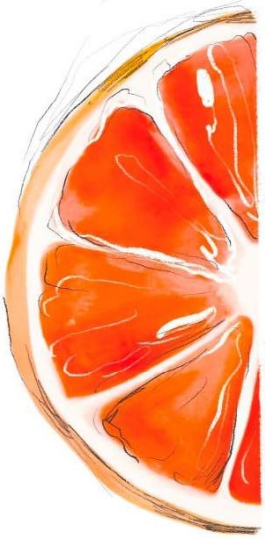
Caracterización fisiológica, bioquímica y molecular de nuevas variedades de naranja de pulpa roja.



PROGRAMA DE DOCTORADO EN CIENCIAS DE LA ALIMENTACIÓN

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Caracterización fisiológica, bioquímica y molecular de nuevas variedades de naranja de pulpa roja

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A mis padres les debo todo lo que soy,

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El objetivo principal de esta Tesis Doctoral ha sido caracterizar en profundidad los frutos de dos nuevas mutaciones de naranja dulce de pulpa roja, denominados Kirkwood Navel y Ruby Valencia, procedentes de las naranjas Washington Navel (cv. Palmer) y Valencia late (cv. Olinda), respectivamente. Para ello, se abordaron las bases fisiológicas y moleculares del metabolismo de carotenoides implicadas en la singular coloración del fruto, la evolución de los componentes de calidad de los frutos durante la maduración, su aptitud para la conservación postcosecha y comercialización en fresco, y el potencial de estas nuevas variedades para la producción y procesado de zumo.

Los resultados bioquímicos y moleculares del metabolismo de carotenoides indican que la alteración en los mutantes Kirkwood y Ruby parece deberse a un bloqueo parcial en la actividad licopeno β-ciclasa como sugiere la acumulación de grandes cantidades de carotenos lineales precursores del licopeno y los menores niveles de productos por debajo del licopeno en la ruta, como la violaxantina y el ácido abscísico. Sin embargo, el inusual contenido y composición de carotenoides en estas variedades no está asociado a diferencias en la expresión de genes del metabolismo de carotenoides o en la secuencia de los genes que codifican para las licopeno ciclasas respecto a las variedades de naranja de referencia (Capítulo 1).

Los resultados del Capítulo 2 revelaron que el contenido en compuestos bioactivos y otras características en relación con calidad del fruto, así como sus cambios durante la maduración, son similares entre las dos variedades de pulpa roja y las respectivas variedades de naranja común. Por tanto, la mutación no parece tener un efecto negativo en los diferentes componentes de la calidad interna del fruto. Sin embargo, los extractos liposolubles de la pulpa de ambos mutantes presentaron una mayor capacidad antioxidante ABTS y frente el radical oxígeno singlete (SOAC), que se correlacionó significativamente con su alto contenido en carotenoides. Además, el estudio comparativo de diferentes variedades de cítricos indica que la naranja Ruby exhibe mayor capacidad antioxidante que los frutos de otras variedades comerciales, como la naranja común Valencia late, los pomelos Marsh y Star Ruby, o las mandarinas Clemenules y Nadorcott. La vitamina C tiene una importante contribución a la capacidad antioxidante hidrosoluble de las diferentes variedades de cítricos, mientras que los carotenoides fitoeno y fitoflueno, debido a sus altas concentraciones, son los principales compuestos que contribuyen a la capacidad antioxidante liposoluble en la pulpa de la naranja Ruby (Capítulo 3).

Los resultados correspondientes al Capítulo 4 muestran que las naranjas Kirkwood y Ruby tienen una buena aptitud para la conservación postcosecha refrigerada y vida útil, con respuestas fisiológicas similares a las de las naranjas comunes y manteniendo sus características de calidad, por lo que pueden alcanzar el mercado y llegar a los consumidores con su valor añadido intacto. Asimismo, ambas variedades de pulpa roja contienen niveles más altos de norisoprenoides, monoterpenos, aldehídos de cadena larga y presentan diferencias significativas en ésteres alifáticos en frutos conservados durante la conservación postcosecha y vida comercial (4 semanas a 2°C más 5 días a 20°C), respecto a las variedades de referencia. El mayor contenido en norisoprenoides está asociado a los altos niveles de sus carotenos precursores. Por tanto, el inusual contenido y composición de carotenoides en la pulpa de Kirkwood y Ruby no solo afectó al color de los frutos, sino también al perfil de compuestos volátiles y, probablemente, al aroma.

Por último, los resultados del Capítulo 5 ponen de manifiesto que las variedades de pulpa roja Kirkwood y Cara Cara presentan una buena aptitud para la elaboración de zumo, con un elevado contenido en carotenoides, mayor contenido en tocoferoles y mayor capacidad antioxidante liposoluble que los zumos de la naranja común Navel que, en cambio, presentaron una mayor concentración de vitamina C. Los tratamientos de pasteurización y homogeneización a altas presiones fueron, en general, efectivos manteniendo la composición de carotenoides y de otros compuestos bioactivos y de calidad respecto a los correspondientes zumos frescos. Finalmente, los subproductos de la extracción industrial del zumo de las naranjas Cara Cara y Kirkwood presentaron un mayor valor añadido en comparación

con la naranja tradicional, debido principalmente a una alta concentración de carotenoides y tocoferoles, y mayor capacidad antioxidante frente al oxígeno singlete. Así, el subproducto de la naranja de pulpa roja representa una fuente importante de compuestos bioactivos con un alto potencial para su revalorización industrial.

ABSTRACT

The main objective of this PhD has been to perform a comprehensive characterization of fruits of the new sweet orange red-fleshed mutants, named as Kirkwood Navel and Ruby Valencia, originated from the common oranges Washington Navel (cv. Palmer) and Valencia late (cv. Olinda), respectively. For that purpose, we tackled the physiological and molecular mechanisms of the carotenoids metabolism involved in the exceptional coloration of the fruit, the evolution of the quality components during fruit ripening, their aptitude for postharvest storage and fresh marketing, and, ultimately, the potential of these new varieties for juice production and processing.

The biochemical and molecular results about the carotenoids metabolism of the orange varieties Kirkwood and Ruby indicate that the alteration in these mutants seem to be due to a partial blockage in the lycopene β-cyclase activity as suggested by the high amounts of linear carotenes upstream lycopene and the lower levels of downstream metabolites as violaxanthin and abscisic acid (Chapter 1).

The results of the Chapter 2 revealed that the content of bioactive compounds and other characteristics related to fruit quality, as well as their changes during fruit maturation are similar between both red-fleshed and their respective standard orange varieties. Therefore, the mutation does not have detrimental effects on the different fruit quality components. Nevertheless, pulp extracts of both red-fleshed mutants showed a higher ABTS lipophilic antioxidant capacity and singlet oxygen absorption capacity (SOAC), and SOAC values were correlated with the high content of carotenoids. Furthermore, the comparative study of different citrus varieties indicate that the Ruby orange exhibits the higher antioxidant capacity compared to fruits of other commercial varieties such as the Valencia late orange, the Marsh and Star Ruby grapefruits, and the Clemenules and Nadorcott mandarins. On the other hand, the vitamin C has a relevant contribution to the hydrophilic antioxidant capacity in all the selected citrus varieties while the carotenoids phytoene and phytofluene, because of their elevated concentrations, are the main compounds contributing to the lipophilic antioxidant capacity in pulp of the Ruby orange (Chapter 3).

In the Chapter 4 the postharvest handling of Kirkwood and Ruby oranges was investigated. Both red-fleshed varieties displayed a good performance for cold storage and shelf-life, showing physiological responses similar to those of the standard oranges. Therefore, the red-fleshed oranges maintain their quality attributes during postharvest storage and may reach market and consumers with their value-added. In addition, the volatile profile of the pulp of both red-fleshed varieties in comparison to the reference varieties showed higher levels of norisoprenoids, monoterpenes, long-chain aldehydes, and significant differences in aliphatic esters, in fruits stored during 4 weeks at 2°C followed by 5 days at 20°C. The high levels of norisoprenoids are associated with the large content of their carotenes precursors. Then, the unusual content and composition of carotenoids in the pulp of Kirkwood and Ruby not only modifies the pulp color but also the volatiles profile and, likely, the aroma.

Eventually, the objective of the Chapter 5 aimed at evaluating the suitability for juice production of the two red-fleshed varieties Kirkwood and Cara Cara, in comparison to a standard orange variety Navel. The juice of Kirkwood and Cara Cara showed a large content of carotenes, and higher levels of tocopherols and lipophilic antioxidant capacity than those of the juices from the standard Navel orange; while Navel presented higher concentration of vitamin C. The pasteurization and high-pressure homogenization treatments were effective maintaining the composition of carotenoids and other bioactive and quality compounds in comparison to their respective fresh juices. The orange by-products generated by the industrial juice extraction from Kirkwood and Cara Cara fruit presented higher added-value compared to that from the traditional orange variety, mainly due to the content of carotenoids, tocopherols and SOAC. Therefore, the orange by-products from the red-fleshed varieties represent an important source of bioactive compounds with a high potential for industrial waste valorization.

$^1O_2$	Oxígeno singlete
ABA	Ácido abscísico
ABA-GE	Ácido abscísico glucosil éster
ABTS	Ácido 2,2'-azinobis (3 etilbenzotiazolín)-6-sulfónico
ABTS-H	ABTS hidrosoluble
ABTS-L	ABTS liposoluble
CAH	Capacidad antioxidante hidrosoluble
CAL	Capacidad antioxidante liposoluble
CCD	Dioxigenasa de corte de carotenoides
CPTA	Hidrocloruro de 2-(4-tioclorofenil) trietilamina
CRTISO	Caroteno isomerasa
DEP	Desoxi-D-xilulosa 5-fosfato
DMAPP	Dimetilalil difosfato
DPPH	2,2-difenil-1-picrilhidrazilo
DXP	Dioxixilulosa-5-fosfato
DXR	Dioxixilulosa-5-fosfato reducto isomerasa
DXS	Dioxixilulosa-5-fosfato sintasa
FRAP	Poder reductor antioxidante férrico (*"Ferric Reducing Antioxidant Power"*)
GGDR	Geranilgeranil difosfato reductasa
GGPP	Geranilgeranil difosfato
GGPPS	Geranilgeranil difosfato sintasa
HDR	Hidroximetil-butenil difosfato reductasa
HMBPP	Hidroximetil-butenil difosfato
HPH	Homogeneización a altas presiones *("High-Pressure Homogenization")*
HPP	Procesamiento a altas presiones hidrostáticas (*"High-Pressure Processing"*)
IDF	Índice de daños por frío
IPP	Isopentenil difosfato
MEP	Metileritritol fosfato
MVA	Ácido mevalónico
NCED	9-cis-epoxicarotenoide dioxigenasa
NSY	Neoxantina sintasa
PDS	Fitoeno desaturasa
PSY	Fitoeno sintasa
PYP	"Pale Yellow Petal"
ROS	Especies reactivas de oxígeno
SOAC	Capacidad de secuestrar oxígeno singlete (*"Singlet Oxygen Absorption Capacity"*)
VDE	Violaxantina de-epoxidasa
XAT	Xantofila acil esterasa

XES	Xantofila esterasa
ZDS	ζ-Caroteno desaturasa
ZEP	Zeaxantina epoxidasa
Z-ISO	ζ-Caroteno isomerasa
β-CHX	β-Caroteno hidroxilasa
β-LCY	β-Licopeno ciclasa
ε-CHX	ε-Caroteno hidroxilasa
ε-LCY	ε-Licopeno ciclasa

1. INTRODUCCIÓN

1.1. Importancia socioeconómica de los frutos cítricos

Los frutos cítricos o género *Citrus*, constituyen uno de los principales cultivos frutales a nivel mundial en términos de producción y valor económico. Su demanda y aceptación por los consumidores se deben principalmente a su atractiva coloración, características sensoriales y propiedades nutricionales y/o saludables (Baldwin et al., 2014; Lado et al., 2018). Todos estos factores contribuyen a la calidad de los frutos cítricos y definen su potencial para el mercado de productos frescos y la industria del zumo. La producción y exportación mundial de cítricos ha crecido progresivamente en las últimas tres décadas, aunque a un ritmo más lento en comparación con otros frutales competidores En el conjunto de la producción mundial, las naranjas (*Citrus sinensis*) representan más del 50%, seguidas de mandarinas (*Citrus reticulata*), limones (*Citrus limon*) y pomelos (*Citrus paradisi*) (FAOSTAT, 2020).

La producción mundial de frutos cítricos en el año 2019 fue de aproximadamente 148 millones de toneladas (FAOSTAT, 2020). Alrededor del 65% del total de la producción mundial se cultiva en el hemisferio norte, principalmente China, India, México, EEUU y países de la cuenca mediterránea. Respecto al hemisferio sur, Brasil, es el mayor productor y responsable de alrededor del 17% de la producción mundial. En este contexto, España es actualmente el sexto productor mundial de cítricos, con una producción de 7,15 millones de toneladas en la campaña 2021-22, que la sitúan como el principal productor en Europa, con un 57% del total. Además, España es el primer exportador mundial de cítricos para el mercado en fresco, destinando un 40% de la producción de naranjas y un 61% de la de mandarinas a la exportación. Debido al alto volumen de exportación de los cítricos españoles, el 25% del mercado mundial de naranjas procede de España, cifra que llega al 31% en el caso de las mandarinas (CGCT, 2016). En cambio, otros grandes productores como Brasil destinan más del 80% de su producción a la industria del procesado de zumo (FAOSTAT, 2020).

La producción general de cítricos en la geografía española se distribuye de la siguiente forma: 53.5% del total correponde a naranjas, seguido de mandarinas con el 29.9%, el 15.4% de limones y el 1.2 % de pomelos (Ministerio de Agricultura, Pesca y Alimentación de España, 2021). Entre las distintas comunidades autónomas, la Comunidad Valenciana es la de mayor mayor volumen de producción en España desde hace décadas, representando

aproximadamente el 49.2% de la producción nacional en el año 2019, seguida de Andalucía (35.1%) y Murcia (13.5%) (Generalitat Valenciana, 2020).

Las variedades de naranjas más cultivadas en España pertenecen al grupo de las naranjas Navel (73.4% de la producción total), seguidas de las naranjas tipo 'blancas' (25.8%) y finalmente las naranjas sanguinas, que representan un porcentaje muy pequeño de la producción total (<1%) (Tabla 1). Las variedades de naranja que integran el grupo Navel se destinan mayoritariamente al consumo en fresco por su apreciada calidad organoléptica y el calibre grande de sus frutos. Las principales variedades dentro del grupo de las naranjas blancas corresponden a la naranja Valencia. Estas variedades se caracterizan por ser de maduración tardía, mayor acidez y porcentaje de zumo, y un calibre del fruto más pequeño (Barry, 2020). Los datos oficiales correspondientes a la campaña 2021 indican que, respecto a la producción total de naranja dulce en España, el 40% de las naranjas Navel se destina a la exportación, el 38.5% al consumo interior y el 17% a la transformación. Por su parte, el 33% las naranjas blancas se destinan a la exportación, el 41.5% al consumo interior y el 23% a la transformación (Tabla 1) (Ministerio de Agricultura, Pesca y Alimentación, 2021).

Tabla 1. Resumen de rendimiento, producción y destino (toneladas) de las principales variedades de naranja dulce en España en el año 2021. Los números entre paréntesis indican el porcentaje respecto a la producción total. Fuente: Ministerio de Agricultura, Pesca y Alimentación de España, 2021.

			Destino de la producción (t)		
Variedades	Rendimiento	Producción	Exportación	Consumo	Transformación
Grupo					
Navelina	29.420	1.182.840	432.748	510.755	186.368 (15.7)
Navel	29.008	256.479	87.713	106.906	50.112 (19.5%)
Navelate	29.195	1.313.000	570.481	442.529	243.786 (18.5%)
Grupo					
Valencia	25.317	606.031	223.300	251.370	106.947 (17.6%)
Otras	82.560	362.728	97.094 (26.8)	145.418	113.661 (31.3%)
Sanguinas	23.309	27.948	10.778	10.369	6.732 (24.1%)

España cuenta con un buen sistema productivo y una industria muy especializada en el transporte de fruta fresca a cualquier destino mundial, aunque su principal mercado es el europeo. Sin embargo, el principal problema de los cítricos en España en la actualidad es la falta de rentabilidad que sufren los agricultores debido a los bajos precios en origen que, unidos a los altos costes de producción, hacen que la rentabilidad de las explotaciones agrarias sea cada vez más baja y en ocasiones se produzcan sustanciales pérdidas económicas y peligre el futuro de las mismas (Caballero & Fernández-Zamudio, 2007; Caballero et al., 2010).

Además, la tendencia en las normativas por parte de las instituciones europeas de restricción y/o prohibición al uso de fitosanitarios, la limitación de los recursos hídricos y las nuevas modalidades productivas, como el cultivo ecológico, son otros de los desafíos a los que se enfrenta la citricultura española en general, y la valenciana en particular. Además, la creciente competencia de terceros países, tanto de la cuenca mediterránea como más alejados, que pueden suministrar frutos cítricos a lo largo de todo el año a más bajos costes de producción, son retos para el sector que obliga a mejorar las condiciones de producción y exportación, introducir innovaciones tecnológicas en las distintas fases de la cadena de producción y transporte hasta la llegada a los consumidores para aumentar la competitividad y la rentabilidad del cultivo (Fernández-Zamudio & Lliso, 2017; Fernández-Zamudio, 2020).

Ante estos crecientes desafíos, uno de los objetivos de la citricultura moderna en el que se están invirtiendo mayores esfuerzos en las últimas décadas en los diferentes países productores es la obtención, caracterización y comercialización de nuevas variedades. Esta estrategia persigue obtener nuevas variedades propias que permitan diferenciarse en los mercados internacionales, ofrecer productos novedosos y diferentes para el consumidor, ampliar el periodo de comercialización de fruta para el consumo en fresco y, al mismo tiempo, poder suministrar fruta con características organolépticas y nutricionales mejoradas, y diferentes a las tradicionales. En este sentido el creciente interés por el consumo de alimentos más saludables y con propiedades nutricionales mejoradas promueve la incorporación en los mercados de nuevas variedades con valor añadido.

