrix effect, accuracy, and precision were obtained by the proposed method. According to our knowledge, no similar HPLC-MS/MS method for the determination of such a large number of analytes has been previously published. In the future, considering the excellent validation results obtained, this method could be used in various applied clinical studies or investigations.

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

Abbreviations

MeOH	Methanol
MTBE	Methyl-*tert*-butyl ether
BHT	Butylated hydroxy toluene
AMAC	Ammonium acetate
AA	Acetic acid
W	Water
CAD	Collision-activated dissociation
DP	Declustering potential
APCI	Atmospheric pressure chemical
EP	Entrance potential
CE	Collision energy
HPLC-MS/MS	High performance liquid chromatography coupled to mass spectrometry in tandem mode
LOD	Limit of detection
LOQ	Limit of quantification
MRM	Multiple reaction monitoring
ME	Matrix effect
MS	Mass spectrometry
CXP	Cell exit potential
RSD	Relative standard deviation
RT	Retention time

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Publicación 4. Cuantificación de compuestos bioactives de ocho genotipos de tomates Serbios y evaluación de su capacidad antioxidante

Quantification of the bioactive compounds of eight Serbian tomato genotypes and evaluation of their antioxidant capacity. Sanja Vlaisavljevic, **Mariel Colmán-Martínez**, Anamarija Stojanovic, Miriam Martínez-Huélamo, Rosa M. Lamuela-Raventós y Neda Mimica-Dukic. *Food & Function* (Enviado).

Research Article

Quantification of the bioactive compounds of eight Serbian tomato genotypes and evaluation of their antioxidant capacity

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ABSTRACT

Tomato, one of the most consumed fruits, is rich in beneficial biomolecules such as carotenoids, polyphenols, vitamin C and tocopherol, which may decrease the risk of developing chronic degenerative diseases. Eight different Serbian genotypes were analyzed for the quantification of polyphenol, carotenoid, and vitamin C content, as well as the evaluation of antioxidant capacity. Among the eight genotypes tested, S606, S616 and S612 showed higher levels of carotenoids, especially lycopene. These three genotypes, as well as S364, also showed a higher phenolic content, with rutin predominating, whilst S606 and S615 presented the highest levels of ascorbic acid. Their content of antioxidant compounds suggested that genotypes S606 and S615 have the best antioxidant capacity, which was confirmed by their greater efficiency in neutralizing DPPH and ABTS radicals, as well as high reducing power. The results obtained in the present study could be of considerable interest for breeding programs wishing to select tomato genotypes with high antioxidant and nutritional properties.

Introduction

Eating a healthy diet, and knowing how to recognize and choose a high quality food are currently topics of great interest. The nutritional quality and many sensorial properties of fruits and vegetables are conditioned by their content of vitamins, minerals, dietary fiber, carbohydrates, proteins and antioxidant phytochemicals (carotenoids, polyphenols and other compounds), which have been associated with the prevention and decreased risk of different diseases. The analysis of biomolecules in plant food, whose levels depend on a number of intrinsic (genus, species, cultivars) and extrinsic (agronomic, environmental, handling, storage) factors (Carbonell-Capella et al., 2014), is therefore very important for an assessment of both sensory qualities and health benefits for the consumer.

Although tomato (*Lycopersicum esculentum* L.) is a seasonal fruit, it can be found in markets and supermarkets throughout the year, because its production is carried out in greenhouses. It is one of the most consumed fruits in Europe, especially in the Mediterranean area, both fresh and in tomato-based products (Ilahy et al., 2011). Tomato contains a high amount of bioactive compounds, notably phenolics, carotenoids and other antioxidants (Raffo et al., 2006), the content depending on the variety, growing conditions, and ripeness stage, among other factors (Domínguez et al., 2012). Tomato fruit ripening is a complex process characterized by various morphological, physiological, biochemical and molecular transformations (synthesis and storage of polyphenols and other compounds), which determine the nutritional quality and antioxidant potential at each stage.

Consumers tend to choose tomatoes on the basis of their visual and functional properties, and may consider fruit with an attractive appearance to be healthier. In the commercialization of tomato production, the most profitable and high-yielding cultivars and hybrids are given predominance, while other cultivars, which might have a higher nutritional value, and be an important source of genes for breeding, are disregarded. In order to increase the nutritional value and the content of bioactive compounds, today a range of cultivars and varieties are being produced. Therefore, the aim of this study was to investigate the phytochemical profiles of both traditional and new tomato cultivars.

Considering phytochemical characteristics of new fruits cultivars, traditional breeding of yield, market life, and consumer taste.

There are many studies related to the phytochemical analysis and antioxidant properties of tomato from different origins (Frusciante et al., 2007; García-Valverde et al., 2013; Kaur et al., 2013; Kotíková et al., 2011; Lenucci et al., 2006; Singh et al., 2015), but to date no reports have been published on Serbian varieties. In Serbia there is a strong consumer demand for traditional fruits and vegetables. The aim of Serbian farmers and breeders is to obtain cultivars with a high content of nutrients and bioactive compounds, and provide food that is both tasty and healthy. For this study, eight tomato cultivars with different color, morphology and sensorial characteristics that are attractive for consumers were selected. The phytochemical profile as well as antioxidant properties of these eight Serbian tomato varieties were determined for the first time.

Material and methods

For the purposes of existing breeding programs and the preservation of traditional cultivars, the Institute of Field and Vegetable Crops in Novi Sad, Serbia (IFVCNS) has established a collection of over 400 different tomato accessions. Eight accessions (genotypes) significantly different in morphological and organoleptic characteristics were chosen for the quantification of polyphenols and carotenoids, determination of vitamin C content, as well as evaluation of antioxidant capacity (Table 1) (Figure 1). The trial was conducted in Rimski Šančevi (45°39′58.02″N 19°04′51.16″E), Serbia in 2015. On March 30th, seeds were sown indoors on a surface of moist compost, covered with a thin covering of vermiculite, and watered with a solution of copper-based fungicide. On April 5th, indoor seedlings with a pair of true leaves each were pricked out into larger individual containers. Two weeks before planting out, young tomato plants were hardened off and gradually acclimatized to the harsher outdoor conditions. On June 2nd, seedlings were manually transplanted outdoors in a randomized block design with three replicates. Each plot was a single 5 m row that bedded 11 plants. Rows were spaced 140 cm apart and within-row spacing was 50 cm. Management of soil, and pest and disease control was carried out according to standard procedures. Tomatoes at the fruit maturity stage were hand-harvested randomly in September 2015, from the middle of 10 plants of each of the eight genotypes.

Table 1. Morphological characteristics and description of eight Serbian tomato genotypes

Accession N°	Predominant fruit shape	Fruit size	Exterior color of mature fruit	Intensity of exterior color	Fruit blossom end shape	Taste	Uses
S 364	Plum-shaped	5-8 cm	Red	Dark	Indented	Not sweet	Processing
S 590	Slightly flattened	8-10 cm	Pink	Light	Flat	Sweet	Fresh/Processing
S 606	Plum-shaped	3-5 cm	Orange	Dark	Flat and pointed	Sweet	Fresh
S 607	Plum-shaped	3-5 cm	Red	Intermediate	Flat	Very sweet	Fresh
S 608	High rounded	<3 cm	Yellow	Intermediate	Flat	Very sweet	Fresh
S 612	Heart-shaped	>10 cm	Pink	Light	Pointed	Very sweet	Fresh
S 615	Rounded	8-10 cm	Yellow	Intermediate	Flat	Very sweet	Fresh
S 616	Long-oblong	5-8 cm	Red	Dark	Indented	Not sweet	Processing

Preparation of plant material

Sampled fruits of each genotype were cut into small pieces and sequentially homogenized in a domestic blender for 2 min. The homogenized fruits were introduced into jars and then lyophilized for 48 hours . The samples were kept at room temperature in a dark and dry place.

Extraction

Tomatoes were extracted by liquid-liquid extraction for the analysis of both polyphenols and carotenoids, and analyzed by UHPLC-MS/MS in the case of polyphenols (Di Lecce et al., 2013) and HPLC-UV for carotenoids (Colmán-Martínez et al., 2016; Vallverdú-Queralt et al., 2012).

For the extraction of polyphenols, 0.2 g of dry tomatoes was weighed and homogenized with 5 mL of ethanol:Milli-Q water (0.1% formic acid) (80:20). 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 portion of ethanol was 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 with up to 2 mL of water containing 0.1% formic acid, filtered through 0.22 μm polytetrafluoroethylene (PTFE) syringe filters (Waters Corporation, United States), and injected into a UHPLC−MS/MS system.

Carotenoid extraction consisted of a double liquid-liquid extraction with 5 mL of ethanol/hexane (4:3 v/v) for each extraction. The homogenate was sonicated for 5 min and centrifuged at 2140 × *g* for 15 min at 4 °C. The two supernatants were combined and evaporated under nitrogen flow. Finally, the residue was reconstituted with MTBE up to 1 mL and filtered through a 0.45 μm PTFE filter (Waters, Milford, MA, USA) into an insert-amber vial for HPLC-UV analysis.

Extractions were performed in triplicate and quantified with the corresponding commercial standards. When standards were not available, the compounds were quantified based on the free form of the corresponding metabolite.

For evaluation of antioxidant activity and determination of total phenol and vitamin C content, 0.5 g of lyophilized tomato samples were weighed, homogenized with 5 mL of 80% ethanol (v/v) and then added to the flask and sonicated continuously for 15 min on an ultrasonicator. The extraction was repeated twice.