Una de las líneas de interés es la obtención de variedades de naranja de pulpa roja, es decir con acumulación de licopeno, que son distintas a las naranjas 'sanguinas', que acumulan antocianos y que adquieren una intensa tonalidad rojiza y morada. El licopeno es un carotenoide que proporciona coloración roja a numerosos frutos como el tomate o la sandía, sin embargo, su presencia en los frutos cítricos es muy inusual, como se describe más adelante en esta sección. Hasta hace pocos años sólo existían variedades de pulpa roja en pomelos (*Citrus paradisi*) y pumelos o zamboas (*Citrus grandis* o *Citrus maxima*), que son frutos cítricos con sabor amargo (da Graça et al., 2004). El interés de numerosos programas de obtención de variedades en el mundo ha sido conseguir variedades de naranja, con fondo genético de sabor dulce, que acumulen licopeno en la pulpa. En este contexto, la obtención y disponibilidad de dos nuevas variedades con estas características (Kirkwood Navel y Ruby Valencia), cuyas

características se describen más adelante en esta sección, han sido el punto de partida para abordar las investigaciones en las que se enmarca esta Tesis Doctoral.

1.2. Carotenoides

1.2.1. Aspectos generales de los carotenoides

Los carotenoides son compuestos isoprenoides de 40 átomos de carbonos que se encuentran ampliamente distribuidos en la naturaleza. Son los pigmentos responsables de la coloración de las flores, frutos y otros tejidos vegetales de numerosas especies. Los carotenoides son sintetizados en los plastidios por todos los organismos fotosintéticos, incluidas las plantas, algas y cianobacterias y, además, por otros organismos no fotosintéticos como archaea, bacterias y hongos (Rodríguez-Concepción et al., 2018). Los carotenoides son compuestos de naturaleza liposoluble y a menudo se encuentran esterificados con ácidos grasos, lo que aumenta su liposolubilidad, mientras que su asociación con proteínas o azúcares los hace más hidrosolubles (Rodríguez-Concepción et al., 2018). La principal característica de la estructura química de los carotenoides es su sistema de dobles enlaces conjugados, que consiste en alternar enlaces carbono-carbono simples y dobles. Esta cadena actúa como cromóforo y es la responsable de la capacidad de estos pigmentos de absorber la luz en el espectro de onda visible y, por tanto, de proporcionar color. Además, esta cadena es fundamental en la reactividad y propiedades fisicoquímicas de los carotenoides.

Los carotenoides se clasifican en dos grupos principales: los carotenos, con una estructura hidrocarbonada, y las xantofilas, que contienen oxígeno en la molécula (Figura 1) (Britton, 1995). La longitud de onda a la que absorben los carotenoides aumenta con el número de dobles enlaces conjugados. El licopeno que es el caroteno más insaturado con 11 dobles enlaces conjugados, es de color rojo, mientras que el β-caroteno, aunque tiene el mismo número de enlaces conjugados que el licopeno, es de color naranja, debido a la presencia de dos anillos β-ionona en los extremos de su estructura (Figura 1) (Britton, 1995). La mayoría de las xantofilas tienen ciclados los extremos de la molécula, influyendo en el cromóforo. Debido a su peculiar estructura, para un mismo carotenoide puede existir diferentes isómeros geométricos, lo que aumenta el grado y la diversidad de estructuras para determinados carotenoides, identificándose tanto los isómeros all-*trans(E)*, mono- y poli-*cis(Z)*. La conversión de la forma *trans* a los posibles isómeros *cis* se produce por la acción de

determinadas enzimas específicas (isomerasas), la exposición a altas temperaturas o por la absorción de luz. La mayoría de los carotenoides se encuentran y se sintetizan en conformación all-*trans*, que habitualmente es la forma termodinámicamente más estable, sin embargo, existen algunas excepciones muy relevantes como el fitoeno, cuya forma mayoritaria y la que sintetizan las plantas es el isómero 15-*cis*, o como el isómero 9-*cis*-violaxantina que es el predominante en la mayoría de los frutos cítricos (Gross, 1987; Clinton, 1998). El fitoeno y fitoflueno (Figura 1) presentan algunas características distintivas respecto al resto de carotenoides, ya que el menor número de enlaces conjugados en su estructura los hace incoloros y absorben la luz en el rango del ultravioleta (Meléndez-Martínez et al., 2015).

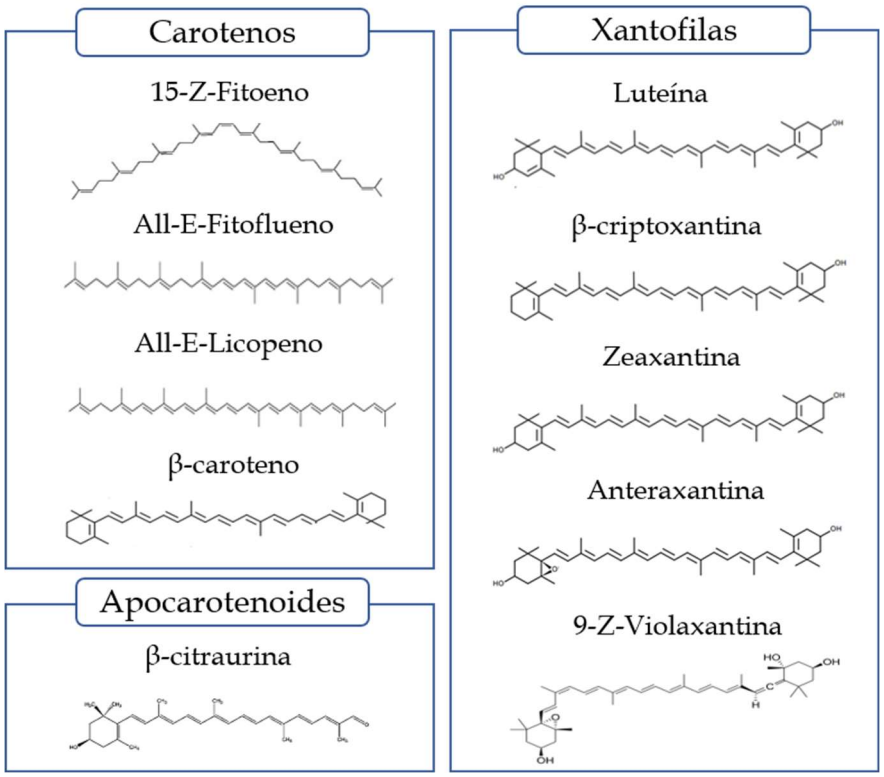

Figura 1. Estructura química de los principales carotenos, xantofilas y apocarotenoides presentes en los frutos cítricos.

Por otro lado, el sistema de doble enlaces conjugados influye de forma importante en las propiedades y/o actividad antioxidante de los carotenoides. Así, en general, cuanto mayor es el número de enlaces conjugados mayor es la capacidad para reaccionar frente a radicales libres. La presencia de anillos y grupos funcionales también modifica esta capacidad, generalmente de manera negativa (Stahl & Sies, 2003).

Los carotenoides desempeñan funciones primarias (esenciales) y secundarias (especializadas) en las plantas. Son indispensables en la fotoprotección de los tejidos fotosintéticos, disipando el exceso de energía en forma de calor y secuestrando radicales libres, protegiendo así a las membranas de la peroxidación lipídica. Los carotenoides forman parte de los complejos pigmentos-proteínas asociados al fotosistema II y juegan un papel esencial en el ciclo de transferencia de electrones a las clorofilas. Parece existir un amplio consenso en que la principal función de los carotenoides en la naturaleza es la protección de la maquinaria fotosintética del exceso de luz y ayudar a la captación de energía como pigmentos accesorios (Pogson et al. 2005; Cazzonelli, 2011). Otras funciones de estos compuestos es que son precursores de importantes fitohormonas, como el ácido abscísico y las estrigolactonas, así como de otras moléculas señalizadoras, las cuales desarrollan funciones reguladoras relevantes en el crecimiento, desarrollo y respuestas al estrés en las plantas (Al-Babili et al., 2015; Felemban et al., 2019). Cómo metabolitos secundarios, los carotenoides tienen funciones importantes en los procesos de comunicación entre plantas y animales. Los carotenoides y también sus derivados los apocarotenoides, bien como pigmentos o volátiles, funcionan como atrayentes de organismos polinizadores y animales dispersores de las semillas (Britton, 2008).

1.2.2. Propiedades de los carotenoides

Numerosos estudios y revisiones exhaustivas han abordado las propiedades de los carotenoides y los efectos beneficiosos de su consumo en relación con la salud. En general, se sugiere que existe una relación entre el consumo de alimentos ricos en carotenoides y la reducción del riesgo a padecer determinadas enfermedades y procesos degenerativos que implican estrés oxidativo y/o procesos inflamatorios, tales como enfermedades cardiovasculares, cáncer, degeneración macular, cataratas o enfermedades relacionadas con el sistema inmunitario, entre otras (Astley ey al., 2004; Krinsky & Johnson, 2005; Perera & Yen, 2007; Berman et al., 2014; Eggersdorfer & Wyss, 2018; Amengual, 2019; Tan & Norhaizan, 2019; Bohn et al., 2021). La mayoría de los animales, incluidos los humanos, no pueden sintetizar carotenoides, por lo que deben ingerirlos a través de la dieta, especialmente en frutas y verduras. Los carotenoides son compuestos no polares que requieren ser incorporados a micelas antes de su absorción por el intestino delgado. Se considera que, en función del tipo de carotenoide, estructura, matriz alimentaria e incluso factores endógenos de cada individuo,

solo entre el 5-50% de los carotenoides son absorbidos y alcanzan el sistema circulatorio (Bohn et al., 2021).

Los efectos beneficiosos de los carotenoides en relación con la salud se han atribuido fundamentalmente a dos propiedades: su capacidad antioxidante y que algunos de ellos exhiben actividad pro-vitamina A (Eggersdorfer & Wyss, 2018; Amengual, 2019). Los carotenoides con al menos un anillo β-ionona no sustituido en su estructura, como el α, β, γ-caroteno y la β-criptoxantina, tienen actividad provitamina A, ya que son precursores para la síntesis de retinol y ácido retinoico (Meléndez-Martínez, 2019). Por otro lado, los carotenoides exhiben una alta capacidad antioxidante, principalmente frente a el oxígeno singlete y otras especies reactivas de oxígeno (ROS) (Stahl & Sies, 2004; Böhm et al., 2012). La eficacia secuestradora (`scavenging´) o desactivadora ('quenching') de los carotenoides frente a los ROS está relacionada con el número de dobles enlaces conjugados presentes en su estructura y que determina el nivel de transferencia de energía (triplete-triplete) que se requiere para el intercambio de electrones y energía entre dos moléculas (Stahl & Sies, 2004). Así, el licopeno es el carotenoide que exhibe mayor capacidad antioxidante debido a que presenta 11 dobles enlaces conjugados. Sin embargo, también se ha demostrado que las xantofilas son eficientes desactivadores del oxígeno singlete (Stahl & Sies, 2004). En este sentido, la capacidad antioxidante de los carotenoides es de particular importancia respecto a sus propiedades biológicas, ya que el estrés oxidativo es un factor crítico en el desarrollo de numerosas enfermedades (Fiedor & Burda, 2014; Rajendran et al., 2014; Barros et al., 2018). Por ejemplo, los carotenoides previenen la oxidación de las proteínas de bajas densidad (LDL) y de lípidos de la retina, inhiben la oxidación de lípidos en células del cerebro y reducen la oxidación de lípidos durante la digestión gastrointestinal (Bohn et al., 2021). En general, se ha demostrado que los carotenoides actúan como antioxidantes en condiciones *in vitro*. Sin embargo, hasta qué punto esta función contribuye a los efectos beneficiosos observados en humanos es todavía motivo de debate. Los motivos que generan estas discrepancias entre los diferentes estudios se deben principalmente al uso de diferentes metodologías, la presencia de otros antioxidantes, el organismo evaluado en los estudios *in vivo* y las aproximaciones empleadas para determinar la contribución de los carotenoides (Krinsky & Johnson, 2005). Por otro lado, otros carotenoides también se han relacionado con un correcto funcionamiento de la función visual, y en el caso de la zeaxantina y luteína, componentes de la mácula de la retina, su

deficiencia se ha relacionado con defectos en la visión e incluso ceguera (Arunkumar et al., 2018).

Entre los diferentes carotenoides, el licopeno presenta un espectro de absorción desplazado hacia longitudes de onda más altas y es considerado el carotenoide con la mayor capacidad para reaccionar frente al oxígeno singlete debido a su estructura lineal y mayor número de dobles enlaces conjugados, lo que le proporciona una mayor reactividad. Además, aunque el consumo de licopeno proviene de un número limitado de productos vegetales, como el tomate, sandía y pomelo rojo o sus derivados (Bailey, 2015), es el carotenoide que más se ha detectado en plasma (Stahl & Sies, 1996; Sies & Stahl, 1998; Böhm & Bitschm, 1999). Así pues, el licopeno es uno de los carotenoides que más interés ha suscitado en la comunidad científica y para el que se han asociado importantes propiedades en relación con la prevención de enfermades y efectos beneficiosos en la salud, especialmente en la reducción del riesgo de determinados tipos de cáncer y enfermedades cardiovasculares (Rao & Agarwal, 1999, 2000; Bramley, 2000; Basu & Imrhan, 2007; Mein et al., 2008; Wang, 2012 Chen et al., 2013; Bailey, 2015). Debido a que el licopeno no presenta actividad pro-vitamina A, sus efectos biológicos en humanos se atribuyen a su capacidad antioxidante, estimulación de la comunicación intercelular, interacción con factores de crecimiento y regulación de la proliferación celular y apoptosis (Sies & Stahl, 1998; Wang, 2012). Sin embargo, su biodisponibilidad depende de diversos factores como el procesado y características de la matriz alimentaria, así como de factores genéticos (Arballo et al., 2021; Mayne et al., 1999).

El fitoeno y fitoflueno son los carotenoides que presentan el menor número de dobles enlaces conjugados, lo que les proporciona unas propiedades fisicoquímicas diferentes respecto a otros carotenoides, especialmente en términos de absorción de luz (espectro y coeficiente de absorción) (Meléndez-Martínez et al., 2019). Clásicamente, estos carotenos no han sido objeto de interés en los estudios sobre carotenoides, debido probablemente a que no proporcionan ninguna coloración ni se les había atribuido ninguna función, a pesar de haber sido detectados en plasma y otros tejidos/órganos. En los últimos años, diferentes estudios han relacionado el consumo o suplementación con fitoeno y fitoflueno con importantes efectos biológicos *in vitro* e *in vivo*, como: 1) protección frente al estrés oxidativo, 2) mejora de la respuesta antiinflamatoria, 3) reducción del colesterol en plasma, 4) efectos positivos en relación con el cáncer de mama y de próstata (Engelmann et al., 2011; Martínez et al., 2014;

Meléndez-Martínez et al., 2015; Meléndez-Martínez et al., 2018). Además, una característica distintiva de estos carotenos es que absorben la radiación ultravioleta (UV). Esta propiedad hace que estos carotenos sean especialmente interesantes respecto a la protección y salud de la piel, así como potenciales ingredientes activos para la industria nutricosmética (Aust et al., 2005; Meléndez-Martínez et al., 2019). El fitoeno y fitoflueno se encuentran presentes en un amplio rango de concentraciones (<0.01-7 mg/100g) en diversas frutas y hortalizas, principalmente el albaricoque, el tomate y productos derivados, la sandía, las zanahorias y el pomelo rojo (Meléndez-Martínez et al., 2018). El concepto 'contenido bioaccesible de carotenoides' hace referencia a la concentración de un carotenoide que es potencialmente absorbible respecto a una porción de alimento (Meléndez-Martínez et al., 2021). En este sentido, diferentes estudios *in vitro* han evaluado la bioaccesibilidad y biodisponibilidad de estos carotenos, detectándose en plasma y en otros tejidos y órganos en concentraciones de ng por g, con importantes variaciones entre órganos, y con mayor presencia del fitoflueno (Khachik et al., 2002; Mapeli-Brahm et al., 2019). Por otro lado, se ha demostrado que la biodisponibilidad del fitoeno y fitoflueno es mucho mayor que cualquier otro carotenoide en frutos de tomate, zanahoria y naranja sanguina (Mapelli-Brahm et al., 2017). Además, en células Caco-2 ambos carotenos incoloros exhiben una considerable mayor incorporación y absorción en comparación con el licopeno (Mapelli-Brahm et al., 2018).

1.2.3. Biosíntesis y regulación de los carotenoides

Los carotenoides derivan de la condensación del precursor universal C5, isopentil difosfato (IPP) y de su isómero dimetilalil difosfato (DMAPP), que pueden interconvertirse por acción de la enzima IPP/DMAPP isomerasa (IDI). Ambos precursores se producen en las plantas a través de dos rutas metabólicas independientes: la ruta del ácido mevalónico (MVA) y la ruta del metil-eritritol-fosfato (MEP). En la ruta MVA, IPP se forma en el citosol a partir de tres moléculas de acetil-CoA y se isomeriza en DMAPP. En cambio, a través de la ruta MEP, el IPP y DMAPP se forman en los plastidios a partir del gliceraldehído- 3-fosfato (revisado en Rodríguez-Concepción et al., 2018). A pesar de que ambas rutas funcionan de manera independiente, se ha demostrado que existe un intercambio o comunicación de precursores entre ambas (Hemmerlin et al., 2003). La reacción inicial de la ruta MEP, catalizada por desoxi-D-xilulosa 5-fosfato sintasa (DXS), es la condensación de gliceraldehído 3-fosfato y piruvato

para producir desoxi-D-xilulosa 5-fosfato (DXP). DXS es el principal punto de control regulador del flujo metabólico en la ruta MEP (Wright et a., 2014). En el segundo paso, la DXP reductoisomerasa (DXR) sintetiza MEP a través del reordenamiento y reducción de DXP. En las reacciones finales de esta ruta, (E)-4-hidroxi-3-metil-but-2-enil pirofosfato sintasa (HDS) sintetiza (E)-4-hidroxi-3-metil-but-2-enil difosfato (HMBPP) y HMPP reductasa (HDR) convierte HMPP en IPP y DMAPP (Figura 2) (Rodríguez-Concepción, 2010). La ruta MEP, por su parte, es la principal fuente de precursores para la síntesis de carotenoides y otras hormonas como las estrigolactonas y el ácido abscísico (Rodriguez-Concepción, 2010). En las plantas superiores la fuente de IPP y DMAPP para la síntesis de carotenoides es totalmente dependiente de la vía MEP. La condensación de tres moléculas de IPP genera geranilgeranil difosfato (GGPP), que es el precursor metabólico directo de los carotenoides, así como otros metabolitos importantes (clorofilas, tocoferoles, monoterpenos, plastoquinonas, giberelinas, entre otros).