HPLC-UV separation of carotenoids

Chromatographic separation was carried out in an HP 1100HPLC system (Hewlett-Packard, Waldbronn, Germany), consisting of a quaternary pump and an autosampler coupled to a diode array detector DAD G1315B. Chromatographic separation was performed on a YMC Carotenoid column, 250 mm × 4.6 mm (Waters, Milford, MA)

connected to a precolumn YMC Guard Cartridge Carotenoid 20 × 4.0 mm i.d. The DAD detector was adjusted at 450 nm for carotenoid detection. The system was controlled by Agilent ChemStation Software. The mobile phase used was Milli-Q water (A), methanol (B), and MTBE (C) with the following linear gradient for B (t (min), %B): (0.0, 90); (40.0, 40); (60.0,6); (62.0, 90); (72.0, 90). Solvent A was used isocratically at 4%. The separation was achieved at a flow rate of 0.6 mL/min. Twenty microliters of the samples were injected in the HPLC-UV system (Colmán-Martínez et al., 2016).

UHPLC-MS/MS separation of polyphenols

The UHPLC analysis was performed using an Acquity UHPLC chromatograph equipped with a Waters binary pump system (Milford, MA, USA). The mobile phase used was water (A) and MeCN (B) with 0.1% formic acid in both solvents. An increasing linear gradient of B was used (t (min), %B), as follows: (0.0, 20); (0.5, 20); (1.5, 30); (2.00, 30); (2.5, 50); (3.0, 100); (3.5, 100); (3.7, 20) and (4.5, 20). The mobile-phase flow rate was 400 µL min^{-1}, and 10 µL of the sample was injected into the UHPLC system. The column was maintained at 30 °C while the autosampler was thermostated at 4 °C.

The UHPLC system was coupled to an API 3000 triple-quadrupole mass spectrometer (Sciex, Foster City, CA, USA) with a Turbo Ion Spray source in negative-ion mode to obtain MS/MS data. TurboIonSpray source settings were as follows: capillary voltage − 3500 V; nebulizer gas (N2) 10 (arbitrary units); curtain gas (N2) 12 (arbitrary units); collision gas (N2) 4 (arbitrary units); entrance potential 10 V; drying gas (N2) heated to 400 °C and introduced at a flow rate of 8000 cm^3 min^{-1}. The phenolic compounds present in tomatoes were detected and quantified by using the multiple reaction monitoring mode (MRM), tracking the transition of the parent and product ion specific to each compound. The system was controlled by Analyst v.1.4.2 software supplied by Sciex (Foster City, CA, USA).

Total phenol content

The total phenolic content was determined according to a previously reported method (Fukumoto & Mazza, 2000), customized for 96-well microplates. Thirty microliters of each extract or standard solution, except in a blank probe, when only the solvent was used, was added to 150 µL of 0.1 mol/L Folin-Ciocalteu (FC) reagent and mixed with

120 µL of sodium carbonate (7.5%) after 10 min. The mixture was incubated in the dark at room temperature (2 h). The absorbance of the resulting solution was measured at 760 nm. The phenolic concentration was determined by comparison with the standard calibration curve of gallic acid, and results were presented as a mean value of triplicate tests. The total phenol value was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry material (dm) calculated according to the standard calibration curve (linear regression).

Ascorbic acid content

Ascorbic acid was determined according to the method of Klein and Perry (1982) modified for 96-well microplates. The samples were re-extracted with *meta*-phosphoric acid (0.1 g/mL) to obtain final concentrations of 50, 70 and 100 mg/mL. The mixture was stirred and left for 45 min at room temperature. Further, the samples (30 µL) were mixed with 2,6-dichlorindophenol and the absorbance was measured within 20 min at 515 nm against a blank. The ascorbic acid content was calculated on the basis of the calibration curve of standard L-ascorbic acid (ranging from 0-320 µg/mL). All measurements were performed in triplicate and the results were expressed as mg of ascorbic acid (AA) per g of dry material (dm).

Reduction of the DPPH radical

The DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a violet solution. This free radical, stable at room temperature, is reduced in the presence of an antioxidant molecule, giving rise to a colorless ethanol solution. The DPPH assay provides an easy and rapid way to evaluate antioxidants by spectrophotometry. Plant extracts were tested for their scavenging effect on the DPPH radical according to Sanchez-Moreno et al. (1998). Ten microliters of the examined extract solutions was added to 100 µL of 90 µmol/L DPPH solution in methanol and the mixture was diluted with 190 µL of methanol. In the control, the exact amount of the extract was substituted with solvent, and in the blank probe, only methanol (290 µL) and extract (10 µL) were mixed. After 1 h, measurements of absorbance were done at 515 nm. Results were expressed as milligrams of Trolox equivalents (TE) per gram of dm of extract calculated according to the standard calibration curve.

ABTS assay

The ABTS assay was performed by a modified previously described procedure (Arnao et al., 2001). Spectrophotometric determination of the scavenging activity of tomato samples was based on transformation of the blue-green solution of radical cation $ABTS^{\bullet+}$ to its neutral colorless form ($ABTS^{2-}$). $ABTS^{\bullet+}$ is generated by the persulfate oxidation of 2,2-azinobis (3-ethylbenzoline-6-sulfonic acid)-($ABTS^{2-}$). An $ABTS^{\bullet+}$ radical cation was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulphate. The mixture was allowed to stand in the dark at room temperature for 16 h before use. Trolox (1mM) was prepared in methanol as an antioxidant standard. The stock solution was diluted in 95 % ethanol on the day of use. Ten microliters of different concentrations of the extracts were added to 290 µL ABTS solution until a final volume of 300µL. After five minutes, the absorbance was read at 734 nm. The results were expressed as Trolox equivalants (TEAC- trolox equivalent antioxidant capacity) per g of dm (TEAC/g dm).

Statistical Analysis

For linear regression analysis, Origin software version 8.0 was used. All the results were expressed as the mean ± SD of three different trials. A comparison of the group means and the significance between the groups were verified by one-way ANOVA followed by Tukey's post hoc test. Statistical significance was set at $p<0.05$.

Results and discussion

The chemical composition of tomatoes can be affected by many factors, such as the cultivar or variety, environmental factors (light, temperature, air composition, mineral nutrition, etc.), cultivation and storage methods (García-Valverde et al., 2013). Among the different tomato varieties tested, considerable differences were found in contents of carotenoids, polyphenols, or vitamin C. The health benefits of tomato and tomato-based products may be due to synergistic interactions of these bioactive compounds.

Quantitative determination of carotenoids

Carotenoids are responsible for the color of many fruits and vegetables, and their concentration depends on the activity of different enzymes and also genetic expression

(Guzman et al., 2010). Several epidemiological studies have provided evidence for the protective effect of carotenoids from tomato, such as a decreasing risk of different degenerative diseases and some types of cancer (Tanaka et al., 2012). The carotenoid content of eight Serbian tomato varieties is shown in Table 2.

Table 2. Quantification of carotenoids in eight Serbian tomato genotypes

Compound	Content of carotenoids in eight tomato genotypes (mg/100g dm)							
	Sample							
	S364	S616	S590	S606	S615	S608	S612	S607
Lutein	1.19±0.06^{e}	1.71±0.04^{c}	1.29±0.02^{e}	4.58±0.03^{a}	1.46±0.04^{d}	1.07±0.03^{f}	2.01±0.02^{b}	0.86±0.04^{g}
Zeaxanthin	n.d. *	n.d.	n.d.	0.13±2.01^{b}	1.30±1.13^{a}	n.d.	n.d.	n.d.
trans-β-Apo-8'-carotenal	n.d.	n.d.	0.85±3.03^{b}	7.20±1.97^{a}	n.d.	n.d.	n.d.	n.d.
Criptoxanthin	n.d.	n.d.	1.36±0.14^{b}	13.92±1.74^{a}	1.37±0.14^{b}	n.d.	n.d.	n.d.
15*cis*-β-Carotene	n.d.	4.88±1.58^{b}	0.99±0.12^{d}	n.d.	0.91±0.08^{d}	n.d.	10.15±0.32^{a}	1.57±1.44^{c}
13 *cis* -β-Carotene	n.d.	n.d.	1.68±1.14^{c}	5.33±0.22^{b}	n.d.	0.57±2.23^{d}	11.36±0.11^{a}	1.62±0.01^{c}
α-Carotene	n.d.	7.79±3.11^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
β-Carotene	n.d.	18.73±0.04^{c}	8.26±0.07^{d}	189.64±0.48^{a}	4.56±0.16^{d}	6.71±0.21^{d}	88.17±0.06^{b}	4.87±0.22^{d}
9- *cis* -β-Carotene	n.d.	5.58±0.13^{d}	n.d.	8.56±0.16^{b}	2.00±0.15^{f}	4.74±0.11^{e}	13.25±0.16^{a}	7.05±0.08^{c}
All-*trans*-Lycopene	n.d.	207.42±0.32^{b}	95.20±0.41^{d}	160.64±0.22^{c}	1.08±0.03^{f}	n.d.	339.53±2.00^{a}	91.62±0.30^{e}
5- *cis* -Lycopene	2.36±0.07^{g}	26.10±0.29^{d}	10.58±0.28^{e}	61.93±0.05^{b}	0.98±0.02^{h}	62.95±0.09^{a}	38.80±0.11^{c}	9.91±0.06^{f}

*n.d. - not detected; § Data are means ± SD of three measurements (n=3). Values with different letters in the same row are significantly different (p<0.05).

These data demonstrate that the concentration of carotenoids can vary considerably according to the genotype, which may affect their biosynthesis (Kaur et al., 2013). The most abundant carotenoid in almost all the varieties of tomato samples was lycopene. Tomato is considered the best dietary source of lycopene, which is one of the most beneficial carotenoids for human health. Agarwal, S.; Rao, A. V. Tomato lycopene and its role in human health and chronic diseases. Can. Med. Assoc. J. 2000, 163, 739- 744. The highest levels of lycopene (mg/100g) were detected in varieties S606, S616 and S612 (160.64, 207.42, 339.53), which are of variable color and shape. Lycopene was not found in S364 and S608.

Numerous studies have reported that dietary intake of foods rich in lycopene results in a decreased incidence of certain cancers, including prostate, lung, and colon cancers, as well as coronary heart diseases and macular degeneration (Dillingham & Rao, 2009). The health benefits of tomato are due to its antioxidant and anti-inflammatory activity, as well as the improvement of the plasma lipid profile, which are associated with the intake of lycopene, *β*-carotene and other carotenoids (Siddiqui et al., 2014). Lycopene occurs in various geometrical configurations, being mainly all-*trans* in tomato fruits. Thermal processing causes some loss of lycopene in tomato and tomato-based foods. Therefore, dehydrated tomatoes have poor lycopene stability (Gomez-Romero et al., 2007).

An isomer of lycopene, 5-*Z*-lycopene, was detected in all the tested samples, with the highest level in S606 and S615. The other dominant carotenoid detected was *β*-carotene, which is nutritionally important because of its provitamin A activity (Fernandez). In this study, concentrations of *β*-carotene in tomato were in the range of 4.57-189.64 mg/100g dm. S606 and S612 contained the highest amount of this compound (189.64; 88.17 mg/100g dm), which was much lower in the other samples. S612 had the highest content of the *β*-carotene isomers 15-*Z*-*β*-carotene and 13-*Z*-*β*-carotene. Interestingly, *α*-carotene was only detected in the "roller" tomato variety S606. Criptoxanthin was found only in S590, S606 and S615, with the highest content in S606 (13.92 mg/100g dm).
Lutein was found in all varieties, with the highest level detected in S606 (4.58 mg/100g dm) and similar amounts in the rest. As can be observed in Table 2, S606 had the highest level of total carotenoids, which is in accordance with its morphological and sensorial characteristics. This variety has an intense orange color with an unusual elongated shape. Therefore, it could be very attractive for both growers selecting high nutrient cultivars, and for consumers interested in a healthy diet. In general, the carotenoid content in Serbian varieties was found to be higher than those previously reported for tomato samples from other origins, for example, Taiwan (lycopene: 1.2-8.9 mg/100 g fw) (Chang et al., 2006), Italy, St. Marzano, (lycopene: 1.86-14.62 mg/100 g fw; lutein: 0.08-0.34 mg/100 g fw; *β*-carotene: 0.11-1.07 mg/100 g fw), Northern India (lycopene: 4.98-30 mg/100 g; *β*-carotene: 1.80-12.01 µg/g)(Kaur et al., 2013), Tunisia (lycopene: 3.90-19.40 µg/g) (Ilahy et al., 2011) and India (West Bengal) (lycopene: 8-23 mg/100g fw; *β*-carotene: 0.8- 3.10 mg/100g) (Siddiqui et al., 2014). Some of the values were very similar to those obtained from Spanish varieties (lycopene: 94-141 mg/g fw; lutein: 76 mg/g fw) (García-Valverde et al., 2013).

Quantification of selected phenols in tomato samples

Dietary phenols are potentially beneficial for health because they may protect the body against major degenerative diseases, aging and some cancers. Phenolic content in fruits and vegetables depends primarily on genetic control, as well as environmental factors (Barros et al., 2012). All the samples tested in the present study were found to be a good source of flavonoids. Predominant phenolic compounds were selected to investigate the differences between the eight varieties of tomato, as shown in Table 3.

Table 3. Quantification of individual phenolic compounds in eight Serbian tomato genotypes

Compound	§Content of selected phenolics (µg/100g dm)							
	S 364	S 616	S 590	S 606	S 615	S 608	S 612	S 607
Caffeic acid	482.90±0.1^{b}	315.36±0.36^{e}	339.07±0.59^{c}	329.74±0.25^{d}	688.54±6.31^{a}	57.63±0.33^{h}	166.09±0.61^{g}	215.07±0.80^{f}
Caffeic acid hexoside I	15.18±0.36^{f}	32.05±0.09^{d}	69.41±.0.10^{b}	30.99±0.06^{e}	71.67±0.012^{a}	n.d.*	6.78±0.12^{g}	40.68±0.01^{c}
Chlorogenic acid	114.44±0.43^{c}	94.79±0.01^{f}	562.06±1.13^{a}	97.47±0.230.11^{e}	140.59±0.62^{b}	57.90±0.12^{g}	48.81±0.03^{h}	110.80±0.05^{d}
Coumaric acid hexoside I	34.24±0.22^{b}	24.46±0.29^{c}	34.44±0.03^{b}	20.44±0.30^{d}	61.44±0.31^{a}	n.d.	13.03±0.09^{e}	5.37±0.24^{f}
Coumaric acid hexoside II	96.62±0.39^{b}	70.55±0.26^{c}	96.75±0.15^{b}	69.41±0.35^{d}	103.99±0.58^{a}	22.18±0.50^{g}	55.28±0.50^{e}	52.93±0.81^{f}
Criptochlorogenic acid	59.46±0.37^{d}	142.58±0.35^{a}	56.48±0.26^{e}	115.48±0.16^{c}	119.55±0.23^{b}	10.22±0.14^{g}	45.44±0.10^{f}	46.13±0.30^{f}
Dicaffeoylquinic acid	25.64±0.40^{d}	16.66±0.22^{f}	36.60±0.31^{b}	21.95±0.08^{e}	62.30±0.32^{a}	5.52±0.24^{h}	10.83±0.15^{g}	26.69±0.22^{c}
Ferulic acid	292.26±0.37^{c}	269.18±0.50^{f}	391.21±0.16^{a}	276.93±0.36^{e}	326.32±0.62^{b}	54.38±0.10^{h}	287.74±1.53^{d}	129.07±0.25^{g}
Ferulic acid hexoside	226.32±0.35^{f}	592.67±0.27^{a}	565.22±2.04^{b}	498.23±0.89^{c}	253.28±0.38^{e}	282.58±0.73^{d}	155.86±0.20^{h}	223.66±20^{g}
Naringenin	10.19±0.18^{f}	49.85±0.26^{e}	8.95±0.13^{g}	60.46±0.17^{d}	153.54±0.23^{c}	182.17±0.31^{a}	7.49±0.29^{h}	154.49±0.27^{b}
Naringenin glucoside	5.83±0.07^{h}	47.95±0.10^{e}	6.82±0.21^{f}	54.19±0.43^{d}	116.89±0.11^{c}	284.23±0.17^{a}	2.70±0.28^{g}	134.91±0.10^{b}
Neochlorogenic acid	5.84±0.9^{c}	n.d.	5.92±0.07^{c}	3.49±0.06^{d}	6.41±0.20^{b}	1.42±0.07^{e}	n.d	7.26±0.17^{a}
p-Coumaric acid	646.50±2.05^{a}	187.87±0.14^{d}	74.04±1.15^{f}	188.01±0.08^{d}	464.25±0.16^{b}	73.70±0.39^{f}	255.21±0.15^{c}	115.38±0.23^{e}
Protocatechuic acid	16.60±0.32^{a}	11.16±0.08^{d}	15.27±0.06^{b}	10.76±0.12^{e}	7.54±0.33^{f}	2.38±0.22^{h}	13.08±0.09^{c}	3.70±0.21^{g}
Quercetin	29.41±0.21^{d}	22.30±0.15^{f}	31.16±0.19^{c}	24.80±0.10	30.96±0.02^{c}	37.40±0.07^{b}	65.06±0.10^{a}	28.39±0.40^{e}
Rutin	619.36±0.4^{f}	751.46±0.24^{d}	359.98±0.58^{g}	861.34±0.34^{b}	1424.30±0.72^{a}	766.28±0.13^{c}	183.44±0.10^{h}	718.13±0.20^{e}

*n.d.-not detected; § Data are means ± SD of three measurements (n=3). Values with different letters (a,b,c,d,e, f, g, h) in the same row are significantly different (p<0.05).

The differences in content of hydroxycinnamoylquinic acid derivatives and flavonoids and their derivatives were quite pronounced among the eight varieties. Rutin was the most abundant polyphenol in almost all samples, ranging from 359.98 to 1424.30 µg/100g dm, with the highest level detected in S615. All the varieties are a good source of phenolic acids (µg/100g dm) and the major acids determined were caffeic acid, ranging from 57.63 to 688.54, followed by chlorogenic acid (48.81-562.06), *p*-coumaric acid (73.70-646.50), ferulic acid (54.38-391.21) and its glycoside ferulic acid hexoside (155.86-592.67). The other phenolic acid derivatives were detected in similar amounts to phenolic acids in all the samples. Protocatechuic acid was found in lower levels and was similar in all the varieties. The samples with the highest levels of individual phenolic acids were S615, S590 and S364.

The highest content of the flavanone naringenin (µg/100 g dm) was found in S608 (182.17 µg/100 g dm) followed by S615 (153.54 µg/100 g dm) and S607 (154.49 µg/100 g dm). Similar levels of naringenin glucoside were also detected. The content of the flavonol quercetin was markedly higher in S612 (65.06 µg/100g dm) in comparison with the other samples, such as those reported by Kaur et al. (2013).

It is difficult to compare the results of polyphenol quantification in this study with those reported in the literature, considering that many factors can affect the phenolic content of tomato, including genetic variability, country of origin, environmental conditions and storage methods. Most studies indicate that phenolic acids (caffeic acid, ferulic acid,

coumaric acid) and their derivatives, and the flavonoid rutin are the most abundant polyphenols in tomato. The content of phenolic acids (ferulic acid <1 µg/1g fw) and flavonoids determined here was lower than those reported in other studies (Barros et al., 2012; García-Valverde et al., 2013). Also, some authors have reported higher levels of polyphenols compared with this study (Gomez-Romero et al., 2010; Li et al., 2012; Vallverdú-Queralt et al., 2011).

Antioxidative activity

The evaluation of antioxidant activity is of great importance in the field of agricultural and food science and technology. Antioxidant activity of tomato fruits has been reported to be mostly caused by the content of polyphenols, carotenoids and vitamin C (Pinela et al., 2012).