El primer paso específico de la síntesis de carotenoides en plantas es la condensación de dos moléculas de GGPP por la fitoeno sintasa (PSY) para producir el primer carotenoide C40, 15-Z-fitoeno. Esta reacción se considera el principal punto de control del flujo metabólico para la síntesis carotenoides (Ruíz-Sola & Rodríguez-Concepción, 2012) (Figura 2). Posteriormente, la enzima fitoeno desaturasa, a través de dos desaturaciones consecutivas produce fitoflueno y ζ-caroteno. La acción secuencial de la ζ-caroteno isomerasa (Z-ISO), ζ-caroteno desaturasa (ZDS) y caroteno isomerasa (CRTISO) produce licopeno a través del intermediario neurosporeno (Moise

et al., 2014). En este punto, la ruta se bifurca en dos ramas principales, la rama ε,β y la rama β,β. La rama ε,β requiere la ciclación del licopeno por la enzima licopeno ε-ciclasa (ε-LCY) en un extremo y por la enzima (β-LCY) en el otro para la formación de un anillo ε- y β-ionona, respectivamente, y producir α-caroteno. En esta rama de la ruta, el α-caroteno es dihidroxilado en ambos anillos ionona por acción de ambas caroteno hidroxilasas, ε-CHX y β-CHX, para generar luteína, el pigmento mayoritario en membranas fotosintéticas de tejidos verdes (Kato et al., 2012). Por su parte, en la rama β,β, la exclusiva actividad de la β-LCY cicla los dos extremos de la molécula de licopeno introduciendo dos anillos β-ionona para formar β-caroteno. A continuación, dos hidroxilaciones consecutivas de la molécula de β-caroteno por parte de la enzima β-caroteno hidroxilasa (β-CHX) genera β-criptoxantina y zeaxantina. La

violaxantina puede ser reconvertida en zeaxantina mediante de-epoxidación catalizada por la vioaxantina de-epoxidasa (VDE). Por último, la enzima neoxantina oxidasa (NSY) cataliza la conversión de violaxantina a neoxantina, la última xantofila de la rama β,β (Rodríguez-Concepción et al., 2018) (Fig. 2).

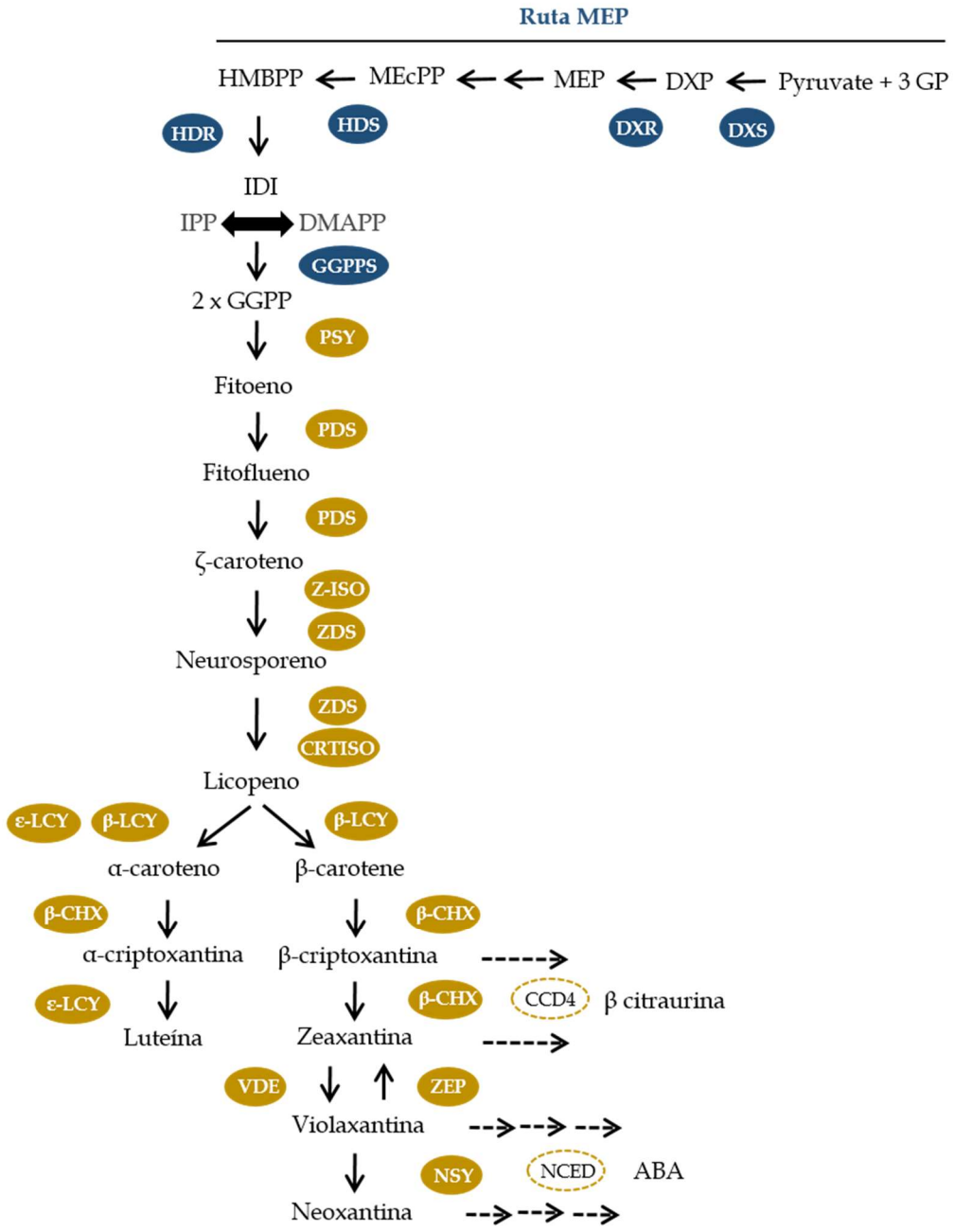

Figura 2. Esquema general de la ruta de biosíntesis de carotenoides en plantas. DXS: 1-deoxi-D-xilulosa 5-fosfato sintetasa; DEP: desoxi-D-xilulosa 5-fosfato; DXR: 1-deoxi-D-xilulosa 5-fosfato reducto isomerasa; MEP: Metil-eritritolfosfato; MEcPP: metil-d-eritritol-2,4-ciclopirofosfato; HDS: (E)-4-hidroxi-3-metil-but-2-enil pirofosfato sintasa; HMBPP: (E)-4-hidroxi-3-metil-but-2-enil difosfato; HDR: 4-hidroxi-3-metilbut-2-enil difosfato reductasa; IPP: isopentil difosfato; DMAPP: dimetilalil difosfato IDI: IPP/DMAPP isomerasa; GGPPS: Geranil geranil fosfato sintasa; PSY: fitoeno sintasa; PDS: Fitoeno desaturasa; Z-ISO: ζ-caroteno isomerasa; ZDS: ζ-caroteno desaturasa; CRTISO: Caroteno isomerasa; β-LCY: β-licopeno ciclasa; ε-LCY: ε-licopeno ciclasa; β-CHX: β-caroteno hidroxilasa; ε-CHX: ε-caroteno hidroxilasa; ZEP: Zeaxantina oxidasa; NSY: Neoxantina oxidasa; VDE: vioaxantina de-epoxidasa; NSY: Neoxantina oxidasa; CCD4: Dioxigenasa de corte de carotenoides; NCED: 9-cis-epoxicarotenoide dioxigenasa; ABA: Ácido abscísico. Círculos azules, marrones y líneas amarillas discontinuas engloban genes precursores de carotenoides de la ruta MEP, genes de biosíntesis de carotenoides y genes del catabolismo de carotenoides, respectivamente.

1.2.4. Carotenoides en cítricos: composición y diversidad

Los carotenoides son los pigmentos responsables del color interno y externo de la mayoría de los frutos cítricos. El color es un atributo determinante de la calidad de los frutos cítricos, tanto para el consumo en fresco como para la producción de zumo, ya que afecta directamente a la calidad del producto final y en la aceptación por el consumidor (Lado et al., 2018). El género *Citrus* se caracteriza por una amplia diversidad genética y la coloración del fruto es uno de los parámetros de mayor variabilidad, ya que abarca desde el amarillo pálido del limón, pomelo blanco o pumelo, al naranja intenso característico de algunas mandarinas, naranjas e híbridos, o incluso hasta las tonalidades rosadas y rojizas de algunos mutantes de pomelos y pumelos o naranjas, (Kato et al., 2012; Ma et al., 2023; Rodrigo et al., 2013a). Este amplio rango de coloraciones, tanto externa como interna, entre los frutos de las diferentes especies y variedades de cítricos se debe al contenido y composición de carotenoides, así como a su distribución específica en el fruto. En general, los frutos maduros de mandarina o híbridos de estas suelen alcanzar mayores contenidos de carotenoides (25-300 μg/g en el epicarpio o flavedo), seguidos por naranja (40-120 μg/g en el flavedo), mientras que los niveles más bajos se encuentran en frutos de limón y pomelo (1-60 μg/g en el flavedo) (Alquézar et al., 2008a, Rodrigo et al., 2013a; Lado et al., 2015a,b; Tadeo et al., 2020).

Una característica común en la mayoría de frutos cítricos es que el flavedo es el tejido con mayor contenido de carotenoides, entre 5 y 20 veces superior respecto a la pulpa (Rodrigo et al., 2013a). En general, la composición cualitativa de carotenoides es similar entre la piel y la pulpa para una misma variedad, y las principales diferencias en cuanto a la composición de carotenoides entre diferentes especies de cítricos se deben a la concentración de carotenoides individuales (Rodrigo et al., 2013a; Ikoma et al., 2016). La mayoría de frutos cítricos coloreados (naranjas y mandarinas) acumulan de forma predominante β,β-xantofilas (principalmente isómeros de violaxantina y β-criptoxantina) cuando alcanza la maduración comercial, aunque en determinadas especies o variedades otros carotenos, como el fitoeno o el licopeno, pueden llegar a alcanzar concentraciones moderadas o elevadas (Alquézar et al., 2008a,b, 2013; Kato et al., 2004). En el flavedo de frutos verdes o inmaduros predominan los carotenoides característicos de tejido cloroplástico, como la luteína y la *trans*-violaxantina, zeaxantina y anteraxantina del ciclo de las xantofilas, y a medida que avanza el proceso de maduración la concentración de estos carotenoides disminuye y aumentan determinadas β-β-xantofilas,

siendo la 9-*cis*-violaxantina la más característica en el flavedo de naranjas y la 9-*cis*-violaxantina y β-criptoxantina en el flavedo de mandarinas (Rodrigo et al., 2013a). Además de violaxantina, las naranjas acumulan concentraciones menores de otras xantofilas, como la anteraxantina y zeaxantina, que también pueden tener un papel importante en la coloración de estos frutos. El contenido total de carotenoides entre las diferentes variedades de naranja varía entre 4-38 μg/g en la pulpa y 40-120 μg/g en la piel. En la pulpa los cambios en el contenido y composición de carotenoides son totalmente independientes del flavedo (Tadeo al., 2020). Así, en la pulpa del fruto inmaduro el contenido de carotenoides es prácticamente nulo y aumenta de forma progresiva con la maduración, siendo también la 9-*cis*-violaxantina la más abundante en naranjas, y la 9-*cis*-violaxantina y la β-criptoxantina en mandarinas (Alquezar et al., 2008a; Gross, 1987; Kato et al., 2004; Lux et al., 2019). Además, en la pulpa de algunas variedades de naranja, como Valencia late o Salustiana, la β-criptoxantina, puede representar hasta el 15% de los carotenoides en la pulpa (Alquézar et al., 2008a). En general, el perfil de carotenoides de las mandarinas es más complejo que el de las naranjas y en el fruto maduro se han detectado más de 20 carotenoides diferentes. En la pulpa de mandarinas Satsumas, la β-criptoxantina representa aproximadamente el 60% de los carotenoides totales (Alquézar et al. 2008a; Kato et al., 2004). Además, los carotenoides C30, β-citraurina y β-citraurineno son especialmente abundantes en la piel de las mandarinas y les proporcionan la coloración rojiza característica que las diferencia de otras especies de cítricos (Alquézar et al. 2008a; Kato et al., 2004; Rodrigo et al., 2013a; Zheng et al., 2019). Recientemente, estudios en distintas variedades de mandarina han reportado niveles de β-criptoxantina muy superiores en la variedad Nadorcott que en los híbridos Fortuna o Nova (Rey et al., 2020).

La acumulación de carotenoides está mayoritariamente condicionada por factores genéticos, sin embargo, las condiciones climáticas y de cultivo ejercen también una fuerte influencia en el contenido de estos (Dhuique-Mayer et al., 2009; Fanciullino et al., 2006). La combinación de todos estos factores es lo que determina la gran variedad en el perfil de carotenoides entre las diversas variedades de cítricos. Una clasificación clásica de los cítricos en función de la composición de carotenoides ha sido la siguiente: variedades con bajo contenido en carotenoides, alto contenido en violaxantina, alto contenido en β-criptoxantina y variedades que acumulan licopeno (Alquézar et al., 2008; Matsumoto et al., 2007).

1.2.5. Regulación de la acumulación de carotenoides en cítricos

La biosíntesis de carotenoides es un proceso complejo que comprende una serie de reacciones de desaturación, isomerización, ciclación, hidroxilación y epoxidación, tal y como se ha descrito anteriormente en el apartado 1.2.3 (Rodríguez-Concepción et al., 2018). En los frutos cítricos, la acumulación de carotenoides está regulada principalmente por cambios transcripcionales de los genes anteriormente mencionados y que explican las variaciones en el contenido y en el perfil de carotenoides que tienen lugar durante el desarrollo y maduración del fruto (Alquézar et al., 2008a; Ikoma et al., 2016; Kato et al., 2004; Rodrigo et al., 2004; Zhang et al., 2012; Ma et al., 2023). En las últimas décadas y con la implementación de las técnicas de secuenciación masiva y los proyectos de secuenciación de genomas de cítricos, se han identificado los genes que codifican para las principales enzimas de biosíntesis de carotenoides, así como su papel en la regulación de la acumulación de carotenoides.

En la ruta de biosíntesis de carotenoides, las enzimas con actividades PSY, β-LCY y β-CHX ejercen un papel regulador fundamental del flujo metabólico de la ruta. En etapas tempranas del desarrollo del fruto se encuentra fundamentalmente activa la rama ε,β de la ruta de carotenoides. Así, mientras la pulpa apenas acumula carotenoides, el flavedo del fruto inmaduro de color verde exhibe una composición de carotenoides típica de tejido cloroplástico, siendo la luteína el carotenoide mayoritario (Rodrigo et al., 2013a; Sun et al., 2018). Con el avance de la maduración, se produce un descenso importante en los niveles de luteína, junto con un aumento del contenido de β,β-xantofilas. Este aumento del contenido total de carotenoides y β,β-xantofilas se produce durante la transición del tejido cloroplástico a cromoplástico, y parece estar coordinada por el incremento en la expresión de genes de biosíntesis de carotenoides. Se ha observado una clara relación entre el mayor contenido de carotenoides en el flavedo y los mayores niveles de transcripción de *PSY* respecto a la pulpa. Por lo tanto, la inducción de la *PSY* durante la maduración del fruto parece ser uno de los factores limitantes de entrada de la metabolitos a la ruta y, junto con el aumento de la expresión de *β-CHX*, activa la síntesis de β,β-xantofilas y la acumulación de carotenoides en los frutos (Alquézar et al. 2008; Fanciullino et al. 2008; Kato et al. 2004; Rodrigo et al. 2004; Tao et al. 2007). Por otro lado, el redireccionamiento desde la rama ε,β a la rama β,β está coordinado en parte, por una marcada disminución en la expresión del gen *ε-LCY*, a la vez que se induce la expresión de *β-LCY*, lo que explica el aumento masivo de xantofilas (Kato et a., 2004; Rodrigo

et al., 2004). En cítricos, se han descrito dos enzimas β-LCY que catalizan la ciclación del licopeno en β-caroteno (Alquézar et al., 2009; Zhang et al., 2012; Lu et al., 2016). El gen *β-LCY1* se expresa en diferentes tejidos y exhibe una expresión relativamente constante durante la maduración del fruto. En cambio, *β-LCY2* se expresa preferencialmente en frutos, se induce fuertemente en el momento del cambio de color y está regulada positivamente durante la maduración. Además, se ha descrito la existencia de dos alelos del gen *β-LCY2* ('a' y 'b') con diferente capacidad para convertir licopeno en β-caroteno. En particular, el alelo 'b' exhibe una actividad para ciclar licopeno prácticamente nula (Alquézar et al., 2009; Lu et al., 2016). Por lo tanto, la expresión diferencial de la cada uno de los alelos de *β-LCY2* tiene un papel regulador esencial en la ruta, controlando el flujo hacia la rama β,β y por ende, la síntesis de β,β-xantofilas. Alteraciones en la expresión o función de este gen pueden conducir a importantes alteraciones en la composición de carotenoides en la pulpa de los frutos cítricos (Alquézar et al., 2009,2013; Lu et al., 2016; Lana et al., 2020; Promkaew et al., 2020; Tatmala et al., 2020). La comparación de frutos de pomelo amarillos (Marsh) y pomelo rojo (Star Ruby) reveló una menor expresión del gen *β-LCY2b* en el segundo que promovería la acumulación de licopeno en la pulpa de esta variedad de pomelo (Alquézar et al., 2013).