Table 4. Total phenolics and vitamin C content and antioxidant capacities of eight Serbian tomato varieties

		S364	S616	S590	S606	S615	S608	S612	S607
Content	Total phenolics (mg GAEg^{-1} of dm)	7.18±1.56^{c}	10.02±0.97^{c}	6.30±0.48^{c}	14.73±1.53^{b}	36.19±5.35^{a}	11.80±1.30^{c}	4.91±2.36^{c}	9.80±0.84^{c}
	Vitamin C (mg AA g^{-1} of dm)	14.45±0.37^{e}	21.81±0.58^{d}	31.85±0.79^{c}	68.54±2.18^{a}	43.40±0.36^{b}	29.44±0.38^{c}	1.52±0.71^{f}	20.52±0.42^{d}
Antioxidant activity	DPPH• (mg TEAC g^{-1} of dm)	9.00±0.01^{c}	5.78±0.22^{e}	2.66±0.18^{f}	48.57±0.50^{a}	15.82±0.29^{b}	6.08±0.073^{e}	7.55±0.12^{d}	5.17±0.04^{e}
	ABTS• (mg TEAC g^{-1} of dm)	15.13±1.36^{c}	8.50±0.92^{f}	5.97±2.11^{h}	126.52±0.47^{a}	23.41±1.88^{b}	11.02±2.01^{e}	14.11±0.75^{d}	6.55±0.21^{g}
	Reducing power (mg EAA g^{-1} of dm)	17.82±0.11^{f}	20.39±1.07^{c}	17.16±0.53^{f}	84.37±0.21^{a}	23.87±0.11^{b}	22.91±0.03^{b}	10.42±0.04^{g}	19.10±0.02^{e}

Data are means ±of three measurements. In each, different letters mean significant differences (p<0.05); dm-dry material; GAE-gallic acid equivalent; EAA-equivalents ascorbic acid

Thus, besides the assessment of the polyphenol and carotenoid profiles of the Serbian tomato varieties, the aim of the present study was to determine the antioxidant capacity and quantify total phenolics and vitamin C. Differences observed in the phytochemical content and antioxidant activity can be primarily related to the genotype.

A high total phenolic content was observed in all samples, but the highest level was found in S615 (36.19 mg GAE/g dm). The values found in the present study are higher than those previously reported by Chang and Liu, 2007 (0.34- 0.39 mg GAE/g dm), Dvila-Avia et al., 2012 (0.17-0.20 mg GAE/g dm); García-Valverde et al., 2013 (1.86-5.58 GEA/kg dm), and Ilahy et al., 2011 (1.29-4.01 mg GAE/g dm), but some values are very similar to those obtained by Li et al., 2012 (4.89-9.97 mg GAE/g dm). These

results were in accordance with the phenolic contents quantified by the UPLC-MS/MS technique, which were highest in S606, S615 and S608.

As a strong antioxidant, ascorbic acid is one of the most important bioactive molecules in tomato fruits, playing an important role in disease prevention (Kaur et al., 2013). The content of ascorbic acid in the samples (1.52-68.54 mg AA/g dm) was similar to previously reported results (2.20-85.00 mg AA/100g dm) (García-Valverde et al., 2013; Kotíková et al., 2011; Pinela et al., 2012; Toor and Savage, 2005). Only in one study which reported on varieties from Italy (Lenucci et al., 2006) some values can be compared with ours. All the samples tested in the present study are a rich source of vitamin C, especially S606, which may be useful data for the promotion of these agricultural products in terms of nutrition.

In accordance with the data presented above, the tomato varieties showed a high antioxidant potential measured by radical scavenging capacity against DPPH and ABTS radicals as well as reducing power (FRAP). The samples with the highest antioxidant capacity were S606 and S615, which could be linked with the higher content of carotenoids, flavonoids and vitamin C found in these varieties. These bioactive compounds, which may act independently or synergistically, are responsible for the health benefits of tomato fruits.

Conclusion

Antioxidant content and activity varied significantly among samples. The varieties S606, S615 and S608 are of particular interest for tomato breeding, as their genotypes could be a source of increased polyphenol, carotenoid, and vitamin C content. Although with completely different morphological and organoleptic characteristics, these three varieties showed similarities in the levels of bioactive molecules and therefore antioxidant potential. Interesting data were obtained for S364, since only two of twelve carotenoids were quantified in this sample, yet it showed a good antioxidant capacity, due to a high level of polyphenols and vitamin C.

Overall, the results obtained in the present study can serve as the basis for increasing the breeding, cultivation, and marketing of nutritionally superior varieties of tomato as a healthy alternative for consumers worldwide, not only in Serbia.

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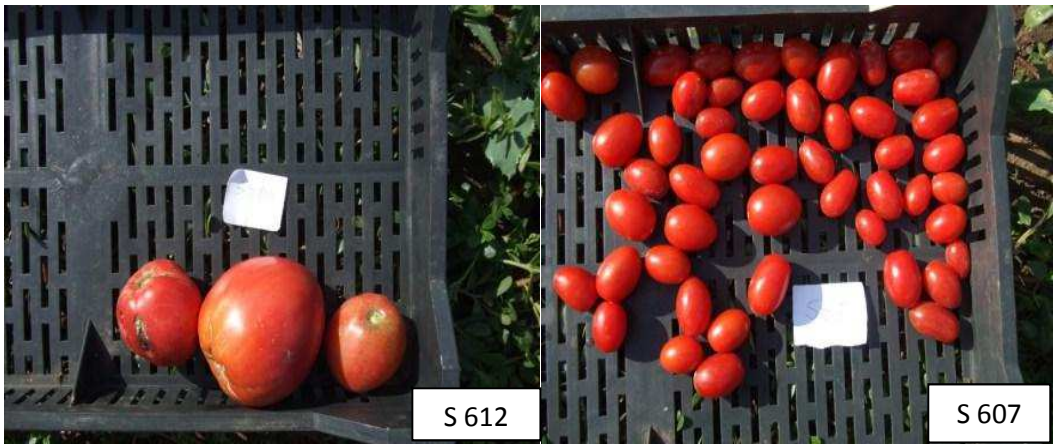
Figure 1. Morphological characteristics of eight Serbian tomato genotypes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B. Comunicaciones en congresos

Comunicación 1. Póster.

Título: "*trans*-Lycopene from tomato juice attenuates immune-inflammatory biomarkers: a dose-response intervention trial"

Autores: **Mariel Colmán-Martínez**; Miriam Martínez-Huélamo; Palmira Valderas-Martínez; Sara Arranz-Martínez; Enrique Almanza-Aguilera; Dolores Corella-Piquer; Ramón Estruch; Rosa M. Lamuela-Raventós.

Congreso: II Workshop anual INSA-UB "Cacao y chocolate: Ciencia

trans - Lycopene from tomato juice attenuates immune-inflammatory biomarkers: a dose-response intervention trial

Mariel Colmán-Martínez[1]*, Miriam Martínez-Huélamo[1,2], Palmira Valderas-Martínez[2,3], Sara Arranz-Martínez[2,3], Enrique Almanza-Aguilera[1], Dolores Corella[2,4], Ramón Estruch[2,3], and Rosa M. Lamuela-Raventós[1,2]

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BACKGROUND AND OBJECTIVES:

TO EVALUATE THE EFFECTS OF CAROTENOIDS FROM TOMATO JUICE (TJ) ON THE EXPRESSION OF INFLAMMATORY BIOMARKERS, BY PERFORMING A 4-WEEK DOSE-RESPONSE NUTRITIONAL TRIAL IN A POPULATION AT HIGH CARDIOVASCULAR RISK.

METHODOLOGY:

The study was an open, prospective, randomized, cross-over, and controlled clinical trial. Twenty-eight volunteers (mean age 69.7 ± 3.1 years; mean BMI 31.5 ± 3.6 kg/m2) at high cardiovascular risk were assigned to consume daily for 4 weeks in random order: 200 mL (low-dose, LD) or 400 mL (high-dose, HD) of TJ made with 5% common olive oil, or water as a control (C). Blood samples were collected at baseline (B) and after each intervention. Endpoints included changes in plasmatic carotenoids, pro-inflammatory cytokines C-reactive protein (CRP) and interferon-γ (IFN-γ), chemokines interleukin-8 (IL-8), eotaxin, and CXCL motif chemokine 10 (CXCL10), and inter-cellular adhesion molecule 1 (ICAM-1), and vascular-cell adhesion molecule 1 (VCAM-1).

RESULTS AND CONCLUSIONS:

Compared to control, both TJ interventions induced significant decreases in ICAM-1, VCAM-1, CRP, and IL-8 ($p < 0.05$), and also showed a trend to reduce eotaxin, IFN-γ and CXCL10, in a dose dependent manner. These decreases were significantly correlated mainly with the *trans* isomeric form of lycopene, while the other carotenoids present in TJ were not associated with any significant changes in these molecules.

INTERVENTION

Table 1. Immune and inflammatory biomarkers measured at baseline and after interventions (n=28)

Plasmatic biomarker	*Baseline*	*Control (Z0)*	*Low-dose (LD)*	*High-dose (HD)*	*p[d]*
ICAM-1 (ng/mL)	3693±1377[a]	3609±1107[a]	318±116[a,b]	159±57[a,b]	<0.001
VCAM-1 (ng/mL)	3993±890[a]	3939±801[b]	400±101[a,b]	218±39[a,b]	<0.001
CRP (ng/mL)	1521±236[a,b,c]	539±200[a]	446±254[a]	532±158[c]	<0.001
IL-8 (pg/mL)	22±9[a]	40±17[a,b,c]	23±16[b]	24±15[c]	0.015
Eotaxin (pg/mL)	135±67	172±114	137±75	181±112	0.172
IFN-γ (pg/mL)	304±80	489±220	399±131	400±144	0.074
CXCL10 (pg/mL)	2908±1598	3397±1489	3366±1671	3606±2185	0.519

Values are expressed as mean ± SD. Values with the same letters are significantly different ($p < 0.05$).
[d]*p* value of the ANOVA for repeated measures from the differences between interventions.