En la pulpa de naranjas y mandarinas, donde el contenido total de carotenoides es más bajo que en el flavedo, también se detecta una menor expresión de la mayoría de genes de la biosíntesis de carotenoides (Kato et al., 2004; Alquézar et al., 2008a; Ma et al., 2023). Estos resultados sugieren una compleja coordinación transcripcional entre las diferentes etapas de la ruta de carotenoides para determinar las variaciones en la concentración de carotenoides en los diferentes tejidos de los frutos y la diversidad del color. Así, por ejemplo, la acumulación de β-criptoxantina, que es uno de los carotenoides más interesantes para la salud y la calidad del fruto, no se explican solamente por los cambios en la expresión de la *β-CHX*, ya que la acumulación del transcrito es menor en las mandarinas que en las naranjas, cuando la acumulación de β-criptoxantina en la pulpa es contraria (Kato et al., 2004). La actividad CHX realiza una doble hidroxilación para formar zeaxantina, y la β-criptoxantina es el producto intermediario de la reacción (Ikoma et al., 2016). La comparación de los niveles de expresión de diferentes genes de carotenoides en frutos de distintas variedades de cítricos ha llevado a la conclusión de que en las mandarinas una mayor expresión de genes tempranos de la ruta (*PSY*, *PDS* y/o *ZDS*) y menor de *CHX* respecto a las naranjas, conduciría a una mayor

acumulación de β-criptoxantina (Kato et al., 2004; Ma et al., 2023). Por otro lado, la disponibilidad de mutantes con alteraciones en el perfil de carotenoides ha sido de gran relevancia para una mejor comprensión de la ruta. Por ejemplo, el mutante de naranja 'Pinalate' con frutos de color amarillo, presenta una inserción de un nucleótido en un exón del gen *Z-ISO* que genera una proteína truncada no funcional y, como consecuencia, produce un bloqueo parcial en la síntesis de ζ-caroteno con la consiguiente acumulación de isómeros *cis* de este caroteno, y de fitoeno y fitoflueno, así como niveles reducidos de xantofilas y de la hormona ácido abscísico (ABA) (Rodrigo et al., 2019).

Por otro lado, la acumulación de carotenoides también depende del balance entre la biosíntesis, acumulación en estructuras especializadas y su catabolismo o degradación. El catabolismo de carotenoides está mediado por una amplia familia de enzimas genéricamente denominadas oxigenasas de corte de carotenoides (del inglés `Carotenoid cleavage dioxyganses´ CCDs), que fragmentan los carotenoides en diferentes posiciones de la molécula para dar lugar a la formación de apocarotenoides (Walter & Strack, 2011). En los frutos cítricos se han establecido diversas subfamilias con esta actividad en función del sustrato y posición de corte en la molécula. La enzima CCD1 se expresa tanto en la pulpa como en flavedo, su expresión aumenta con la maduración y no parece tener un papel relevante regulando el contenido de carotenoides en frutos (Kato et al., 2006). Por su parte, la enzima CCD4b de cítricos cataliza la síntesis de los apocarotenoides de color naranja intenso C30, β-citraurina y β-apo-8'-apocarotenal, a partir de β-criptoxantina y zeaxantina, y su expresión aumenta de forma considerable en el flavedo de frutos cítricos con alta pigmentación, por lo tanto, su actividad está directamente relacionada en el color externo de naranjas y mandarinas (Rodrigo et al., 2013b; Zheng et al., 2019). La subfamilia de enzimas NCED reconoce exclusivamente 9-*cis*-epoxicarotenoides, como la *9-cis*-violaxantina y 9-*cis*-neoxatina, para dar lugar a la xantoxina (C15). Esta actividad enzimática es de gran importancia en el metabolismo celular vegetal, ya que la xantoxina es el precursor de la fitohormona ABA. En los cítricos se han identificado dos genes con expresión diferencial en el fruto, tanto durante la maduración como en distintas situaciones de estrés (Rodrigo et al., 2006).

La coloración naranja característica de las diferentes variedades de naranjas y mandarinas se debe a la acumulación de β,β-xantofilas, principalmente, que en su mayoría se encuentran esterificadas con ácidos grasos en los frutos maduros (Philip, 1973; Lux et al., 2019). La

esterificación de xantofilas con ácidos grasos aumenta su carácter lipofílico, lo que parece que es un requerimiento crucial para su almacenamiento en los cromoplastos, mediando en la estabilidad y biodisponibilidad de las xantofilas (Mariutti & Mercadante, 2018). En el genoma de los cítricos se ha descrito la presencia de 7 genes con elevada homología a las enzimas PYP/XES/XAT (Pale Yellow Petal/Xanthophyll Esterase) de tomate y pimiento implicadas en la esterificación xantofilas. Entre ellos, tan solo tres, *PYP1*, *PYP2* y *PYP6*, mostraron un patrón de expresión concordante con la acumulación de xantofilas esterificadas en la pulpa y, por lo tanto, representan los principales candidatos para la esterificación de xantofilas en cítricos (Zacarías-García et al., 2021).

1.3. Acumulación de licopeno en cítricos

Los cítricos pertenecen a la familia Rutaceae y son nativos de las regiones tropicales y subtropicales de Asia (Liu et al., 2012; Wu et al., 2018). A lo largo de la historia y domesticación del cultivo se han producido y seleccionado numerosas especies y variedades por hibridaciones espontaneas o dirigidas, o mutantes espontáneos por su alto grado de pigmentación, tanto en la piel como en la pulpa, que es una de las características más atractivas para los productores y los consumidores (Barry et al., 2020). Algunos de estos mutantes se distinguen por la acumulación de antocianos y se denominan naranjas sanguinas o de sangre (`blood oranges´) como, por ejemplo, la naranja Moro, Tarocco, Sanguinelli, entre otras. El otro grupo de cítricos pigmentados corresponde a aquellos con pulpa de color roja, por acumulación del caroteno licopeno.

La acumulación de licopeno es una característica inusual en los frutos cítricos y solamente se ha descrito en diferentes variedades de pumelo o zamboas (*Citrus grandis*), pomelos (*Citrus paradisi*), y más recientemente en algunas mutaciones de naranja (*Citrus sinensis*) y un mutante variegado de limón (*Citrus limon*). En la citricultura moderna y con la creciente competencia por variedades distintivas, una característica deseada es la acumulación de licopeno en la pulpa. La presencia de este caroteno, al margen de proporcionar una coloración roja, supone un valor adicional respecto a los frutos de las variedades tradicionales, ya que el licopeno exhibe una alta capacidad antioxidante, entre otros beneficios para la salud. Barry et al. (2020) en una revisión exhaustiva en el libro 'The genus *Citrus*' describen las principales variedades comerciales de cítricos en la actualidad, así como su origen y domesticación. En general, la

información sobre cítricos que acumulan licopeno y su origen es escasa, y a continuación, se describen las principales variedades y características de frutos de coloración roja en las diferentes especies de *Citrus*, así como las que se encuentran comercialmente disponibles en la actualidad.

1.3.1. Pumelos, pomelos y limones

El pumelo o zamboa (*Citrus grandes* o *Citrus maxima*) es una de las especies de cítricos descritas como ancestrales, y las hibridaciones acontecidas durante la evolución y domesticación han dado lugar a los pomelos, naranjas dulces y las mandarinas modernas (Wu et al., 2014, 2018). Las diferentes variedades de pumelos existentes en la actualidad son originales y se producen en su mayoría en China y países del sureste asiático. Las diferentes variedades de pumelos varían en cuanto al color interno y externo, tamaño y forma, entre otras características. De manera práctica, las variedades de pumelos se clasifican en función de la pigmentación de la pulpa: no pigmentadas o blancas y pigmentadas. La mayoría de las variedades comerciales actuales derivan de un ancestro común no pigmentado, probablemente de origen siamés/tailandés (Wu et al., 2018). Las variedades de pomelo pigmentadas se clasifican en función del grado de pigmentación, como ligera, pigmentada o altamente pigmentada. Dentro de las ligeramente pigmentadas, Pingshan es una las variedades más comunes y conocidas en China. Su cultivo se remonta a la dinastía Song, hace 600 años, y en la actualidad se produce principalmente en la provincia de Fujian, de donde es original. En la actualidad las variedades más pigmentadas de pumelo son las que gozan de mayor interés comercial. Así, Hongroumi, mutante de pulpa roja de Guanximi, ha reemplazado a la variedad original como más producida en la provincia de Fujian, debido a una mayor preferencia por la pulpa de color rojo por parte del consumidor. La variedad Chandler, es una de las variedades de pumelo más conocidas en el mundo y fue originada a partir de un híbrido de las variedades parentales Siamese Sweet y Siamese Pink en 1961 en California. Por otro lado, y en ese mismo año, la variedad Hirado Buntan fue importada desde China a Estados Unidos y una selección actual de la variedad original representa el cultivar de pumelo más común en el estado de Florida.

Existen claras evidencias que indican que el pomelo (*Citrus paradisi*) es la única especie de cítrico originada en América, como un híbrido interespecífico entre pumelo y naranja dulce,

posiblemente en las Islas del Oeste en el Caribe. La primera mención al pomelo data de 1750 en las islas Barbados, pero su nombre y características las define Macfadyen en 1830, a partir del árbol cítrico de Jamaica referido como 'el olvidado'. A nivel comercial y en comparación con otras especies de cítricos, el pomelo es relativamente reciente. Gmitter (1995) ha establecido un árbol de la filogenia del origen y descendencia de gran número de variedades de pomelos de los que se tiene certeza, desde su introducción en EEUU, entre los que se incluyen las distintas variedades y mutaciones que se han identificado y seleccionando tanto de forma espontánea como en los distintos programas de mejora y de obtención de variedades. De forma resumida se puede indicar que la variedad de pomelo Duncan se introdujo en Estados Unidos en 1823 y representa el origen del que han surgido posteriormente todas las variedades de pomelo desarrolladas. En este sentido, en el estado de Texas se han desarrollado la mayoría de cultivares de pomelos que acumulan licopeno, disponibles en la actualidad. A partir del pomelo Duncan se desarrollaron dos pomelos blancos, Walters, de gran relevancia histórica en cuanto al desarrollo de subsiguientes variedades rojas, pero de casi nula implantación comercial actual; y el pomelo Marsh (1860), de mayor relevancia comercial. Aunque existen ciertas dudas, se considera que la primera descripción de un pomelo rojo se produjo en una plantación de pomelo Marsh en 1913 y se denominó Thompson Pink o Marsh Pink. Otra variante roja fue el pomelo Shambar (1936) del que no se han descrito nuevas variedades derivadas. A partir del pomelo coloreado Thompson surgieron otras mutaciones espontaneas con mayor acumulación de licopeno en la pulpa, como Ruby Red, que posteriormente fue el origen de otras variedades con diferente intensidad de coloración en la pulpa, que a su vez generaron otras selecciones más recientes; como Hendenson del que se originó Flame, Ray Ruby del que derivó Nel Ruby o A&I 1-48 que por irradiación originó Rio Red. Por otro lado, una mutación espontánea del pomelo blanco Walters dio lugar a la variedad Foster, ligeramente coloreada, que a su vez en 1930 dio lugar a otra mutación espontánea, Hudson, con una coloración de la pulpa roja intensa, pero con muchas semillas. Posteriormente, Hensz en 1959, irradiando semillas de pomelos Hudson, desarrolló una variedad sin semillas y con un alto nivel de pigmentación en la pulpa a la que se denominó Star Ruby. Esta variedad fue propagada en 1970 y se ha convertido en la principal variedad comercial de pomelo del mundo. Además, en las últimas décadas se han ido desarrollando y

propagando nuevas selecciones de pomelo en la búsqueda de nuevas variedades con mayor grado de coloración (Gmitter, 1995).

El limón (*Citrus limon*), un híbrido entre naranja amarga y cidro (Curk et al., 2016; Wu et al., 2018), se cultiva mayoritariamente en regiones más cálidas de países de la cuenca mediterránea, California y Sudáfrica, o Argentina, que en la actualidad es el mayor productor mundial de limones. Debido a la naturaleza poliembriónica de la semilla del limón es extremadamente difícil obtener nuevas variedades por hibridación y, por tanto, la mayoría de las variedades comerciales actuales de limón se han obtenido por selección de mutaciones naturales o inducidas de las variedades originales. La variedad Eureka se describió por primera vez en California en 1858 y, se seleccionó y propagó posteriormente (Barry et al., 2020). En la actualidad se tiene constancia de una sola variedad de limón con coloración rojiza, el limón variegado de pulpa rosa, mutación espontánea de la variedad Eureka descrita por primera vez en 1932 (Shamel, 1932). La pulpa de esta variedad exhibe una coloración rosada pálida en plena madurez, contiene pocas semillas y alta acidez, y la piel es de tipo variegada, con sectores longitudinales verdes y amarillos en frutos inmaduros, que tras el cambio de color durante la maduración pasan a sectores de tonalidad amarilla y rosada, respectivamente (Lana et al., 2020).

La coloración roja en los frutos cítricos, generalmente en la pulpa o vesículas de zumo y en menor medida en la piel o flavedo, se debe a la acumulación de licopeno. Los trabajos pioneros de Kahn & Mackinney (1953) y Lime et al. (1957) reportaron que los principales carotenoides en el pomelo rojo Ruby Red eran licopeno y β-caroteno. Posteriormente, numerosos trabajos han estudiado el perfil y contenido en carotenoides en la piel y la pulpa de las diferentes variedades de pomelo, confirmando los resultados iniciales y determinando los cambios cuantitativos y cualitativos durante la maduración en frutos de diferentes localizaciones, zonas productivas, condiciones climáticas, etc., tal como se recoge en diferentes revisiones (Gross et al., 1987; Rouseff et al., 1992; Alquézar et al., 2008a). El efecto de la temperatura ambiental es una de las características más particulares en cuanto a la acumulación de licopeno en los cítricos, ya que entre 21 °C y 35 °C se promueve la acumulación del pigmento y se pierde a temperaturas entre 20 y 4 °C (Meredith & Young, 1969). Esta característica puede explicar las diferencias entre las determinaciones en carotenoides y licopeno en los distintos trabajos, y entre una misma variedad crecida en diferentes localizaciones, y explica el concepto de que

los pomelos de pulpa roja cultivados en zonas más cálidas alcanzan una coloración roja más intensa y mayor contenido en licopeno.

Debido al interés que han suscitado las nuevas variedades de cítricos, en los últimos 10-15 años numerosos estudios han evaluado el contenido y composición de carotenoides en comparación con las variedades parentales y/o tradicionales. Una selección reciente de variedades de pumelos o zamboas del Banco de Germoplasma del Instituto Valencia de Investigaciones Agrarias de Valencia (IVIA) refleja la variabilidad en cuanto a la coloración interna entre las variedades rojas y blancas (Figura 3, cedida por la Dra. Gema Ancillo, IVIA).

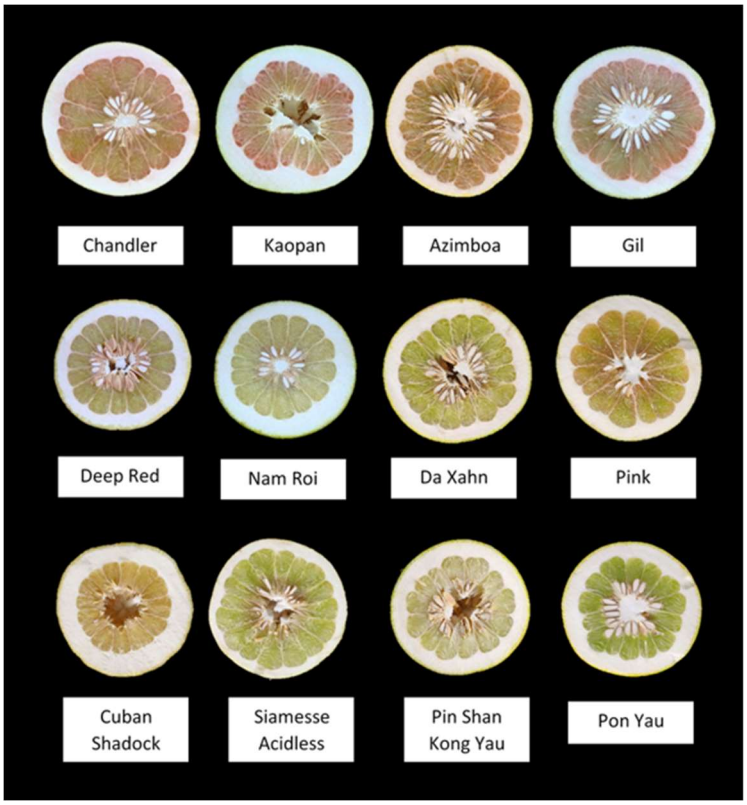

Figura 3. Aspecto de la pulpa en secciones transversales de frutos en una selección de 12 variedades de pumelo/zamboa (*Citrus grandis*), donde se aprecia la diversidad en la coloración entre variedades pigmentadas y no pigmentadas procedentes del Banco de Germoplasma del Instituto Valenciano de Investigaciones Agrarias. Imagen cedida por Dra. Gema Ancillo (IVIA).

1.3.2. Composición de carotenoides en variedades de pumelo y pomelo de pulpa roja

Los diferentes estudios en variedades de pumelo y pomelo indican que el perfil de carotenoides en el flavedo de las variedades de pulpa roja y blanca es relativamente similar, siendo el fitoeno, β-caroteno, violaxantina y luteína los carotenoides principales (Alquézar et al., 2008; Rodrigo et al., 2013a). Sin embargo, mientras que el contenido total de carotenoides en el flavedo de pumelos y pomelos blancos es superior respecto a la pulpa; en las variedades de pulpa roja ocurre lo contrario y la pulpa presenta niveles de carotenoides sustancialmente más altos que la piel, especialmente de carotenos (Xu et al., 2006; Yan et al., 2018). En cuanto a los niveles de carotenoides en la pulpa, los pumelos rojos acumulan cantidades notablemente más elevadas que las variedades blancas, especialmente licopeno y β-caroteno. Así, mientras que los pumelos blancos acumulan niveles bajos de carotenoides (<1-3 μg/g peso seco), los pumelos de pulpa roja, como por ejemplo Guanxi, Chuzou early red y Chuhong, acumulan licopeno y β-caroteno, seguido de fitoeno y muy bajas concentraciones de xantofilas (Xu et al., 2006; Liu et al., 2016; Yan et al., 2018). Además, la acumulación de carotenoides durante la maduración sigue patrones diferentes entre las variedades pigmentadas y no pigmentadas. Así, el contenido de carotenoides desciende durante la maduración en el pumelo blanco, mientras que en el rojo se observa un incremento drástico de los niveles de licopeno, β-caroteno y fitoeno. El licopeno se detecta a niveles muy bajos desde estados tempranos del desarrollo y su contenido aumenta de forma regular a lo largo de la maduración, alcanzándose niveles máximos en fruto maduro (Liu et al., 2016; Yan et al., 2018; Tatmala et al., 2020).

Los diferentes estudios realizados respecto al contenido y composición de carotenoides en frutos de pomelo revelan profundas diferencias entre las variedades blancas o no pigmentadas y las pigmentadas, pero también entre las diferentes variedades de pulpa roja, debido principalmente al amplio rango en la acumulación de licopeno (Gross et al., 1987; Alquézar et al, 2013). La Figura 4 ilustra las diferencias en la coloración de la pulpa entre una selección de 10 variedades de pomelo y 2 híbridos de pomelo x pumelo (Melogold y Oroblanco) del Banco de Germoplasma del IVIA. De forma general, el contenido de carotenoides entre variedades de pomelo con distinta pigmentación pone de manifiesto que, tanto en el flavedo como en la pulpa de los pomelos rojos, los carotenos son los pigmentos más abundantes, principalmente fitoeno, fitoflueno, licopeno y β-caroteno. En general, las variedades de pomelo rojo más

conocidas como Star Ruby, Ray Ruby y Flame acumulan concentraciones de carotenoides mucho más elevadas en la pulpa que las variedades no pigmentadas, como Marsh (Khan & Mackinney, 1953; Fanciullino et al., 2006; Xu et al., 2006; Alquézar et al., 2009, 2013; Lado et al., 2015). En función del contenido de licopeno, se ha descrito que los pomelos Flame y Star Ruby son los que presentan los niveles más alto de este caroteno, mientras que Ray Ruby acumula concentraciones significativamente inferiores (Fanciullino et al., 2006; Xu et al. 2006; Zhang et al., 2019). El análisis de carotenoides a lo largo del proceso de desarrollo y maduración en variedades de pomelo revela cambios notables. Así, el estudio comparativo entre el pomelo rojo Star Ruby y el blanco Marsh, indica que el flavedo de ambos en estados tempranos del desarrollo presentaba un perfil de carotenoides típico de tejido cloroplástico, mayoritariamente formado por xantofilas, siendo la luteína el más abundante. Con el cambio de color y la pérdida de clorofilas también disminuyó el contenido de xantofilas y, en el caso de Marsh, de carotenoides totales, pero los carotenos lineales aumentaron significativamente en la piel del pomelo Star Ruby.

Figura 4. Aspecto de la pulpa en secciones transversales de frutos en una selección de 10 variedades de pomelo (*Citrus paradisi*) y dos híbridos de pomelo (Oroblanco y Melogold) donde se aprecia la diversidad en la coloración entre variedades pigmentadas y no pigmentadas. Todos los frutos proceden del Banco de Germoplasma del Instituto Valenciano de Investigaciones Agrarias. Imagen cedida por Dra. Gema Ancillo (IVIA).

Por su parte, la pulpa de ambos pomelos presentaba carotenoides a niveles de trazas en estados iniciales del desarrollo, que en Marsh apenas sufrieron cambios durante la maduración. Sin embargo, en el pomelo Star Ruby se detectó un incremento notable de fitoeno y fitoflueno, pero sobre todo de licopeno, que alcanzó niveles especialmente elevados en frutos cercanos a su estado de madurez comercial (Alquézar et al., 2013; Lado et al., 2015). A diferencia de lo que se ha descrito en pumelos, los resultados de los estudios en variedades de pomelo rojo parecen indicar que la acumulación de licopeno se encuentra asociada al aumento en el contenido de carotenoides característico durante la maduración del fruto, proceso que no se produce en los pomelos blancos.

1.3.3. Naranjas

La naranja dulce (*Citrus sinensis*) se originó a consecuencia de un retrocruzamiento entre un híbrido de mandarina (ancestral extinto) y pumelo, y a su vez con otro pumelo (Xu et al., 2013). La amplia gama de variedades y cultivares de naranja actuales son el resultado de la selección y domesticación durante los últimos miles o cientos de años. En la actualidad, todos los genotipos de naranjas existentes son fruto de mutaciones seleccionadas y desarrolladas a través de mecanismos naturales o inducidos (Barry et al., 2020). El primer caso documentado de naranja con coloración rojiza en la pulpa, denominada como Sarah, data del año 1961 y se originó mediante una mutación espontánea de la naranja Shamouti (Monselise & Halvey, 1961). Posteriormente, en 1976 se identificó en Venezuela una nueva mutación espontánea de la naranja común Washington Navel, denominada Cara Cara (Barry et al., 2020), y la naranja Puka, mutante de naranja Valencia, identificada en Argentina, pero de la que se dispone muy escasa información (Barry et al., 2020). En la actualidad, Cara Cara es la única variedad de naranja de pulpa roja disponible para su libre producción y comercialización. Años más tarde, Liu et al. (2007) describieron un nuevo mutante espontáneo de pulpa roja derivado de la variedad de naranja china Anliu, denominado Hong Anliu o Red-Anliu.

El creciente interés por nuevas variedades de naranja pigmentadas ha llevado a diferentes grupos de investigación a estudiar en profundidad el contenido y composición de Cara Cara y Red Anliu, ya que han sido las únicas disponibles en bancos de Germoplasma o plantaciones comerciales hasta hace pocos años. Diferentes estudios han analizado el perfil de carotenoides, así como su patrón de acumulación en los diferentes tejidos del fruto en comparación con las

variedades parentales u otras variedades tradicionales. Considerando el rango de carotenoides descrito para diferentes variedades de naranja, la variedad Anliu y su mutante rojo acumulan concentraciones consideradas como bajas o intermedias. En estados iniciales del desarrollo, los niveles de carotenoides fueron muy similares entre ambos genotipos y aumentaron ligeramente durante la maduración en la naranja parental Anliu por la acumulación de las xantofilas β-criptoxantina y violaxantina. En la pulpa de Red Anliu, sin embargo, el contenido de carotenoides se multiplicó por 4-5 veces, principalmente por la acumulación de licopeno. Así, el mutante Red Anliu presentaba alrededor del doble de carotenoides totales en la pulpa del fruto maduro respecto a la variedad parental. A diferencia de otras variedades de cítricos de pulpa roja, en este mutante no se detectaron concentraciones elevadas de otros carotenos lineales (Liu et al., 2007).

Washington Navel es una de las principales variedades de naranjas del grupo Navel en el mundo y a partir de ella se han obtenido diferentes selecciones que constituyen algunas de las principales variedades cultivadas a nivel mundial y en la Comunidad Valenciana, como Navelina, Lane Late, Newhall, Palmer, Powell, Foios, entre otras (Barry et al., 2020). En la naranja Cara Cara (Figura 5), derivada de Washington Navel, a pesar de que el contenido individual de carotenoides oscila entre los distintos trabajos, todos coinciden en que la pulpa contiene niveles de carotenoides totales muy superiores respecto a las variedades estándar Navel. Entre estos, el fitoeno es el carotenoide mayoritario, acumulando también cantidades menores de fitoflueno y licopeno, y bajas concentraciones de β-caroteno. En particular, solo el fitoeno puede llegar a representar más 80% del total, y la suma de los carotenos lineales representa entre el 90-99% del contenido total de carotenoides (Alquézar et al., 2008b; Cilla et al., 2018; Lu et al., 2017; Rodrigo et al., 2015). Sin embargo, en los diferentes trabajos se encuentran discrepancias en cuanto al contenido de xantofilas, ya que algunos trabajos han detectado concentraciones ligeramente inferiores de violaxantina en Cara Cara (Alquézar et al., 2008; Xu et al., 2016), mientras que otros no han observado diferencias significativas (Lu et al., 2017; Rodrigo et al., 2015, Cilla et al., 2018). Aunque las principales diferencias entre Cara Cara y las variedades de naranja común se localizan en la pulpa, también es importante destacar que el flavedo de Cara Cara presenta cantidades de fitoeno inusualmente elevadas (Alqúezar et al., 2008; Lu et al., 2017; Xu et al., 2006).

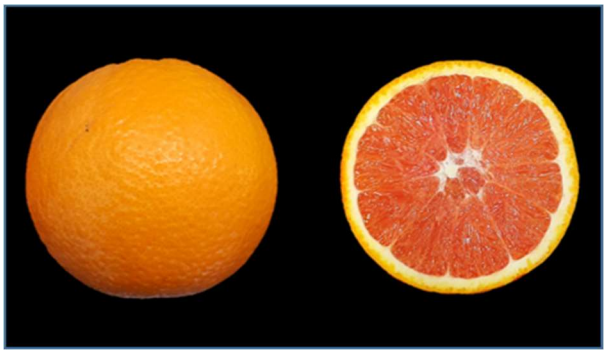

Figura 5. Aspecto externo e interno de la variedad de naranja dulce (*Citrus sinensis*) Cara Cara.

Otros trabajos han analizado el contenido y composición individual de carotenoides a lo largo del proceso de desarrollo y maduración del fruto en los frutos de Cara Cara (Alquézar et al., 2008; Lu et al., 2017; Liu et al., 2021). En comparación con las naranjas comunes, Cara Cara presenta una coloración sustancialmente distinta en la pulpa desde estados muy tempranos del desarrollo (Alquézar et al., 2008; Lu et al., 2017). Esta inusual pigmentación temprana de la pulpa está asociada a la acumulación de licopeno, pero también a importantes concentraciones de carotenos incoloros. Así, el contenido de fitoeno (10-60 μg/g), fitoflueno (1-6 μg/g) y licopeno (1-12 μg/g) aumentó progresivamente durante la maduración, alcanzando los máximos niveles en el fruto maduro (Alquézar et al., 2008; Lu et al., 2017). Por otro lado, estudios complementarios han evaluado el efecto de la conservación postcosecha en frío (4 °C) y a temperatura ambiente (20 °C) en el contenido de carotenoides en Cara Cara. Así, el contenido de carotenoides en la piel se incrementa a temperatura ambiente, mientras que en la pulpa permanece inalterada hasta un mes de conservación. En cambio, a bajas temperaturas la pulpa experimenta un aumento considerable de la concentración de fitoeno y β-caroteno a la par que no hay cambios significativos en otros carotenoides, incluido el licopeno (Tao et al., 2012).

A pesar de que las naranjas Navel se destinan mayoritariamente para el consumo en fresco, la llamativa coloración y el alto contenido en carotenoides de la pulpa de Cara Cara ha suscitado el interés en caracterizar las propiedades y la composición del zumo de esta variedad. Tanto el zumo fresco como procesado (homogeneizado a altas presiones hidrostáticas y pasteurizado) de Cara Cara presenta un perfil de carotenoides muy similar al descrito anteriormente para la pulpa, y contiene entre 4 y 5 veces mayor concentración de carotenoides respecto a zumos de otras naranjas Navel. Los valores de carotenoides oscilan

ligeramente entre los diferentes análisis, sin embargo, en todos los casos, el fitoeno es el carotenoide mayoritario en zumos de Cara Cara (8-14 μg/ml), seguido de licopeno (2-5 μg/ml), xantofilas (2-5 μg/ml) y fitoflueno (0.5-4 μg/ml). En cambio, el zumo de naranjas Navel comunes como Washington o Bahía se componen casi exclusivamente de xantofilas (4-7 μg/ml). Estos resultados evidencian que el zumo de Cara Cara tiene un perfil de carotenoides similares al de la pulpa, con concentraciones inferiores de carotenoides de color naranja, como las xantofilas, respecto a las naranjas estándar, y altas concentraciones de carotenos lineales (Rodrigo et al., 2015; Brasili et al., 2017; De Ancos et al., 2017, 2020).

1.4. Mecanismos moleculares de la acumulación de licopeno en los frutos cítricos

El licopeno es un intermediario en la ruta de carotenoides que conduce a la síntesis de xantofilas y finalmente ácido abscísico. El licopeno se cicla en sus extremos por la acción de las enzimas ε-LCY/ β-LCY y β-LCY/ β-LCY, para generar α- y β-caroteno, respectivamente. Esta reacción es un paso clave en la síntesis y regulación de carotenoides en plantas, en general, y en cítricos en particular (Cunningham, 2002; Alquézar et al., 2009). El número limitado de variedades de pulpa roja y la baja frecuencia con la que surgen nuevas mutaciones espontáneas que acumulan licopeno sugiere que la reacción de ciclación de licopeno es altamente eficaz y estrictamente regulada en los cítricos. Por tanto, es lógico asociar la acumulación de este caroteno a una menor o alterada/deficiente actividad licopeno ciclasa u otros procesos asociados a la misma. Sin embargo, cabe destacar que la acumulación de licopeno no es la única consecuencia, aunque sí la más llamativa, en los frutos cítricos de pulpa roja, especialmente en las naranjas, ya que también acumulan concentraciones inusualmente elevadas de otros carotenos lineales anteriores en la ruta de biosíntesis, como el fitoeno, fitoflueno y, en menor medida, β-caroteno. Así, la mutación que genera el fenotipo rojo no solo produce acumulación de licopeno sino también otros carotenos tempranos de la ruta que se acumulan en bajas concentraciones o incluso no se detectan en las variedades originales. Los resultados obtenidos en los diferentes mutantes rojos de cítricos indican que tanto el contenido como la composición de carotenoides es distinta entre los diferentes mutantes, así como las diferencias respecto a las variedades parentales. Por tanto, es razonable asumir que los mecanismos bioquímicos y moleculares que llevan a estas alteraciones en la composición de carotenoides pueden ser diferentes entre los distintos mutantes y que, alternativamente,

distintas mutaciones (o alteraciones moleculares) pueden conducir a la misma característica fenotípica de acumulación de licopeno y otros carotenos (Alquézar et al., 2008b, 2009, 2013; Lu et al., 2017; Yan et al., 2018; Lana et al., 2020; Tatmala et al., 2020).

Con el ánimo de dilucidar y caracterizar estos mecanismos, diferentes trabajos han llevado a cabo análisis comparativos de la expresión de genes del metabolismo de carotenoides entre variedades rojas y variedades tradicionales, así como la secuenciación de los genes que codifican para las enzimas licopeno ciclasas en cítricos. En este apartado se exponen los principales resultados de estos estudios transcriptómicos en diferentes mutantes de pulpa roja, así como los potenciales mecanismos moleculares asociados a la acumulación de licopeno.

1.4.1. Pumelos, pomelos y limones

La principal característica que diferencia a los pumelos de pulpa roja de los de pulpa blanca es la acumulación de licopeno y β-caroteno en los primeros (Alquézar et al. 2009; Xu et al., 2006; Yan et al., 2018). Liu et al. (2016) observó diferencias en 4 aminoácidos en la secuencia del gen *β-LCY* entre la pulpa del pumelo red Guanxi y su variedad parental, además de menores niveles de expresión de este gen en el pumelo rojo. Yan et al. (2018) sugiere que la combinación de niveles superiores de mRNA del gen *ZDS* junto con la menor abundancia de transcritos de los genes *β-CHX* y *ZEP* son los responsables de las diferencias en el contenido de carotenoides entre el pumelo rojo Chuhong y el pomelo blanco Feicui. Así, los estudios transcriptómicos en pumelos rojos indican que el equilibrio entre una regulación positiva de genes de biosíntesis de carotenos, junto con una regulación negativa de la transcripción de genes por debajo del licopeno en la ruta, especialmente *β-LCY* y *β-CHX*, durante la maduración, puede ser la principal causa de la acumulación de licopeno en pumelo (Liu et al., 2016; Promkaew et al., 2020; Tatmala et al., 2020).

Los pomelos son junto con sus ancestros pumelos/zamboas las especies de cítricos con mayor número de mutantes/variedades de coloración roja. Así, el color de las diferentes variedades de pomelos varía entre rosado tenue y rojo intenso (Figura 4). Además, en ocasiones la piel también exhibe matices rojos, aunque de menor intensidad que la pulpa. El gen *β-LCY2* se expresa preferencialmente en tejidos cromoplásticos, debido a que se induce fuertemente durante el proceso de diferenciación de los cromoplastos que se asocia con el cambio de color y el aumento de carotenoides durante la maduración (Alquézar et al., 2009;

Sadali et al., 2019). En general, parece que los pomelos rojos presentan una menor expresión del gen *β-LCY2* en comparación con los pomelos blancos. Además, los pomelos rojos Star Ruby y Flame muestran una expresión preferencial por el alelo no funcional *β-LCY2b*, aproximadamente un 70% superior respecto al alelo de mayor actividad *β-LCY2a* (Mendes et al., 2011; Alquézar et al., 2013). La combinación de estos dos factores parece ser una característica común en los pomelos rojos. En cambio, las naranjas y mandarinas poseen, en general, una mayor expresión del gen *β-LCY2* y del alelo de mayor actividad 'a', en particular, lo que confiere una mayor y más eficiente capacidad para la conversión de licopeno hacia xantofilas (Alquézar et al. 2009; Zhang et al. 2012). Por tanto, la combinación de estas dos evidencias sugiere que los niveles de expresión de *β-LCY2* y la expresión diferencial de los alelos es un factor clave en la acumulación de licopeno en los pomelos rojos.

1.4.2. Naranjas

Hasta la realización de la presente Tesis Doctoral, solo se han caracterizado en profundidad los mutantes de naranja dulce Red Anliu y Cara Cara. Sin embargo, el análisis de la composición de carotenoides entre ambas variedades es completamente distinto. Así, mientras que Red Anliu acumula concentraciones bajas de licopeno y casi exclusivamente al final de la maduración (Lui et al., 2007), Cara Cara muestra un perfil mucho más complejo y acumula niveles muy superiores de carotenoides totales. Esta naranja acumula licopeno (trazas) y concentraciones inusualmente elevadas de carotenos fitoeno y fitoflueno desde el inicio del desarrollo del fruto que aumentan progresivamente durante la maduración (Alquezar et al., 2008b). Por tanto, los resultados obtenidos respecto al contenido y composición de carotenoides en ambos mutantes de pulpa roja indican que los mecanismos bioquímicos responsables de la alteración en la composición de carotenoides parecen ser distintos entre ambos genotipos. En este apartado se exponen los principales resultados de los estudios transcriptómicos y moleculares transcriptómicos y moleculares realizados en ambas variedades, así como las hipótesis variedades, así como las hipótesis sugeridas para explicar los mecanismos implicados en la acumulación de licopeno.