Table 2. Correlations between *cis* and *trans*-lycopene and inflammatory molecules after and before adjusting by *cis* and *trans*-lycopene isomers

Adjusting for	*Biomarker*		*Pearson's coefficient test*	*p value*
	CRP	*trans*-Lycopene	-0.227	0.042
		Total *cis*-Lycopenes	-0.157	0.154
	VCAM-1	*trans*-Lycopene	-0.697	<0.001
		Total *cis*-Lycopenes	-0.628	<0.001
	ICAM-1	*trans*-Lycopene	-0.625	<0.001
		Total *cis*-Lycopenes	-0.551	<0.001
Total *cis*-Lycopenes	CRP	*trans*-Lycopene	-0.414	<0.001
	VCAM-1	*trans*-Lycopene	-0.501	<0.001
	ICAM-1	*trans*-Lycopene	-0.453	<0.001
trans-Lycopene	CRP	Total *cis*-Lycopenes	0.374	0.002
	VCAM-1	Total *cis*-Lycopenes	0.356	0.003
	ICAM-1	Total *cis*-Lycopenes	0.309	0.010

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Comunicación 2. Póster.

Título: “Development of an advance HPLC-MS/MS method for the determination of carotenoids and fat-soluble vitamins in human plasma”

Autores: Miriam Martínez-Huélamo; Barbora Hrvolová; **Mariel Colmán-Martínez**; Sara Hurtado-Barroso; Jiri Kalina; Rosa M. Lamuela-Raventós.

Congreso: II Workshop anual INSA-UB “Cacao y chocolate: Ciencia y Gastronomía”, Sta. Coloma de Gramenet, España, 2016

Development of an advanced HPLC-MS/MS method for the determination of carotenoids and fat-soluble vitamins in human plasma

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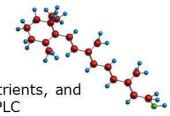
BACKGROUND AND OBJECTIVES

Carotenoids, xanthophylls and carotenes, are natural fat-soluble, red, yellow and orange pigments characterized by a wide distribution, structural diversity and numerous physio-chemical and biological properties. Another group of interesting and useful compounds are fat-soluble vitamins and their metabolites such as retinol, retinol acetate, cholecalciferol, and α-tocotrienol. These compounds have free radical scavenging properties that allow them to function as antioxidants.



Available methods can determine only a few representatives of the aforementioned fat-soluble micronutrients, and few of them can be applied for the simultaneous analysis of compounds in biological samples. Most use HPLC separation coupled to UV–VIS or DAD detection, but with these methods it is extremely challenging to obtain the sensitivity required for the analysis of human fluids, in which the concentration of fat-soluble micronutrients is very low. The problem of sensitivity can be solved by usage of tandem mass spectrometry detection, although finding general ionization conditions suitable for all targeted analytes is very difficult.

The **aim** of this research was to develop and validate a new HPLC-MS/MS method for the quantification of selected carotenoids and fat-soluble vitamins in human plasma.

METHODOLOGY

EXTRACTION OF CAROTENOIDS AND VITAMINS

Extraction
- 200 µL plasma + 200 µL EtOH + 0.5 mL Hexane (BHT)
- Vortex 1 min

Centrifugation
- 2070 x g
- 5 min
- 4°C

Evaporation
- Under N_2 flow

Reconstitution
- 100 µL MeOH

Analysis
- Column: YMC Carotenoid (C30)
- Injection volume: 20 µL
- Flow: 0,6 mL/min

APPLICATION METHOD

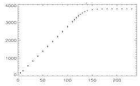
8 Volunteers (18-32 years)

2 weeks with High Antioxidant Diet

Plasma in the morning at 08:00 after fasting

VALIDATION METHOD PARAMETERS

Accuracy
- Intraday
- Interday



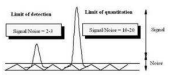
Linearity

Recovery

Precision
- Intraday
- Interday

Matrix Effect

Sensitivity
- LOD
- LOQ



RESULTS

Table 1. Limit of detection (LOD), limit of quantification (LOQ), recovery, matrix effect, linearity range, interday and intraday precision, interday and intraday accuracy and concentration obtained in the study with high antioxidant diet by the HPLC-MS/MS method.

Analyte	Transition MRM	LOD (nM)	LOQ (nM)	Recovery (%)	Matrix effect (%)	Linearity Range (µg/mL)	Interday Precision (% RSD)	Intraday Precision (% RSD)	Interday Accuracy (%)	Intraday Accuracy (%)	Concentration plasma (nM)
retinol	269 › 181	7.0	17.5	103 ± 9	109 ± 9	17.5-34909	6.7	11.5	101 ± 4	105 ± 4	115 ± 11
25-hydroxycholecalciferol	383 › 365	7.5	27.5	92 ± 3	87 ± 1	27.5-12480	3.3	11.5	102 ± 3	105 ± 6	190 ± 33
retinol acetate	329 › 269	6.1	24.4	103 ± 2	95 ± 2	24.4-30442	1.3	11.1	105 ± 1	106 ± 7	<LOQ [a]
α-tocotrienol	411 › 165	266.1	885.4	99 ± 5	90 ± 3	885.4-11774	10.4	9.5	104 ± 4	108 ± 3	n.d. [b]
cholecalciferol	385 › 367	13.0	46.8	103 ± 3	90 ± 1	46.8-25998	2.0	12.7	103 ± 1	104 ± 7	n.d. [b]
astaxanthin	597 › 147	1.7	5.0	102 ± 3	100 ± 3	5.0-1675	8.8	10.3	103 ± 3	105 ± 6	<LOQ [a]
lutein	551 › 429	14.1	49.2	86 ± 1	91 ± 2	49.2-17579	4.1	12.1	106 ± 2	109 ± 6	260 ± 139
zeaxanthin	568 › 476	741.8	2471.5	86 ± 2	87 ± 3	2471.5-17578	5.5	10.8	107 ± 5	113 ± 2	n.d. [b]
cantaxanthin	565 › 363	3.5	10.6	100 ± 3	104 ± 8	10.6-1770	6.2	11.5	104 ± 6	109 ± 11	28 ± 12
E-β-apo-8′-carotenal	417 › 325	7.2	24.0	104 ± 2	100 ± 1	24.0-24002	13.1	11.5	107 ± 8	114 ± 2	<LOQ [a]
cryptoxanthin	553 › 535	441.3	1468.8	105 ± 3	94 ± 2	1468.8-18088	4.4	14.0	101 ± 1	103 ± 8	<LOQ [a]
13-*Z*-β-carotene	536 › 444	104.3	348.3	101 ± 2	88 ± 1	348.3-18626	9.1	13.6	101 ± 9	109 ± 2	n.d. [b]
α-carotene	536 › 444	41.0	136.0	104 ± 5	103 ± 4	136.0-9313	5.9	12.1	107 ± 4	112 ± 1	101 ± 19
β-carotene	537 › 413	76.4	257.0	101 ± 2	96 ± 5	257.0-9313	3.4	12.2	100 ± 3	103 ± 4	2634 ± 1870
9-*Z*-β-carotene	537 › 413	545.8	1816.1	97 ± 7	92 ± 2	1816.1-18626	4.9	14.0	101 ± 5	107 ± 4	n.d. [b]
5-*Z*-lycopene	537 › 413	352.0	1175.3	105 ± 8	97 ± 3	1175.3-18626	5.2	13.4	102 ± 3	107 ± 9	n.d. [b]

[a]: under limit of quantification; n.d. b: not detected

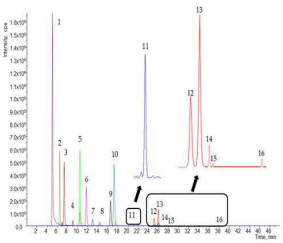


Figure 1. Chromatogram of working standard solutions obtained by HPLC-MS/MS analysis. Peaks: (1) retinol; (2) 25-hydroxycholecalciferol; (3) retinol acetate; (4) α-tocotrienol; (5) cholecalciferol; (6) astaxanthin; (7) lutein; (8) zeaxanthin; (9) cantaxanthin; (10) E-β-apo-8′-carotenal; (11) cryptoxanthin; (12) 13-Z-β-carotene; (13) α-carotene; (14) β-carotene; (15) 9-Z-β-carotene; and (16) 5-Z-lycopene.

CONCLUSIONS

A unique HPLC-MS/MS method for the simultaneous quantification of 16 carotenoids and fat-soluble vitamins in human plasma was designed and fully validated. Good quality values of LOD, LOQ, recovery, linearity, matrix effect, accuracy, and precision were obtained by the proposed method. According to our knowledge, no similar HPLC-MS/MS method for the determination of such a large number of analytes has been previously published. In the future, considering the excellent validation results obtained, this method could be used in various applied clinical studies or investigations.

ACKNOWLEDGMENTS

This work was supported by CICYT (AGL2013-49083-C3-1-R and AGL2016-70113-R), the Instituto de Salud Carlos III, ISCIII (CIBEROBN) from the Spanish Ministry of Economy and Competivity (MEC), Generalitat de Catalunya (GC) 2014 SGR 773, and by the Project LO1208 (TEWEP) of the National Feasibility Programme I of the Czech Republic. Hrvolová, B. also thanks the student grant n. SGS04/PřF/2016 from University of Ostrava, Czech Republic.

Comunicación 3. Póster.

Título: “Biodisponibilidad de los carotenoides del sofrito en varones tras una dieta rica en alimentos con componentes antioxidantes”

Autores: Sara Hurtado-Barroso; Miriam Martínez-Huélamo; **Mariel Colmán-Martínez**; José Fernando Rinaldi-Alvarenga; Rosa M. Lamuela-Raventós.

Congreso: VIII Seminario sobre Alimentacióny Estilos de Vida Saludables, Palma de Mallorca, España, 2016

Biodisponibilidad de los carotenoides del sofrito en varones tras una dieta rica en alimentos con componentes antioxidantes

Sara Hurtado-Barroso[1,2], Miriam Martínez-Huélamo[1,2], Mariel Colmán-Martínez[1], Jose Fernando Rinaldi Alvarenga[1], Rosa María Lamuela-Raventós[1,2]

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[2]CIBER Fisiopatología de la Obesidad y Nutrición (CIBERobn), Instituto de Salud Carlos III, Madrid, España

INTRODUCCIÓN

El sofrito es una salsa típica de la dieta mediterránea que contiene tomate como componente principal y otros ingredientes de origen vegetal (aceite de oliva, cebolla y ajo) ricos en componentes bioactivos. Entre ellos cabe destacar los carotenoides, que están presentes principalmente en el tomate. Sin embargo, la biodisponibilidad de los mismos es baja y varios factores como el procesamiento y la matriz del alimento pueden interferir positiva y/o negativamente en su absorción.

OBJETIVO

Evaluar la biodisponibilidad de los carotenoides del sofrito tras una dieta rica en antioxidantes

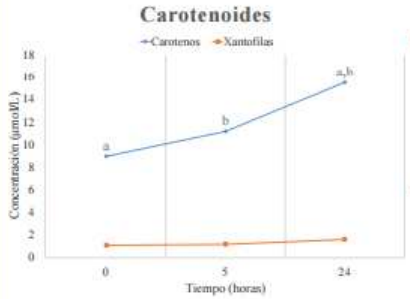
DISEÑO DEL ESTUDIO

Estudio piloto
22 varones
18-32 años
No fumadores
Sanos

ANÁLISIS CROMATOGRÁFICO

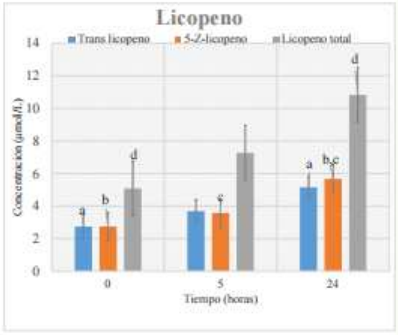
HPLC-UV
HP 1100HPLC

RESULTADOS



- El licopeno es el carotenoide mayoritario en el plasma antes y después de la ingesta de sofrito, alcanzando su máximo a las 5 horas después de la ingesta.
- A las 5 horas, el licopeno representa el 75% de los carotenoides presentes en el plasma.

- En todos los casos, el licopeno aumenta de las 0 a las 24 horas. Además el aumento de 5 a 24 horas del isómero *cis* también es significativo.
- El 5-*cis*-licopeno se encuentra en la misma proporción que el *trans*-licopeno a las 0 y 5 horas, pero a las 24 horas el isómero *cis* aumenta con respecto al *trans* un 4,7%, aunque sin diferencias significativas.



(a-d): diferencias significativas con un p-valor <0,05

CONCLUSIONES

El contenido de carotenoides aumenta significativamente, tras la ingesta de sofrito, debido en gran parte al aumento de licopeno a las 24 horas de su administración.

FINANCIACIÓN

Este estudio ha sido financiado por el Ministerio de Economía y Competitividad (AGL2013-49083-C3-1-R) y por el Centro de Investigación Biomédica en Red-Fisiopatología de la Obesidad y la Nutrición (CIBEROBN).

Comunicación 4. Póster.

Título: “Polifenoles y carotenoides del zumo de tomate en plasma y orina, tras 4 semanas de intervención”

Autores: Miriam Martínez-Huélamo; **Mariel Colmán-Martínez**; Palmira Valderas-Martínez; Sara Arránz-Martínez; Dolores Corella; Ramón Estruch; Rosa M. Lamuela-Raventós.

Congreso: XI Congreso de Dieta Mediterránea, Barcelona, España, 2016

POLIFENOLES Y CAROTENOIDES DEL ZUMO DE TOMATE, EN PLASMA Y ORINA, TRAS 4 SEMANAS DE INTERVENCIÓN

Miriam Martínez-Huélamo[1,2], Mariel Colmán-Martínez[1], Palmira Valderas-Martínez[2,3], Sara Arranz-Martínez[2,3], Dolores Corella[2,4], Ramón Estruch[2,3], Rosa María Lamuela-Raventós[1,2]

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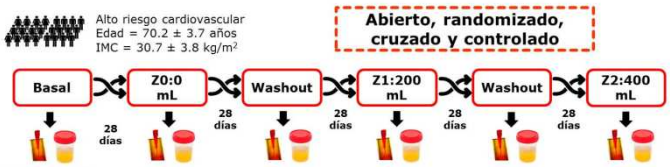
[3]Departmento de Medicina Interna, Hospital Clinic, Instituto de Investigaciones Biomédicas August Pi i Sunyer (IDIBAPS), Universidad de Barcelona, España.
[4]Departmento de Medicina Preventiva y Salud Pública, Universidad de Valencia, España.

OBJETIVO

El objetivo de este trabajo fue estudiar la correlación entre el consumo durante 4 semanas de diferentes dosis de zumo de tomate con 5% de aceite de oliva refinado y las concentraciones de compuestos fenólicos y carotenoides en orina y plasma de 34 participantes sanos, pero con alto riesgo cardiovascular.

DISEÑO DEL ESTUDIO



PREPARACIÓN DE MUESTRA

RESULTADOS

POLIFENOLES

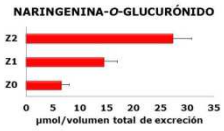


Figura 1. Excreción urinaria de la naringenina-*O*-glucurónido tras las intervenciones.

Tabla 1. Concentración de polifenoles en plasma tras el estudio crónico.

PLASMA

COMPUESTOS FENÓLICOS	Z0 (nmol/mL)	Z1 (nmol/mL)	Z2 (nmol/mL)
Ác. 3,3-Hidroxifenilpropiónico	0.63 ± 0.62	1.21 ± 1.14	0.46 ± 0.38
Ác. 4-Hidroxibenzoico	0.13 ± 0.11	0.16 ± 0.16	0.15 ± 0.13
Ác. Cafeico	0.77 ± 0.50	0.81 ± 0.54	0.70 ± 0.44
Ác. Hipúrico	40.91 ± 31.78	37.64 ± 25.48	35.32 ± 25.65
Ác. Hidroferúlico	0.19 ± 0.20	0.17 ± 0.15	0.14 ± 0.13
Ác. Hidroxihipúrico	0.88 ± 0.66	1.17 ± 0.93	0.82 ± 0.44
Ác. p-Cumárico	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.01
Ác. Fenilacético	3.27 ± 3.06	4.22 ± 4.11	2.93 ± 3.00
Ác. Protocateico	0.02 ± 0.04	0.01 ± 0.03	0.01 ± 0.02

CAROTENOIDES

PLASMA

Tabla 2. Carotenoides en plasma tras el estudio crónico.

CAROTENOIDES	Z0 (µg/mL)	Z1 (µg/mL)	Z2 (µg/mL)
Retinol	1.857±0.355	1.613±1.279	2.062±0.897
Astaxantina	0.800±0.879	0.985±0.941	1.024±1.027
Luteína	0.033±0.046	0.047±0.071	0.046±0.070
apo-β-8-Carotenal	0.575±0.028	0.411±0.283	0.602±0.039
Criptoxantina	0.084±0.112	0.149±0.175	0.188±0.202
15*Z*-β-Caroteno	0.000±0.000	0.001±0.005	0.002±0.005
13*Z*-β-Caroteno	0.043±0.066	0.083±0.069	0.151±0.023
β-Caroteno	0.651±0.317	0.981±0.826	1.436±0.760
Licopeno	1.311±1.093	6.690±6.214	9.972±4.423

Figura 2. Suma de carotenoides en plasma tras las intervenciones a diferentes dosis de zumo de tomate con aceite de oliva refinado.

CONCLUSIONES

Existe un claro efecto dosis/respuesta de los carotenoides en plasma tras la ingesta crónica de zumo de tomate. Sin embargo, estos efectos no son tan evidentes en los compuestos fenólicos, no observándose variaciones en plasma y en orina, a excepción de la naringenina-*O*-glucurónido, pero sin diferencias significativas.

AGRADECIMIENTOS

Los autores desean expresar su agradecimiento por el apoyo financiero a CICYT (AGL2013-49083-C3-1-R) y el Instituto de Salud Carlos III, ISCIII (CIBERobn) del Ministerio Español de Economía y Competitividad (MEC) y la Generalitat de Catalunya (GC) 2014 SGR 773.

Comunicación 5. Póster.

Título: “Effect of the irrigation, the cultivation method and the olive variety on the phenolic content of olive oils”

Autores: **Mariel Colmán-Martínez**; Paola Quifer-Rada; Ruth Martínez-Las Heras; Raquel Callejón-Fernández; Brígida Jiménez; Rosa M. Lamuela-Raventós.

Congreso: 7th International Conference on Polyphenols and Health (ICPH), Tours, Francia, 2015

Effect of the irrigation, the cultivation method and the olive variety on the phenolic content of Olive Oils

M. Colmán-Martínez[1], P. Quifer-Rada[1,2], R. Martínez-Las Heras[3], R. Callejón-Fernández[4], B. Jiménez[5], R. Lamuela-Raventós[1,2]

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[3]Institute of Food Engineering for Development, Universitat Politècnica de València, Spain
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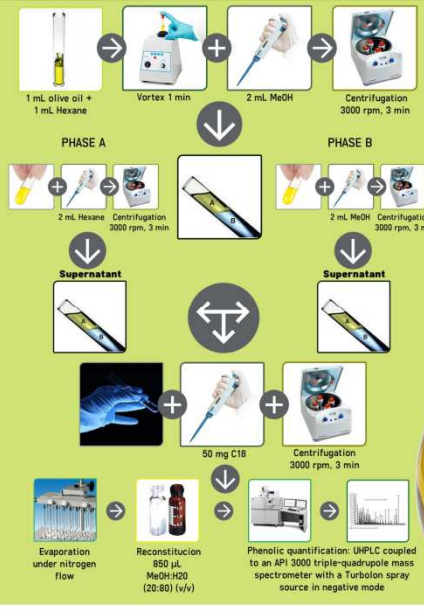
[5]Institute for Agricultural and Fisheries Research and Training. Ministry of Agriculture and Fisheries. Junta de Andalucía, Córdoba, Spain

Introduction

Olive oil is considered one of the healthiest dietary fats due to its fatty acids profile and its rich phenolic composition. Nevertheless, the nutritional quality of the olive oils is affected by agronomic and technological factors, the variety of olive, the geographical area of production, the harvest period and the extraction process, as well as the climatic conditions prevailing in the production year. Irrigation positively influences the composition and organoleptic characteristics of olive oil. The aim of this work was to study the phenolic profile of oils produced with two different types of olives harvested in Andalucía, Spain, *Hojiblanca* and *Picual*, and to determine whether there are differences in the phenolic content of these oils according to the type of irrigation received and the cultivation method (conventional or organic).