Lu et al. (2016) mediante ensayos de complementación y secuenciación del gen *β-LCY2b* en diferentes especies, sugirió que mutaciones en dos aminoácidos, en particular en la posición 72 y 359, suponen modificaciones críticas para la correcta actividad enzimática licopeno ciclasa

en la naranja Red Anliu. Además, la naranja Red Anliu presenta bajos niveles de transcritos del gen *β-LCY2* y una expresión preferente del alelo no funcional 'b' respecto a la variedad parental, similar a la situación descrita para los pomelos rojos. A pesar de estas evidencias, la secuencia de los promotores es idéntica entre ambas, por tanto, la diferencia en los niveles de expresión entre genotipos podría estar regulada por otros mecanismos adicionales, como metilación del DNA o factores de transcripción (Lu et al., 2016). En consecuencia, la combinación de niveles de expresión reducidos de *β-LCY2a*, junto con una expresión predominante del alelo 'b' sobre el 'a' parece ser el principal mecanismo de acumulación de licopeno en la pulpa del mutante Red Anliu.

Otros estudios transcriptómicos y proteómicos realizados en la naranja Red Anliu han aportado datos interesantes sobre potenciales diferencias en el metabolismo de este genotipo respecto al parental Anliu. Debido al carácter pleiotrópico de la mutación, se han identificado diversas rutas y procesos metabólicos alterados en el mutante, como la biosíntesis de isoprenoides, ácidos tricarboxílicos, glucólisis/gluconeogénesis y otros procesos del metabolismo primario que tienen lugar en la mitocondria como la cadena de transporte de electrones y la fosforilación oxidativa (Liu et al., 2019; Liu et al., 2022; Pan et al., 2009; Xu et al., 2009; Yu et al., 2012). Genes asociados a la ruta de las pentosas fosfato también se encuentran inducidos en el mutante. Esta ruta es una fuente importante de NADPH para muchos procesos biosintéticos, incluidos la biosíntesis de carotenoides (Yu et al., 2012). Pan et al. (2009) identificó 74 proteínas que se expresaban diferencialmente entre Red Anliu y su variedad parental. La mayoría de ellas se encuentran relacionadas con la respuesta al estrés, regulación de carbohidratos y metabolismo energético, y modificación/degradación de proteínas. Además, las actividades de 5 importantes enzimas antioxidantes (ascorbato peroxidasa, catalasa, superóxido dismutasa, glutatión peroxidasa y glutamato descarboxilasa) se encontraban aumentadas en Red Anliu, sugiriendo la existencia de un mayor estrés oxidativo. El tratamiento de muestras de pulpa de ambos genotipos con terc-butilo hidroperóxido (tBOOH) (inductor de ROS) incrementó significativamente el contenido de licopeno en el mutante, pero no en la variedad parental. Los autores sugieren que el mayor estrés oxidativo observado en el mutante Red Anliu puede ser la causa principal de la alteración en la carotenogénesis y, por ende, de la acumulación de licopeno (Pan et al., 2009).

La pulpa del fruto maduro Cara Cara contiene entre 10 y 25 veces mayor contenido total de carotenoides que la de variedades Navel tradicionales (Alquézar et al., 2008; Lu et al., 2017; Rodrigo et al., 2015) (Figura 5). No obstante, Cara Cara presenta un perfil de carotenoides totalmente alterado desde estados muy tempranos del desarrollo, con concentraciones muy elevadas de fitoeno y trazas de licopeno, cuyo contenido aumenta a lo largo de la maduración. La enzima fitoeno sintasa (PSY) cataliza la producción del primer caroteno de la ruta, fitoeno, y está considerada como el principal punto de control metabólico de la síntesis de carotenoides (Rodriguez-Concepción et al., 2018). Estudios transcripcionales del gen *PSY* indican que este gen no se encuentra sobre-expresado en Cara Cara y, por tanto, no parece la causa de la acumulación de las altas concentraciones de fitoeno (Alquézar et al. 2008; Lu et al. 2017). Además, ensayos con inhibidores enzimáticos de la actividad fitoeno desaturasa (PDS) y β-LCY, norflurazona y CPTA, respectivamente, han obtenido fenotipos similares entre Cara Cara y naranjas Navel, demostrando que la actividad PSY tampoco es superior en la naranja de pulpa roja (Lu et al., 2017). Otros autores han sugerido que la acumulación de licopeno podría generar una retroalimentación positiva y conducir a la acumulación de fitoeno (Tao et al., 2007; Alquézar et al., 2008), cómo ha sido descrito en tomate (Giuliano et al., 1993). Por otro lado, una mayor expresión de genes de la ruta MEP podría incrementar el flujo metabólico y disponibilidad de precursores isoprenoides para la síntesis de carotenoides (Alquézar et al., 2008; Wang et al., 2021). Además, se han detectado niveles reducidos de ABA no solo en Cara Cara (Alquézar et al., 2008; Liu et al., 2021) sino también en el pomelo Star Ruby (Alquézar et al., 2013). Por tanto, niveles inferiores de esta hormona parecen ser una característica común en los mutantes de pulpa roja. En cambio, no hay un consenso claro de si la acumulación de licopeno en las naranjas rojas está asociada con una reducción del contenido de β,β-xantofilas, ya que se han observado resultados contradictorios entre los diferentes trabajos (Liu et al., 2007; Alquézar et al., 2008; Lu et al., 2017; Liu et al., 2021; Rodrigo et al., 2015; Xu et al., 2006). En definitiva, se han descritos varios mecanismos moleculares como posibles causas de las diferencias en el contenido y composición de carotenoides en estas variedades, como alteraciones en la regulación de la expresión génica, mutaciones en las secuencias génicas de enzimas de biosíntesis y/o modificaciones postranscripcionales. En general, los estudios de expresión de genes del metabolismo de carotenoides han apuntado que no parecen haber importantes diferencias entre Cara Cara y las naranjas Navel comunes (Alquézar et al., 2008;

Lu et al., 2017). En la misma línea, los análisis de las secuencias de los genes que codifican para las enzimas LCY no han mostrado diferencias entre los distintos genotipos (Lu et al., 2017). En su conjunto, estos resultados indican que la alteración en el contenido de carotenoides en Cara Cara no parece estar asociada ni con diferencias de expresión, ni en las secuencias genómicas de genes del metabolismo de carotenoides, lo que hace especular con otros factores y/o mecanismos alterados que, en la actualidad, son desconocidos.

1.5. Caracterización de compuestos de calidad y estudios sobre las propiedades nutricionales de las naranjas de pulpa roja

Además de los carotenoides, la naranja dulce es reconocida por ser una importante fuente de micro- y macronutrientes como minerales, azúcares, ácido fólico y vitaminas C y E, y otros compuestos bioactivos de interés para la salud como compuestos fenólicos (ácidos fenólicos y flavonoides) y limonoides, entre otros (Lv et al., 2015). Existen escasos trabajos en los que se haya evaluado el contenido de otros compuestos bioactivos en la pulpa y el zumo de naranjas de pulpa roja, especialmente la vitamina C y compuestos fenólicos, y su capacidad antioxidante en relación con variedades de naranja común (De Ancos et al., 2017, 2020; Brasili et al., 2017; Cilla et al., 2018). En especial, la variedad de naranja de pulpa roja Cara Cara ha sido la que ha recibido mayor atención en los últimos años. En general, la naranja Cara Cara presenta niveles similares, a excepción de carotenoides, de compuestos bioactivos en comparación con otras naranjas Navel. Por ejemplo, en algunos trabajos el contenido de vitamina C en la pulpa y zumo de Cara Cara osciló entre un 10-15% inferior respecto al de Navel (De Ancos et al., 2017, 2020) y hasta un 40% en otros estudios (Kafkas et al., 2011).

Por otro lado, el perfil de flavonoides en Cara Cara es muy similar al descrito para las naranjas comunes, siendo las flavononas los principales compuestos fenólicos En particular, la hesperidina, es el flavonoide mayoritario seguido de la narirutina y de otros flavonoides minoritarios como la rutina, eriocitrina y didimina (Gattuso et al., 2007, Chen, et al., 2015, Zhang et al., 2021). Sin embargo, en otros trabajos se han observado ligeras diferencias en cuanto al contenido total e individual de flavonoides entre Cara Cara y la naranja Navel. Así, Cara Cara presenta un contenido ligeramente superior de flavonoides totales y en particular, los niveles de la flavonona mayoritaria, hesperidina, fueron un 28% superiores respecto a Navel (De Ancos et al., 2017). En cambio, Brasili et al. (2017) detecta un contenido superior de

flavononas, entorno al 10%, en la naranja Bahia Navel, mientras que De Ancos et al. (2020) no observa diferencias significativas ni en el contenido individual ni total de flavonoides. Otros trabajos, sin embargo, detectaron menor contenido de flavonoides en las naranjas Cara Cara y Red Anliu, y concluyeron que la acumulación de licopeno está directa o indirectamente asociada con la síntesis de flavonoides (Pan et el., 2014; Chen et al., 2015). Muchas de estas discrepancias se pueden deber a la influencia de la zona de cultivo o el estado de maduración del fruto, que no es comparable en todos los casos. Respecto a la capacidad antioxidante de extractos hidrosolubles, se ha observado mayor capacidad antioxidante por los métodos DPPH y ABTS en la pulpa y zumo de Navel respecto a Cara Cara que, además, se correlacionaron con el mayor contenido en vitamina C en la variedad tradicional (De Ancos et al., 2017; 2020).

Los azúcares son los principales nutrientes en los frutos cítricos y su contenido y equilibrio, junto con el de los ácidos orgánicos, influye de forma importante en el sabor del fruto y, por tanto, sus características organolépticas (Lado et al., 2014). Así, el contenido de azúcares individuales (sacarosa, glucosa y fructosa) y totales detectado en Cara Cara no mostró diferencias significativas respecto al de Washington Navel (Kafkas et al., 2011) y Bahía Navel (Brasili et al., 2017). Además, mientras algunos trabajos han detectado niveles de ácido cítrico inusualmente bajos en Cara Cara (Kafkas et al., 2011), otros análisis han reportado concentraciones muy similares a las descritas para variedades comunes de naranja dulce (Wu et al., 2021). Estos metabolitos, sin embargo, están sustancialmente alterados en la pulpa roja de las naranjas Red Anliu. Por un lado, los niveles de ácidos son considerablemente mucho más bajos que en los frutos de su correspondiente parental, y se han observado alteraciones importantes en la expresión de genes de los transportadores de citrato y de transportadores de electrones al ciclo de los ácidos tricarboxílicos (Guo et al., 2016; Hussain et al., 2017). Además, en estas mismas variedades se detectaron concentraciones superiores de mono- y disacáridos en los frutos que acumulan licopeno (Pan et al., 2014). Estos resultados indican que el mutante de pulpa roja Red-Anliu, además de la alteración en el contenido de carotenoides, presenta diversas anomalías a nivel de metabolitos primarios, reforzando la hipótesis de que la naturaleza de la mutación es diferente en comparación con las naranjas del grupo Navel.

Más recientemente, otros estudios han detectado niveles inferiores de terpenos (mono- y sesquiterpenos) en la pulpa y hojas de Cara Cara en comparación con la variedad estándar Seiku Navel (Liu et al., 2021, 2022). Sin embargo, esta menor capacidad de síntesis de terpenos en Cara Cara no parece ser una característica común en los diferentes mutantes de cítricos de pulpa roja (Liu et al., 2015; 2019; 2021). El análisis metabolómico de terpenoides en la naranja Red Anliu ha mostrado que contiene mayor concentración de compuestos volátiles totales y, en particular, mayor concentración de monoterpenos, incluido limoneno, y de aldehídos, y menor concentración de sesquiterpenos, lo que se ha interpretado como una tendencia hacia la estabilización del balance del metabolismo de terpenoides (Liu et al., 2019).

Hasta la fecha, solo existen dos estudios en los que se han abordado los efectos del consumo de naranjas de pulpa roja en organismos in vivo. En el primer estudio, se utilizó una línea del nemátodo Caenorhabditis elegans que sobre-expresa péptidos β-amiloides y se emplea como modelo para el estudio de enfermedades como el Alzheimer. El suplemento del 1% de zumo pasteurizado de la naranja de pulpa roja Cara Cara al medio de crecimiento produjo una reducción de los niveles de ROS y aumentó significativamente la vida media del gusano respecto a los suplementados con zumo de la naranja común Bahía Navel. La suplementación con el zumo de ambas variedades retrasó la parálisis inducida por los péptidos β-amiloides, pero el zumo de Cara Cara retrasó en mayor medida la aparición de estos síntomas respecto al zumo de Bahía (De Oliveira et al., 2019).

El único estudio realizado en humanos con naranjas de pulpa roja que acumulan licopeno fue llevado a cabo por Silveira et al. (2015). En este trabajo, dos grupos de individuos, voluntarios con sobrepeso y peso normal, ingirieron durante 8 semanas 750 ml de zumo Sanguínea de Mombuca (variedad de naranja de pulpa roja originaria de Brasil). Los niveles de colesterol LDL, colesterol total y proteína C reactiva (indicador de procesos inflamatorios) se redujeron significativamente en ambos grupos y, además, la capacidad antioxidante del suero aumentó después del consumo del zumo de naranja de pulpa roja. Además, la presión sanguínea diastólica se redujo en los individuos con sobrepeso después de la intervención. El conjunto de estos resultados indica que el consumo de zumo de naranja de pulpa roja podría ser beneficioso para la prevención del síndrome metabólico y, en particular, capacidades antiinflamatorias, antioxidantes y reductoras de los niveles de lípidos (Silveira et al., 2015). Por otro lado, un estudio de la microbiota fecal y metabolómico no dirigida en humanos sanos que

han ingerido zumo de la naranja Navel Bahia y la naranja roja Cara Cara, ha mostrado que el mayor cambio en la composición de la misma se produjo después de la ingesta de zumo de Cara Cara, con un mayor aumento de especies de las familias Mogibacteriaceae, Tissierellaceae, Veillonellaceae, Odoribacteraceae y Ruminococcaceae (Brasili et al., 2019).

1.6. Nuevas variedades de naranja de pulpa roja

Los dos mutantes de naranja de pulpa roja que hasta la fecha han sido más intensamente estudiados son la naranja dulce Cara Cara, una mutación de la naranja Washington Navel originaria de Venezuela (Lee, 2001), y la naranja Hong Anliu o Red Anliu, mutante espontáneo de la variedad parental Anliu, originaria de China (Liu et al., 2007). La naranja Cara Cara es la única variedad de naranja de pulpa roja que en la actualidad está disponible comercialmente para el cultivo, mientras que Red Anliu está limitada al país de origen y sin difusión internacional. La disponibilidad de nuevas variedades de naranja de pulpa roja es un objetivo importante en la Citricultura moderna, ya que supone, por un lado, disponer de marcas propias para diferenciarse de otros países productores y comercializadores, y especialmente, nuevas variedades con valor añadido, ya que la presencia de licopeno en la pulpa las diferencia de las naranjas tradicionales y su consumo podría proporcionar beneficios adicionales para la salud. La obtención de este tipo de variedades es complicada, ya que se basa en la mayoría de los casos, especialmente en naranjas del grupo Navel, en la identificación y selección de mutantes espontáneos o procedentes de programas de inducción de mutaciones, con una característica deseada, que al tratarse del color de la pulpa es de difícil detección en etapas tempranas y supone un proceso destructivo.

A pesar de estas limitaciones, en diferentes países se han desarrollado programas de obtención de nuevas variedades, entre los que cabe mencionar la selección de dos mutantes de pulpa roja por parte de la compañía Biogold S.L. en Sudáfrica. La primera de ellas se originó en Kirkwood (Eastern Cape) y por ello se denominó 'Red Kirkwood'. El obtentor de esta variedad fue André Potgieter en 1992, a partir de una rama independiente de un árbol de la variedad Palmer Navel. La segunda variedad, se denominó 'Ruby Valencia Seedless', procedente de una mutación natural de un árbol de Olinda Valencia encontrada en Crocodile Valley Estate, cerca de Nelspruit (Mpumalanga) por Dennis Salomon en el año 1992. Ambas variedades se seleccionaron por el color rojo de su pulpa y fueron propagadas

vegetativamente en años sucesivos en diferentes plantaciones locales y sobre diferentes patrones, y después de varias generaciones se comprobó que las características de la mutación se mantuvieron estables por propagación asexual. Ambas variedades pertenecen al grupo Biogold (https://www.citrogold.co.za/) y fueron registradas inicialmente en Sudáfrica y, posteriormente, tanto Kirkwood Red Navel (US PP24,778 P3) como Ruby Valencia Seedless (US PP31,794 P2) se patentaron internacionalmente. Después de su propagación, saneamiento, y del proceso de autentificación varietal, a partir de año 2012 se hicieron disponibles bajo los acuerdos legales correspondientes entre viveristas y campos experimentales de diversos países para comprobar su adaptación, características y potencial para su comercialización. En España ambas variedades corresponden a la compañía Biolgold-EM (https://www.biogold-em.com/) filial de la anterior sudafricana dedicada a la experimentación y comercialización de variedades protegidas, que puso a disposición de la cooperativa de primer grado ANECOOP S.L., el cultivo de varios árboles de ambas variedades (injertadas sobre patrón Citrange Carrizo (*Citrus sinensis* L. Osb. x *Poncirus trifoliata* L. Raf) en sus campos experimentales en Museros (Valencia), para comprobar su desarrollo vegetativo y reproductivo en las condiciones climáticas y de cultivo valencianas. A partir del año 2015, el grupo de investigación donde se ha realizado esta Tesis Doctoral tuvo acceso a ambas variedades, en árboles adultos con buen desarrollo vegetativo y producción en la parcela experimental de ANECOOP S.L. en Museros (Valencia) (Figura 6). A partir de la campaña 2015-16 y durante 4-5 campañas se ha recolectado y utilizado gran parte del material vegetal utilizado en esta investigación, frutos en diferentes estados del desarrollo y maduración, frutos en su momento de

madurez comercial, hojas y flores, en función de las distintas experiencias realizadas.