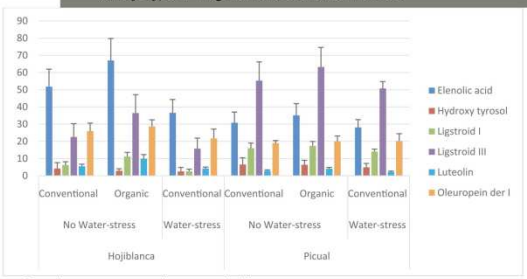
Sample Preparation



Results

Thirty-two phenolic compounds were identified in the two types of olive oils. The major compounds found in both types of oils were elenolic acid, ligstroid derivatives, luteolin, oleuropein derivatives and also tyrosol derivatives.

Figure 1. Main significantly different phenolic compounds identified in the two types of olive oils, classified by olive variety, type of irrigation and cultivation method.



Results are expressed as mean ± SD

Conclusion

Oils richest in phenolic compounds can be obtained by olives from organic farming. Organic cultivation increased mainly the concentration of elenolic acid in the *Hojiblanca* variety, whilst in the *Picual* variety the main compound found was a ligstroid derivative. Likewise, the type of irrigation influences the content of phenolic compounds in the olives, being the "no water-stress" technique which more increases the concentration of phenolic compounds in the olives and thereby, providing oils richest in phenolic compounds than those obtained by the "water stress" technique.

Financial Support

CICYT (AGL2013-49083-C3-1-R) from the Spanish Ministry of Economy and Competitiveness (MEC), Generalitat de Catalunya (GC)SGR 773 FI-DGR 2013/FI-DGR 2014, the Instituto de Salud Carlos III, ISCIII (CIBEROBN-CB06/03) from the Spanish Ministry of Science and Innovation (MICINN) and Manuel Heredia Halcón, owner of the organically grown farm *Suerte Alta*.

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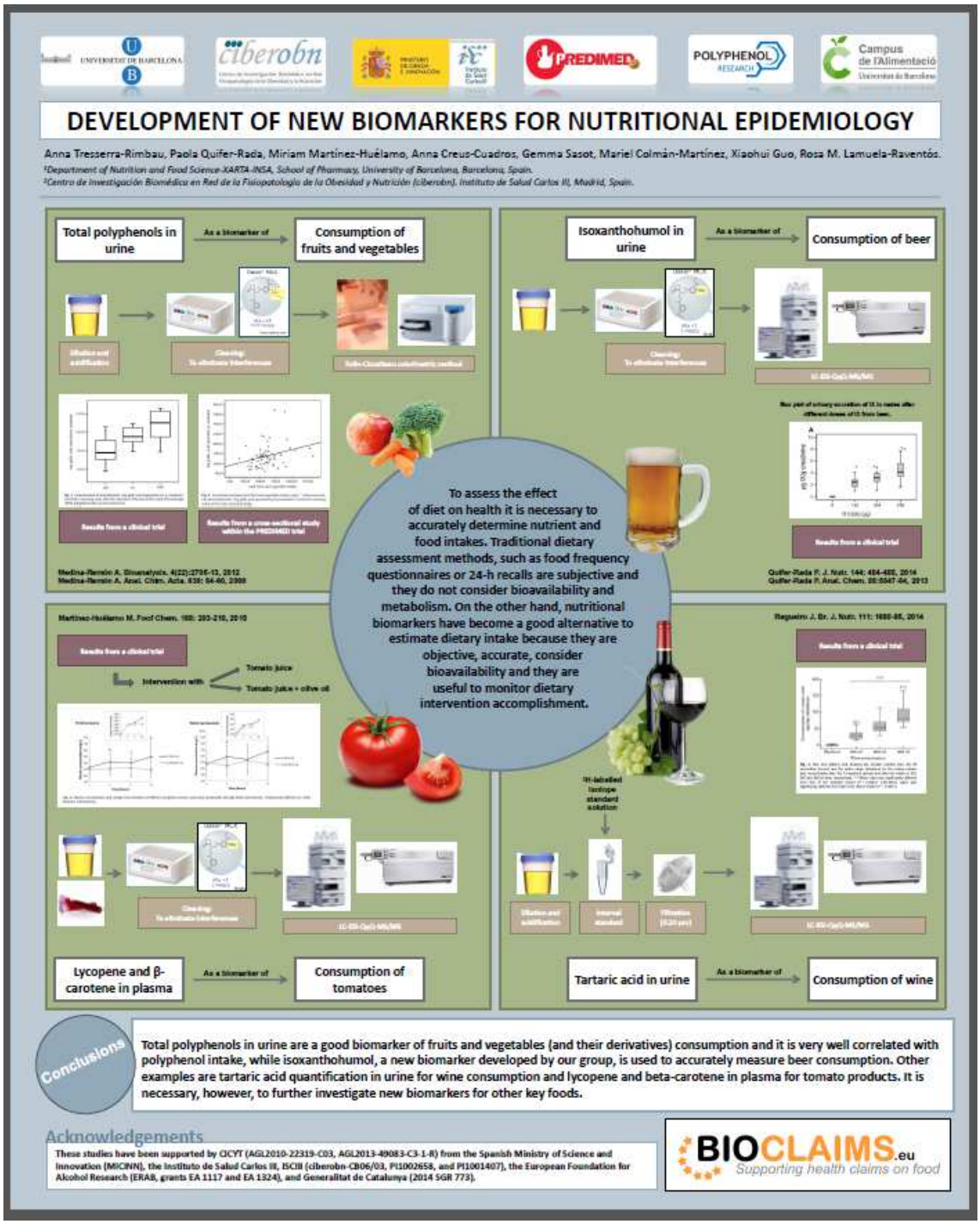
Comunicación 6. Póster.

Título: “Development of new biomarkers for nutritional epidemiology”

Autores: Anna Tresserra-Rimbau; Paola Quifer-Rada; Miriam Martínez-Huélamo; Anna Creus-Cuadros; Gemma Sasot-Flix; **Mariel Colmán-Martínez**; Xiaohui Guo; Rosa M. Lamuela-Raventós.

Congreso: Biomarkers and Health Claims on Food: BIOCLAIMS Meeting with stakeholders, Palma de Mallorca, España, 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
Isoxanthohumol in urine
As a biomarker of
Consumption of beer
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.
Intervention with
Tomato juice
Tomato juice + olive oil
Lycopene and β-carotene in plasma
As a biomarker of
Consumption of tomatoes
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).
BIOCLAIMS.eu
Supporting health claims on food

Comunicación 7. Póster.

Título: “A new method for simultaneous identification of carotenoids, xantophylls and fat soluble vitamins in human plasma samples”

Autores: **Mariel Colmán-Martínez**; Miriam Martínez-Huélamo; Rosa M. Lamuela-Raventós.

Congreso: 22nd Young Research Fellow Meeting, París, Francia, 2015

A NEW METHOD FOR SIMULTANEOUS IDENTIFICATION OF CAROTENOIDS, XANTHOPHYLLS AND FAT SOLUBLE VITAMINS IN HUMAN PLASMA SAMPLES

Mariel Colmán-Martinez[1,2], Miriam Martínez-Huélamo[1,2] and Rosa M. Lamuela-Raventós[1,2]*

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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.

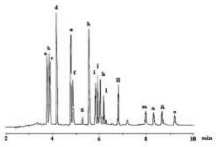
The aim of this word was validate a method for the identification of mainly carotenoids, xantophylls and fat soluble vitamins in human plasma, capable of be useful for dietary habit studies and for antioxidants status investigations.

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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(4) Mc. Call MR. *et al. Free Rad. Biol. Med.* 26 (1999) 1034–1053.

ACKNOWLEDGMENTS

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22nd Young Research Fellow Meeting 2015

Comunicación 8. Póster.

Título: “Screening the polyphenolic metabolic differences between wine and dealcoholized wine in plasma by high resolution mass spectrometry”
Autores: Anna Creus-Cuadros; Paola Quifer-Rada; Xiaohui Guo; **Mariel Colmán-Martínez**; Mercè Mercader-Martí; Rosa M. Lamuela-Raventós.
Congreso: 22nd Young Research Fellow Meeting, París, Francia, 2015

SCREENING THE POLYPHENOLIC METABOLIC DIFFERENCES BETWEEN WINE AND DEALCOHOLIZED WINE IN PLASMA BY HIGH RESOLUTION MASS SPECTROMETRY

Anna Creus-Cuadros[1], Paola Quifer-Rada[1,2], Guo Xiaohui[1], Mariel Colmán-Martínez[1], Mercè Mercader Martí[3], Rosa M. Lamuela-Raventós[1,2]

[1]Nutrition and Food Science Department, XaRTA, INSA. Pharmacy School, University of Barcelona, Av. Joan XXIII s/n Barcelona, Spain
[2]CIBER Fisiopatología de la obesidad y nutrición (ciberobn) and RETICS RD06/0045/0003. Institute of Health Carlos III, Spain
[3]Miguel Torres, Vilafranca del Penedès, Spain

Introduction

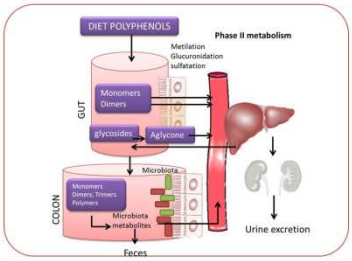
Red wine is a rich source of polyphenols. Its moderate intake is associated with beneficial effects on health, such as cardiovascular disease prevention. Up to now, only a few studies have focused on the bioavailability of polyphenols considering the matrix of wine, such as alcohol. Nowadays, there is no consensus if alcohol increases polyphenols bioavailability by improving polyphenol's solubility or increases the elimination as a result of the diuretic effect. The aim of this study was to analyze the phenolic profile and their metabolites in plasma after an acute intervention of wine and dealcoholized wine (DW) in order to test if the alcohol present in the wine matrix affected phenolic absorption and metabolism.