Las características generales de las variedades y de sus frutos se han descrito por los obtentores (https://www.citrogold.co.za/; https://www.biogold-em.com/) y más recientemente por los viveristas y comercializadores de las mismas en los distintos países. En general, la variedad 'Kirkwood Red Navel' es productiva, no presenta problemas de vecería, su desarrollo y producción se ha comprobado en diferentes patrones y, en general, no presenta problemas de compatibilidad ni de otro tipo, y se considera como una Navel ordinaria. El fruto es de forma redondeada y tamaño medio, con buena calidad organoléptica y alto contenido en zumo. Su principal característica es el color rojo intenso de la pulpa, mientras la piel tiene un color anaranjado normal o ligeramente más intenso que otras Navel. Suele madurar al

mismo tiempo o con ligero retraso respecto a otros clones de Washington Navel o Cara Cara, y con una pérdida de acidez similar o un poco más lenta. Por ello, puede permanecer en el árbol más tiempo (4 y 5 semanas) que otras variedades análogas como Cara Cara, lo que permite extender su campaña de comercialización. A pesar de que no hay datos oficiales hasta la fecha, las campañas y experiencias de campo sugieren que es menos propensa al desorden fisiológico de la piel denominado comúnmente como clareta o 'creasing' (en inglés) que los frutos de Cara Cara.

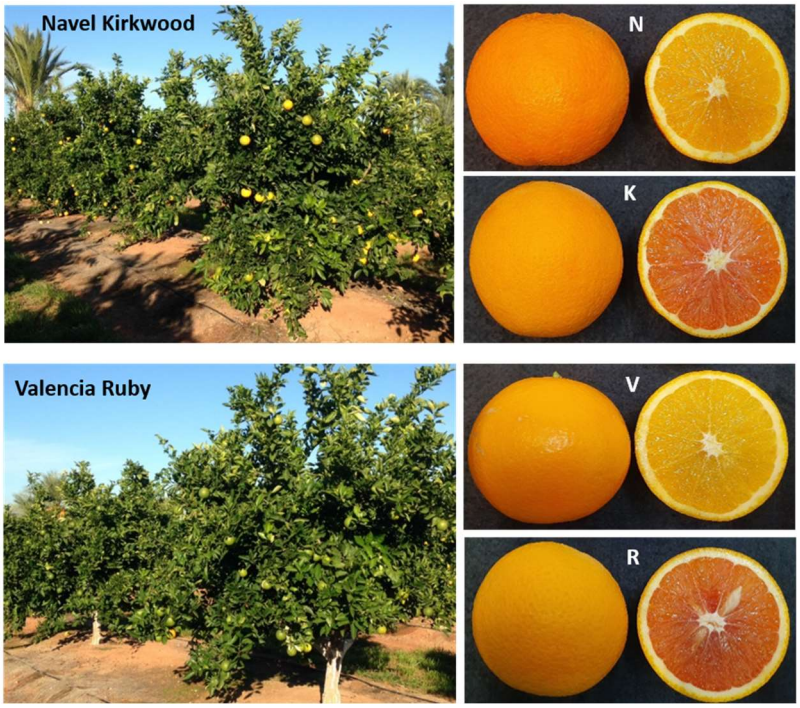

Figura 6. Aspecto general de árboles de las variedades Kirkwood Navel y Ruby Valencia, y de frutos maduros de dichas variedades (K and V, respectivamente) y de las naranjas de las variedades comunes de referencia Washington Navel (cv. Foios) (N) y Valencia late (cv. Midknight) (V) de la parcela experimental de ANECOOP (Museros, Valencia) utilizadas en este trabajo.

La variedad 'Ruby Valencia Seedless', se denominó originalmente así por la ausencia de semillas, pero se ha comprobado que en campos comerciales y en proximidad de otras variedades de tiempo de floración similar, los frutos pueden contener pocas semillas (como los de la imagen de la Fig. 6). Por ello, en este trabajo, así como diferentes operadores la reconocen como 'Ruby Valencia'. El fruto es de tamaño medio-alto para este tipo de variedades, pero el árbol suele ser muy productivo y tendente a grandes producciones, por lo que si no se controla con técnicas agronómicas adecuadas los frutos pueden ser de menor tamaño. Otras características vegetativas y de producción son similares a otras variedades

Valencia. El fruto presenta una buena calidad organoléptica y junto con el alto porcentaje de zumo y el color más intenso del mismo, representan características de interés para la industria de procesado.

La disponibilidad de estas dos nuevas variedades, de las que no existía previamente resultados publicados en revistas científicas, nos permitió plantear una serie de objetivos científicos que se desarrollan y conforman esta Tesis Doctoral. Las principales razones para ello fueron que, la disponibilidad de nuevos mutantes de naranja que acumulan licopeno en la pulpa pueden ser buenas herramientas experimentales para investigar y discernir las bases bioquímicas y moleculares que conducen a esta característica, que hoy en día todavía no han sido esclarecidas. Este objetivo principal se complementa con otros objetivos más aplicados o de interés industrial/comercial, ya que empresas del sector productivo de cítricos apoyan y tienen interés en conocer las características de calidad y propiedades de estas variedades, y su potencial para la comercialización en fresco y el procesado de zumo. Asimismo, en este caso, se dispone de una variedad de naranja del grupo Navel, cuyo destino principal es el consumo en fresco, y otra variedad del grupo Valencia, que son las variedades que se emplean mayoritariamente en la elaboración del zumo, lo que cubriría los dos destinos principales de los frutos cítricos en la alimentación. En base a estos objetivos, esta Tesis Doctoral se ha realizado gracias a la obtención de un contrato predoctoral del programa FDEGENT (FDEGENT/2018/007) de la Generalitat Valencia para desarrollar un proyecto de investigación de interés industrial y en colaboración con la empresa Citrus Rosso S.L.

2. OBJETIVOS

Considerando los antecedentes anteriores, el objetivo general de esta Tesis Doctoral fue analizar y caracterizar en profundidad los frutos de dos nuevas mutaciones de naranja dulce de pulpa roja, denominados Kirkwood Navel y Ruby Valencia, procedentes de las naranjas Washington Navel (cv. Palmer) y Valencia late (cv. Olinda), respectivamente. Para ello, se estudiarán con especial atención las bases fisiológicas y moleculares del metabolismo de carotenoides implicadas en la singular coloración del fruto de estos mutantes, la evolución de los componentes de calidad de los frutos durante la maduración, su aptitud para la conservación postcosecha y comercialización en fresco, y el potencial de estas nuevas variedades para la producción y procesado de zumo.

Para abordar este objetivo general se plantearon los siguientes objetivos específicos:

1. Estudiar el metabolismo de carotenoides en la piel y la pulpa de las naranjas de pulpa roja Kirkwood Navel y Ruby Valencia, en comparación con dos variedades de naranja común de referencia, Navel Foios y Valencia Midknight, respectivamente, a lo largo de la maduración. Para ello, se analizará el contenido y composición de carotenoides, los niveles de expresión de genes del metabolismo de carotenoides, las características estructurales de los orgánulos celulares que acumulan carotenoides (plastidios), así como los niveles de ácido abscísico.
2. Analizar los cambios en componentes relacionados con la calidad organoléptica y nutricional en la pulpa de las variedades rojas en comparación con las respectivas variedades de referencia, durante la maduración del fruto. En particular, se evaluarán los cambios en el contenido de azúcares, ácidos orgánicos, vitamina C (ácido ascórbico) y E (tocoferoles), fenoles, flavonoides, capacidad antioxidante de extractos hidro- y liposolubles y capacidad antioxidante específica frente al oxígeno singlete.
3. Estudiar el comportamiento y las respuestas de las naranjas de pulpa roja, en comparación con las respectivas variedades de referencia a la conservación postcosecha a bajas temperaturas (4 semanas a 2 °C) y vida comercial (5 días a 20 °C). Para ello, se analizarán diferentes parámetros fisiológicos de los frutos como la pérdida de agua, firmeza y daños por frío, así como cambios en compuestos bioactivos, capacidad antioxidante y compuestos volátiles.
4. Evaluar la aptitud de naranjas Navel de pulpa roja, Kirkwood y Cara Cara, frente a la variedad de naranja común, Washington Navel (cv. Foios) para la producción y procesado de zumo. Para dicho objetivo, se analizarán los efectos de diferentes sistemas extracción (manual e

industrial) y de procesado (pasteurización y homogenización a altas presiones) en el contenido y composición de diferentes componentes de calidad, compuestos bioactivos y capacidad antioxidante, entre las diferentes variedades. Asimismo, se analizará la composición del residuo generado durante la extracción industrial de zumo y su potencial como subproducto para la industria alimentaria y/o afines.

3. RESULTADOS

3.1. CAPÍTULO 1

A comprehensive analysis of carotenoids metabolism in two red-fleshed mutants of Navel and Valencia sweet oranges (*Citrus sinensis*)

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Abstract

Kirkwood Navel and Ruby Valencia are two spontaneous bud mutations of the respective parental lines of sweet orange (Citrus sinensis) Palmer Navel and Olinda Valencia, showing an atypical red pigmentation of the pulp. These red-fleshed varieties are commercially available and highly attractive for consumer but their carotenoid metabolism and the basis of the mutation have not been investigated. The red colour of Kirkwood and Ruby pulp was observed from the very early stages of fruit development until full maturity and associated with an altered carotenoid profiling. The red-fleshed varieties accumulated from 6- up to 1000-times more total carotenoids compared to the standard oranges. Specifically, the pulp of Kirkwood and Ruby accumulated large amounts of phytoene and phytofluene, and moderate contents of lycopene. Moreover, the red-fleshed oranges contained other unusual carotenes as d-carotene, and lower concentrations of downstream products such as b,b-xanthophylls, abscisic acid (ABA) and ABA-glucosyl ester. This peculiar profile was associated with chromoplasts with lycopene crystalloid structures and round vesicles likely containing colourless carotenes. The flavedo and leaves of Kirkwood and Ruby showed minor changes in carotenoids, mainly limited to higher levels of phytoene. The carotenoid composition in Kirkwood and Ruby fruits was not explained by differences in the transcriptional profile of 26 genes related to carotenoid metabolism, covering the main steps of biosynthesis, catabolism and other processes related to carotenoid accumulation. Moreover, sequence analysis of the lycopene cyclase genes revealed no alterations in those of the red-fleshed oranges compared to the genes of the standard varieties. A striking event observed in Kirkwood and Ruby trees was the reddish coloration of the inner side of the bark tissue, with larger amounts of phytoene, accumulation of lycopene and lower ABA content. These observations lead to the conclusion that the mutation is not only manifested in fruit, affecting other carotenogenic tissues of the mutant plants, but with different consequences in the carotenoid profile. Overall, the carotenoid composition in the red-fleshed mutants suggests a partial blockage of the lycopene b-cyclization in the carotenoid pathway, rendering a high accumulation of carotenes upstream lycopene and a reduced flow to downstream xanthophylls and ABA.

Keywords: citrus fruit, red orange, carotenoids, lycopene, colourless carotenes, gene expression, abscisic acid

1. Introduction

Citrus is one of the major fruit-tree crops in the world (Talón et al., 2020) with an estimated production of more than 140 million tons and is highly demanded worldwide for fresh consumption and juice processing (https://www.fao.org/faostat/en/#data/QCL). The health-related and sensorial properties of Citrus and the consumers perception of their benefits are closely associated with the content of certain phytochemicals such as vitamins, carotenoids, minerals, flavonoids, phenolic acids, limonoids, volatiles and sugars, among others (Ma et al., 2020).

Fruit colour is one of the most remarkable features among the different citrus species and varieties, and a key factor in quality and consumer acceptance (Tadeo et al., 2020). The colouration of most citrus fruits is due to carotenoids, a large family of isoprenoid-derived compounds, which play essential roles in many physiological processes of the plant (Rodriguez-Concepción et al., 2018). The variability in carotenoid content and composition determines the distinctive pigmentation of citrus fruits, ranging from yellow or pale-yellow to orange and the pink/red of specific varieties of pummelos, grapefruits and sweet oranges (Xu et al., 2006; Ikoma et al., 2016; Liu et al., 2016; Lu et al., 2017; Tadeo et al., 2020).

Carotenoids are recognized for their benefits in human nutrition and health (Eggersdorfer and Wyss, 2018; Amengual, 2019; Ma et al. 2020). They are efficient antioxidants scavenging singlet molecular oxygen ($^1O_2$) and peroxyl radicals (Stahl and Sies, 2003; Müller et al., 2011) and specific carotenoids such as α-carotene, β-carotene and β-cryptoxanthin are the main source of vitamin A from plant-based food (Eggersdorfer and Wyss, 2018). In addition, many studies pointed out that the regular intake of carotenoids from fruit and vegetable products has important health-related benefits and reduces the risk of specific degenerative diseases (Rao and Rao et al., 2007; Rajendran et al., 2014; Böhm et al., 2021; Meléndez-Martínez et al., 2021). Consequently, the colouration of citrus fruits, determined by the content and composition of individual carotenoids, has a remarkable impact not only in their organoleptic quality but also regarding their nutritional and health properties.

The biosynthesis of carotenoids in plants occurs exclusively in plastids through the methylerythritol 4-phosphate (MEP) pathway, which provides precursors for carotenoid production. This pathway involves the participation of several enzymes, such as 1-deoxy-D-

xylulose-5-phosphate synthase (DXS), which is the first reaction of the MEP pathway and catalyses the synthesis of 1-deoxy-d-xylulose 5-phosphate from pyruvate and D-glyceraldehyde 3-phosphate. Then, the terminal reactions of the MEP pathway are catalysed by 4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS), which synthesize (E)-4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBPP), and HMPP reductase (HDR), an enzyme that converts HMBPP into isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The condensation of one DMAPP (DMAP) and three IPP molecules by the geranyl geranyl pyrophosphate synthase (GGPS) produces geranylgeranyl diphosphate (GGPP) (Figure 1).

The first committed step for carotenoid biosynthesis in plants is the head-to-head condensation of two GGPP molecules by phytoene synthase (PSY) to produce the first C40 carotenoid product 15-Z-phytoene. This reaction is generally considered the major rate-limiting step of carotenogenesis. Sequential desaturations and isomerizations by phytoene desaturase (PDS), ζ-carotene isomerase (Z-ISO), ζ-carotene desaturase (ZDS) and carotenoid isomerase (CRTISO) produce lycopene, through the intermediates ζ-carotene and neurosporene (Moise et al., 2014). At this point, the pathway splits into two branches by cyclization of lycopene at both ends to give α-carotene (α,ε-branch) and β-carotene (β,β-branch). The ε,β-branch requires both lycopene ε-cyclase (ε-LCY) and lycopene β-cyclase (β-LCY) activities for the formation of ε- and β-ionone rings, respectively, at both ends to generate α-carotene. In the ε,β-branch, α-carotene is di-hydroxylated at both ionone rings by carotene hydroxylases (β-CHX/CYP97A and ε-CHX/CYP97C) to render lutein, the predominant carotenoid pigment in the photosynthetic membranes of green plant tissues (Kato, 2012). In the β,β-branch, a single activity β-LCY is required to introduce β-ionone ring at both ends of lycopene to form β-carotene (Figure 1).

In citrus fruit, two different β-LCY enzymes catalyse the cyclization of lycopene to β-carotene (Alquezar et al., 2009; Zhang et al., 2012). β-LCY1 is expressed in a large variety of tissues showing a fairly constant expression during fruit ripening, while β-LCY2 is preferentially expressed in fruits and is up-regulated during ripening at the onset of colouration (Alquezar et al., 2009; Zhang et al., 2012).

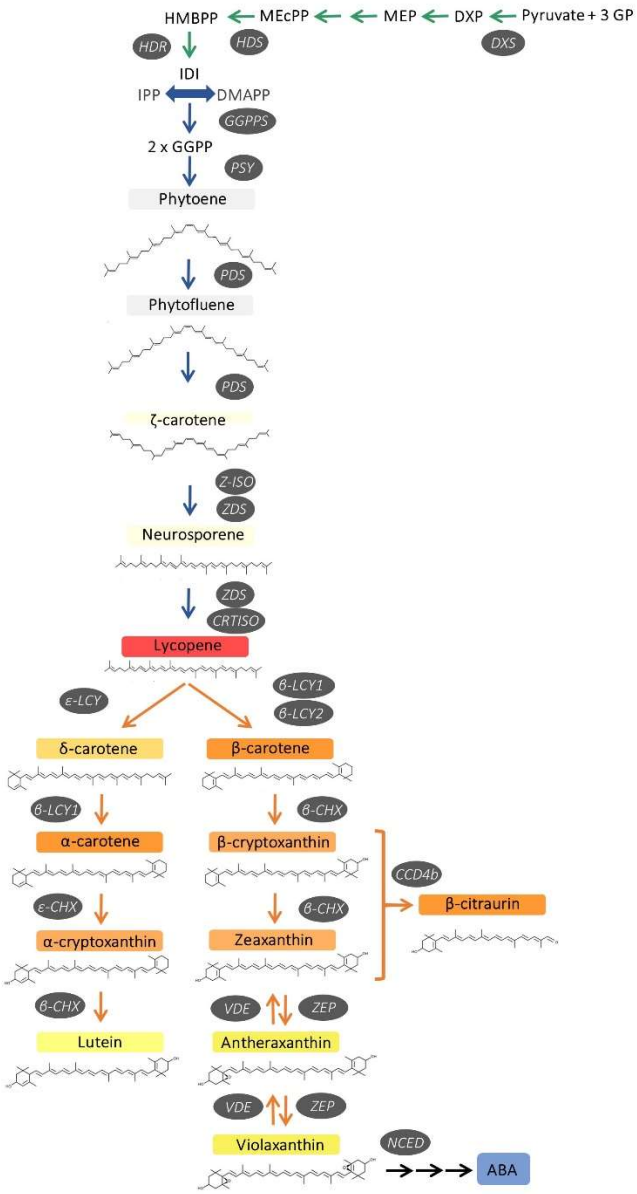


Figure 1. Schematic representation of carotenoid biosynthesis in citrus fruits, indicating main enzymes and genes of the pathway. 1-deoxy-D-xylulose-5-phosphate synthase (*DXS*), hydroxymethylbutenyl diphosphate synthase (*HDS*) and reductase *(HDR*), geranylgeranyl diphosphate synthase (*GGPPS*), phytoene synthase (*PSY*), phytoene desaturase (*PDS*), ζ-carotene isomerase (*Z-ISO*), ζ-carotene desaturase (*ZDS*), carotene isomerase (*CRTISO*) ε-lycopene cyclase (ε-LCY), β-lycopene cyclase (*β-LCY1*/2), ε-carotene hydroxylase (*ε-CHX*), β-carotene hydroxylase (*β-CHX*), carotenoid cleavage dioxygenase 4b (*CCD4b*), zeaxanthin epoxidase (*ZEP*), violaxanthin de-epoxidase (*VDE*) and 9-cis-epoxy-carotenoid dioxygenase (*NCED*).