Objective

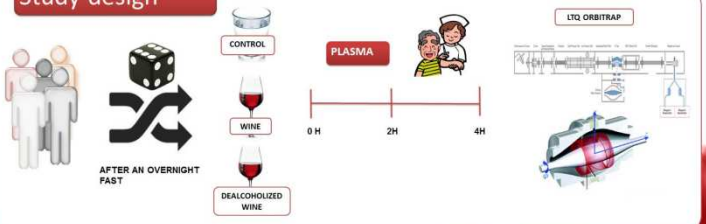
To analyze the phenolic profile and their metabolites in plasma after an acute intervention of wine and dealcoholized wine (DW) in order to test if the alcohol present in the wine matrix affects the phenolic absorption and metabolism.

Method

Liquid chromatography coupled to high resolution mass spectrometry was used for an accurate identification of polyphenols and polyphenolic metabolites in plasma at 0h, 2h and 4h after an acute consumption of wine and dealcoholized wine.



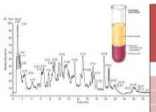
Study design

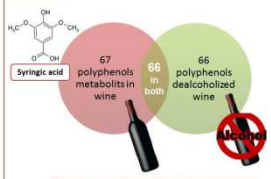


Results

Table1. Table shows the Total Polyphenol concentration in wine and DW expressed in equivalents of Gallic acid.

Total polyphenols	[Mean Conc.] meq GA/L	SD	CV
WINE	2686.2	138.0	5.1
DW	2179.9	114.5	5.3





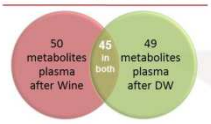


Table2. Table only shows phenolic metabolites that were different between RD and DRW interventions.

POLYPHENOLIC METABOLIC DIFFERENCES	PLASMA AFTER INTERVENTION			
	WINE		DEALCOHOLISED WINE	
	2H	4H	2H	4H
HYDROXYBENZOIC ACIDS				
Protocatechuic acid glucoside		✓	✓	✓
4-hydroxyhippuric acid *		✓	✓	✓
Syringic acid	✓	✓		
Vanillic acid sulfate 1		✓	✓	✓
Vanillic acid sulfate 2		✓	✓	✓
Gallic acid ethyl ester*	✓		✓	✓
PHENYLACETIC ACIDS				
Homovanillic acid sulfate		✓	✓	✓
Homoprotocatechuic acid *		✓	✓	✓
Homovanillic acid*				✓
CYNNAMIC ACIDS				
Dihydrocaffeic acid sulfate		✓	✓	✓
Caffeic acid*				✓
Ferulic acid 4-*O*-glucuronide				✓
p-coumaric acid *		✓	✓	✓
o-coumaric acid*		✓		
STILBENES				
trans-resveratrol-4'-*O*-glucuronide*		✓		
trans-resveratrol*		✓	✓	✓
FLAVONOLS				
kaempferol-3-glucoside*	✓		✓	✓
kaempferol*	✓			
FLAVANOLS				
Epicatechin*				✓
(Epi)catechin sulfate		✓	✓	✓
taxifolin*	✓			

Metabolites of benzoic acids, hydroxycinnamic acid, stilbenes, flavones, flavonols and their derivatives were identified; including metabolites derived from the microbiota metabolism such as propionic acids, phenylacetic acids, hydroxyphenylpentanoics acids and valerolactones. Also, several conjugated metabolites of the phase II metabolism were identified such as glucuronidated and sulfated metabolites. For example protocatechuic acid was observed in wine and its conjugated form protocatechuic-*O*-glucoside acid was identified in plasma. In addition, methylated, methyl-glucuronidated, diglucuronidated, di- and trisulfated and sulfated-glucuronidated conjugated forms occurred during the phase II metabolism were also identified.

Conclusions

- Dealcoholized wine had lower concentration of polyphenols than Wine.
- 67 and 66 phenolic compounds were identified in wine and dealcoholized wine, respectively. Syringic acid was only identified in wine sample.
- 50 and 49 polyphenolic metabolites were identified in plasma after wine and DW, respectively.
- Plasma polyphenols remained longer after the DW intervention (at least 2h), possibly due to the diuretic effect of the ethanol.

References

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2. Tresserra-Rimbau, A. *et al.* 2014.
3. Chiva-Blanch, G. *et al.* 2012.
4. Chiou, Y.-S. *et al.* 2014.
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6. Ortuño, J. *et al.* 2010.
7. Manach, et al. 2004.

Comunicación 9. Comunicación Oral.

Título: “La variedad de oliva, el estrés y el cultivo ecológico condicionan los niveles de polifenoles del aceite de oliva virgen”

Autores: Paola Quifer-Rada; Ruth Martínez-Las Heras; **Mariel Colmán-Martínez**; Raquel Callejón-Fernández; Brígida Jiménez; Rosa M. Lamuela-Raventós.

Congreso: I Workshop Anual INSA-UB. El universo del aceite de oliva, Barcelona, España, 2015


Universitat de Barcelona
B:KC Barcelona Knowledge Campus
Campus d'Excel·lència Internacional
Campus de l'Alimentació
Universitat de Barcelona
HUB
I Workshop Anual INSA UB
El Universo del Aceite de Oliva
11 Noviembre 2015
Campus de la Alimentación Torribera UB, Santa Coloma de G.
Presentación
Los workshops anuales INSA nacen con el objetivo de ser un punto de encuentro de los miembros del INSA y el resto de la comunidad científica entorno a un tema central abordado de forma transversal y multidisciplinaria.
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Programa Preliminar
9.30 Bienvenida y presentación INSA. Dr. J.M. Llobet
10 Autenticidad del aceite de oliva. Dr. L. Conte, Univ. di Udine (Italia)
10.30 Presentación proyectos Foment de la Recerca Interna 2013 (FRI 2013–INSA UB)
11 Café
11.30 Comunicación 1 . Seleccionada entre comunicaciones
11.45 Comunicación 2. Seleccionada entre comunicaciones
12 Análisis sensorial de aceite. Técnica, fiabilidad, problemas actuales y tendencias de futuro. Dr. A. Romero, IRTA-Centro Mas de Bover (Reus)
12.30 Reinterpretando la calidad: desde su origen bioquímico hasta su percepción sensorial. Dr. D.L. García, Instituto de la Grasa (Sevilla)
13 El papel de la restauración en la evolución del aceite de oliva. Sr. J. Biarnés, Fundación Alicia
13.15 Cultura del aceite y alimentación saludable en las escuelas Taller cata aceite oliva. Servicio Educativo del CCOC, Centre de la Cultura de l'Oli de Catalunya (La Granadella, Lleida)
14 Comida
15 El aceite de oliva en la prevención del cáncer de pecho. Dra. M. Solanas, Univ. Autónoma Barcelona
15.30 Papel del aceite de oliva en el tratamiento de patologías con un importante componente inflamatorio. Dra. C. Alarcón de la Lastra, Univ. Sevilla
16 Comunicación 3. Seleccionada entre comunicaciones
16.15 Comunicación 4. Seleccionada entre comunicaciones
16.30 Comunicación 5. Seleccionada entre comunicaciones
16.45 El aceite de oliva en la prevención de la enfermedad cardiovascular. El estudio PREDIMED. Dr. R Estruch, Univ. Barcelona

Comunicación 10. Póster.

Título: “Use of high resolution mass spectrometry tools for the screening of the polyphenolic metabolic differences between wine and dealcoholized wine”
Autores: Anna Creus-Cuadros; Paola Quifer-Rada; Xiaohui Guo; **Mariel Colmán-Martínez**; Mercé Mercader-Martí; Rosa M. Lamuela-Raventós.
Congreso: Jornàda Tècnica sobre la Recerca en Enologia i Viticultura en Catalunya, Tarragona, España, 2015

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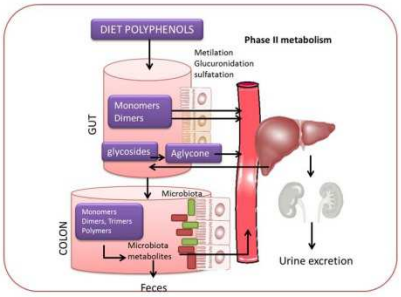

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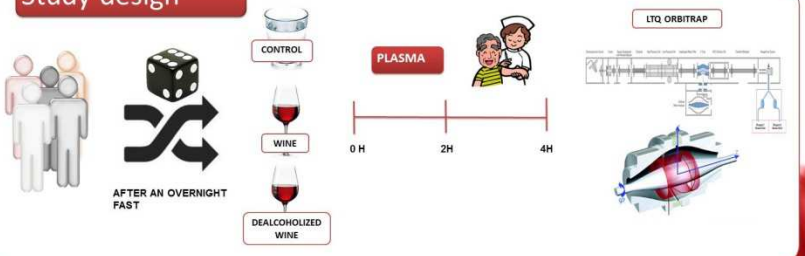

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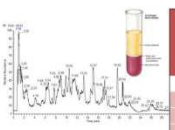

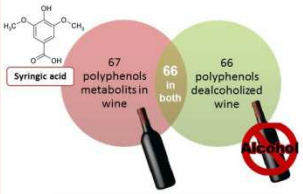

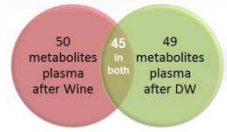

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