In some citrus species, including sweet orange, two alleles of *β-LCY2* (a and b) with different activity to convert lycopene into β-carotene have been identified (Alquézar et al., 2009; Zhang et al., 2012; Lu et al., 2016). Subsequently in the pathway, β-carotene is converted to zeaxanthin via β-cryptoxanthin by two-step hydroxylation catalyzed by the carotene β-ring hydroxylase (β-CHX). Furthermore, zeaxanthin is converted to violaxanthin via antheraxanthin by zeaxanthin epoxidase (ZEP) (**Figure 1**). The accumulation of carotenoids depends on a tight relationship between synthesis and catabolism. In this context, carotenoid cleavage dioxygenases (CCDs) are a group of enzymes that catalyse the oxidative cleavage of carotenoids and, in citrus fruits, NCEDs (9-Z-epoxycarotenoid dioxygenases), CCD1 and CCD4b have been studied in relation to the catabolism of carotenoids (Kato et al., 2006; Ma et al. 2013; Rodrigo et al., 2013a). Moreover, the esterification of β,β-xanthophylls with fatty acids by xanthophylls acyl esterases (XES/PYP) is an active process in orange-pigmented citrus fruits which may enhance their stability and accumulation (Mariutti and Mercadante, 2018; Lux et al., 2019; Zacarias-Garcia, 2021).

Carotenoid synthesis and colour development in citrus fruit occur concomitantly with the differentiation of chloroplast into chromoplasts (Lado et al., 2015; Lu et al 2017, Zhang et al., 2019), leading to the development of sink structures organized to store carotenoids, and in which fibrillins (FIB) may play structural roles organizing carotenoids in lipoprotein complexes (Simkin et al., 2007). Globular chromoplasts are the most representative in mature sweet orange fruits, characterized by abundant plastoglobules containing the β,β-xanthophylls (Lado et al., 2015; Schweiggert and Carle, 2017). By contrast, in tissues of varieties and mutants that accumulate lycopene, other type of chromoplasts containing needle-like crystals are observed (Lado et al., 2015; Zeng et al., 2015; Zhang et al., 2019).

The peel of immature-green sweet orange shows the characteristic carotenoid profile of a chloroplast-containing tissue, being lutein the main carotenoid, while the content of carotenoids in the pulp is almost negligible. At the onset of fruit colouration, a progressive decrease in lutein takes place in parallel with an accumulation of specific β,β-xanthophylls, being 9-Z-violaxanthin the main carotenoid in tissues of mature sweet oranges (Ikoma et al., 2016; Lux et al., 2019; Tadeo et al., 2020). On the contrary, accumulation of lycopene in citrus fruits is an unusual feature only described in certain varieties of pummelo (*Citrus grandis, Citrus maxima*), grapefruit (*Citrus paradisi*), or mutants of lemon (*Citrus limon*) and sweet

orange (*Citrus sinensis*). Currently, only three red-fleshed sweet orange varieties have been characterized: Shara (Monselise and Halvey, 1961), Cara Cara (Saunt et al., 2000) and Hong Anliu (Liu et al., 2007). Exhaustive research has focused on the pattern of carotenoid accumulation in these red-fleshed citrus fruits with the aim of elucidating the molecular mechanisms responsible for this trait. The profile of carotenoids suggests that the basis of the alteration leading to lycopene accumulation may differ among citrus species and varieties (Liu et al. 2007; Alquézar et al. 2008, 2013b; Yu et al., 2012; Zeng et al., 2015; Lu et al., 2016; Yan et al 2018; Lana et al., 2020; Promkaew et al., 2020). In the red Hong Anliu, a spontaneous mutant of the Anliu orange, the concentration of carotenoids in the juice sacs was 2-times higher than in the standard variety, mainly by the accumulation of lycopene (~2 μg g^{-1}) at expenses of xanthophylls, which are strongly reduced (Liu et al., 2007). In contrast, carotenoid compliment in the Cara Cara orange, a bud mutation of Washington Navel, has a different biochemical phenotype characterized by: 1) accumulation of considerable amounts of phytoene from early stages of development, that may reach up to 25-80 μg g^{-1} FW in mature fruits, 2) lycopene content in the pulp can be as high as ~9 μg g^{-1} FW, and 3) xanthophyll levels lower or similar than in the standard Navel (Xu et al., 2006, Alquézar et al., 2008; Rodrigo et al., 2015; Lado et al., 2015; Lu et al., 2017; Liu et al., 2021).

Overall, the molecular basis of lycopene accumulation in *Citrus* is not fully understood. In the red Star Ruby grapefruit pulp, for instance, a reduced expression of the *β-LCY2* gene compared to the white Marsh grapefruit has been suggested as the main feature for the lycopene accumulation (Mendes et al., 2011; Alquézar et al., 2013). The comparison of several pummelos varieties with different pulp pigmentation indicated that lycopene is associated with a reduced expression of genes encoding for enzymes operating downstream lycopene (Liu et al., 2016; Yan et al., 2018; Promkaew et al., 2020). On the contrary, in the pulp of Pink lemon, a spontaneous bud mutant of Eureka lemon, accumulation of lycopene was associated with an up-regulation and down-regulation of the genes upstream and downstream lycopene β-cyclase, respectively (Lana et al., 2020). While in Cara Cara orange, an enhancement of the expression of specific MEP pathway genes and an increased channelling of isoprenoid precursors into the carotenoid pathway, has been hypothesized to be related to its red-fleshed phenotype (Alquézar et al., 2008; Lu et al., 2017; Zhang et al., 2021), even though other mechanisms may be involved (Alquézar et al., 2008; Ye et al., 2010; Lu et al., 2017). Finally, in

the Hong Anliu mutant, an up-regulation of genes upstream of lycopene and the preferential expression of the afunctional allele *β-LCY2b*, are suggested to enhance the accumulation of lycopene (Xu et al., 2009; Lu et al., 2016).

The availability and comparison of new lycopene-accumulating mutants may be a very valuable tool to dissect and understand the biological basis of lycopene accumulation in the pulp of sweet orange. Recently, two independent bud mutations, referred to as 'Kirkwood Navel' (Kirkwood, K) and 'Ruby Valencia'(Ruby, R), from the standard Palmer Navel in Kirkwood Eastern Cape and the Olinda Valencia in Crocodile Valley (Mpumalanga), respectively, were identified in South Africa and selected for their red pigmentation of the pulp of mature fruits (https://www.citrogold.co.za/) (Barry et al., 2020). These mutants have been further propagated and the red-fleshed phenotype remained stable under commercial conditions. The accessibility of these two mutants is of genetic interest since each belongs to two distinctive orange groups: Navel and Valencia oranges. Moreover, both mutants are relevant for the commercial citrus industry since Navel are the main oranges for fresh-fruit consumption, whereas Valencia cultivars are the major source for juice production worldwide (Barry et al., 2020). Thus, this work aimed to perform a comparative physiological, biochemical and molecular characterization of the carotenoid metabolism in the red-fleshed mutants Kirkwood Navel and Ruby Valencia orange during fruit development and maturation. We report a comprehensive analysis of carotenoid content and composition in the pulp of both mutants and the corresponding standard varieties from the initial stages of fruit development to full maturity. The carotenoid data were complemented with the analysis of the expression of genes related to carotenoid metabolism, covering the MEP pathway (*DXS*, *HDS*, *HDR* and *GGPS*), carotenes and xanthophylls biosynthesis (*PSY*, *PDS*, *Z-ISO*, *ZDS*, *ε-LCY*, *β-LCY1*, *β-LCY2*, *β-CHX*), carotenoids catabolism (*NCED, CCD1* and *CCD4b*), storage and accumulation (*HSP21* and *FIB*), and xanthophylls esterification (*PYPs*).

2. Materials and Methods

2.1. Plant material, fruit size, colour index and internal maturity

Trees of the standard oranges (*Citrus sinensis* L. Osbeck) i.e., Foios Navel (referred as Navel, N) and Midknight Valencia (Valencia, V) as well as the two red-fleshed mutants Kirkwood Navel and Ruby Valencia were planted in the experimental orchard of the Foundation ANECOOP (Museros, Valencia, Spain; 39º34'10.8"N 0º21'28.8"W), growing in a loamy-sand soil with drip irrigation, and subjected to the same environmental and growing conditions (https://anecoop.com/en/leading-the-industry/new-varieties-and-new-products/the-doctors farm/). Adult trees of standard and mutant genotypes were located in continuous rows in the same evaluation block, and both were budded onto Carrizo citrange [*Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf.] rootstock. Fruit of each genotype were periodically harvested starting at the initial stages of fruit development (June) until full maturation in January for Foios Navel and Kirkwood Navel, and April for Midknight Valencia and Ruby Valencia (**Figure 2**). For each sampling point, at least 20-30 fruit from three trees of each genotype were harvested and immediately delivered to the laboratory. Fruit were selected for uniform colour and size, and absence of any lesion or defect. Flavedo tissue (outer coloured peel layer of the fruit) was collected with a scalpel, and pulp juice vesicles were excised from central segments. Tissue was immediately frozen in liquid nitrogen, ground to a fine powder and stored at -80 °C until analysis. Young-full developed leaves (3-4 months old) from the four genotypes were also collected from young shoots from the same trees. Leaves were rinsed with distilled water, dried before removing the central vein and freezing the leaves in liquid nitrogen before grounding it to a fine powder, and stored at -80 °C.

Initial analysis of carotenoid content and composition were performed in mature fruits harvested from South Africa sampled from mature Palmer and Kirkwood Navel, and Valencia late and Ruby Valencia trees located in a cultivar evaluation orchard in Citrusdal (Western Cape, South Africa). All trees were budded onto Carrizo citrange [*Citrus sinensis* (L.) Osb. × *Poncirus trifoliata* (L.) Raf.] rootstock. The mature fruit of the Navel varieties were harvested in July and those of Valencia's in October. The fruit were immediately delivered to the Department of Horticultural Sciences of the University of Stellenbosch (South Africa) and processed as stated above until analysis.

Branches of around 1 year-old (about 1 cm diameter) were removed from both mature Ruby Valencia and standard Valencia trees in the cultivar evaluation block in Citrusdal. At initial inspection, the Ruby Valencia shoots showed red colouration of the inner side of the bark. Sections around 5-10 cm long were excised and maintained in plastic bags to be transported to the University of Stellenbosch. Each section was cut into 0.5 cm long sections and the bark and xylem/wood tissues were separated with a scalpel, frozen in liquid nitrogen and lyophilized.

Colour of the peel and pulp was measured using a CR-400 Minolta chromameter (Konica Minolta, Japan) at three different positions on the fruit. Hunter parameters *a* (negative to positive corresponds from green to red) and *b* (negative to positive, from blue to yellow) were determined, and colour was expressed as the *a/b* Hunter ratio, a classical relationship for colour measurement in citrus fruits (Stewart and Wheaton, 1972). The data of fruit colour index for each developmental stage are the means ± SD of 10 fruits.

The juice was extracted from the pulp using a household electric hand squeezer (Citromatic MPZ22, Braun, Spain), filtered through a metal sieve with a pore size of 0.8 mm, and immediately frozen in liquid nitrogen and stored at -20°C until analysis. The total soluble solids (TSS) and total titratable acidity (TA) of the juice of each variety was determined using a digital refractometer PAL-BX/ACID1 (ATAGO, Japan). TSS was expressed as °Brix and TA as mg of citric acid/100 ml of juice. The maturity index (MI) was calculated as the TSS/TA ratio.

2.2. Chlorophylls and carotenoids extraction

Chlorophylls and carotenoids were extracted and analysed as described by Rodrigo et al. (2015). Briefly, freeze-ground material of flavedo (0.5 g) and pulp (2 g), and lyophilized wood or bark (0.2 g) were weighed in screw-capped polypropylene tubes, 4 Ml of methanol (MeOH) and 5 Ml Tris-HCl (50 Mm, Ph 7.5) containing 1 M NaCl were added and each sample was sonicated 5 min in XUBA3 ultrasonic water bath (Grant Instruments, England) at room temperature. Dichloromethane (DCM) (10 Ml) was added to the mixture, stirred for 5 min at 4°C and centrifuged at 3,000 g for 10 min at 4°C. The hypophase was recovered and the aqueous phase re-extracted with DCM until it was colourless. The extract was reduced to dryness by rotatory evaporator at 35°C and redissolved in diethyl ether to determine the chlorophyll (a+b) content by measuring the absorbance at 644 and 662nm (Smith and Benitez,

1955). Subsequently, samples were saponified in methanolic KOH (6%, w/v) overnight at room temperature. Saponified carotenoids were recovered from the upper phase after adding water and petroleum ether:diethyl ether (9:1) to the mixture. Extracts were dried and kept at −20 °C until further analysis. Each sample was extracted at least twice, and results are mean ± SD.

2.3. Carotenoids analysis by HPLC-DAD

The carotenoid composition of each sample was analysed by HPLC with a Waters liquid chromatography system equipped with a 600E pump and a photodiode array detector (DAD) model 2998, and Empower3 software (Waters, Spain). A C30 carotenoid column (250 × 4.6 mm, 5 μm) coupled to a C30 guard column (20 × 4.0 mm, 5 μm) (YMC, Teknokroma, Spain) was used. The chromatographic conditions are described in Lado et al. (2015) and Rodrigo et al. (2015). Absorbance spectra and retention time identified each carotenoid, peaks were integrated at their individual maximal wavelength, and their contents were calculated using the appropriate calibration curves, as described elsewhere (Lado et al., 2015; Rodrigo et al., 2015).

2.4. Transmission electron microscopy

Sample fixation and preparation were carried out following the protocol described by Lado et al. (2020) with slight modifications. Briefly, juice vesicles were manually excised from freshly harvested fruit and fixed in modified Karnovsky fixative (3% glutaraldehyde solution K_2PO_4 0.2 M, pH 7) followed by a post-fixation in OsO_4 2%. Subsequently, the tissue was dehydrated in ethanol and embedded in Spurr resin. Ultra-thin sections (0.1 mm) were cut and contrasted with a solution of uranyl acetate (4%) and lead citrate (0.5%). The samples were visualized on a JEOL JEM1010 transmission electron microscope (60 kV) (Microscopy Facility of the SCSIE-UV, Valencia, Spain).

2.5. Gene expression analysis by quantitative real-time PCR

Total RNA extraction from flavedo and pulp was performed as described previously (et al. 2004; Alòs et al., 2014). cDNA synthesis and gene expression analyses were performed as described by Rodrigo et al. (2013), and subsequently treated with Dnase treatment (Ambion, Madrid, Spain). Total RNA was quantified in a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Madrid, Spain). Briefly, 2 μg of total RNA were reverse transcribed using the SuperScript III Reverse Transcriptase (Invitrogen, Madrid, Spain) following the

manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on a LightCycler 480 instrument (Roche, Madrid, Spain), using the LightCycler 480 SYBRGreen I Master kit (Roche, Madrid, Spain). Twenty ng of cDNA was used for each amplification reaction in a total volume of 10 µl. The cycling protocol consisted of 10 min at 95°C for pre-incubation, followed by 35 cycles of 10 s at 95°C for denaturation, 10 s at 59°C for annealing and 10 s at 72°C for extension. Fluorescence data were acquired at the end of extension phase and reactions specificity was checked by post-amplification dissociation curve. Amplification efficiency € and correlation coefficient (r^2) of each primer were calculated using the standard curve method and the formula E = 10(−1/slope). For expression measurements, a LightCycler 480 Software release 1.5.0, version 1.5.0.39 (Roche, Madrid, Spain) was used, and the expression levels were relative to values of a reference sample calculated using the Software tool REST-MCS (Rest Multiple Condition Solver v2, http://rest.gene-quantification; Pfaffl et al. 2002). The selected reference sample to calculate expression levels was the standard Valencia pulp harvested in November, which was arbitrarily given the expression value of 1. The *Actin* gene expression was chosen to normalize raw Cp's based on a previous selection of reference genes (Alós et al., 2014). The results were the average of three independent sample replicates and the relative expression Software tool REST 2009 (http://www.gene-quantification.de/ rest-2009.html) was used to determine statistical significance between varieties ($P < 0.05$) The list of genes analysed and primers used for amplification are in Table S4.

2.6. Sequencing of ε-LCY, β-LCY1 and β-LCY2 genes

Genomic DNA from pulp of immature fruits of Navel Foios, Valencia Midknight, Kirkwood Navel and Ruby Valencia was isolated using DNeasy Plant Mini Kit (Qiagen), and was used to amplify the genomic sequences of *β-LCY1* and *β-LCY2* from each genotype by PCR. Since genomic DNA of *ε-LCY* contains introns, cDNA from immature flavedo of the four varieties was used to amplify this gene. The primers used for amplification and sequencing were designed based on orange1.1g014377m.g for *β-LCY1*, orange1.1g010693m.g for *β-LCY2* (www.phytozome.com) and XM_025097735.1 for *ε-LCY* (www.ncbi.nlm.nih.gov). The full coding sequence of each gene and each variety was amplified using AccuPrime™Taq DNA Polymerase High Fidelity (Invitrogen, Massachusetts, USA). For *β-LCY1* and *β-LCY2*, the cycling program consisted of 30 s at 98°C, then 35 cycles of 10 s at 98°C for denaturation, 30 s

at 58°C for annealing and 1 min at 72°C for extension. For *ε-LCY*, the cycling program consisted of 3 min at 98°C, 35 cycles of 30 s at 98°C for denaturation, 30 s at 56°C for annealing and 90 s at 72°C for extension. List of primers used for amplification and sequencing is supplied in Table S4. The single nucleotide polymorphisms in the sequences were analysed using Chromas 2.6.6. Software (Technelysium Pty Ltd; http://technelysium.com.au/), and the multiple sequence alignments were performed with DNAMAN sequence analysis software (Lynnon, Quebec, Canada).

2.7. Analysis of abscisic acid (ABA) and its catabolites

Determination of ABA, its glycosylated form (ABA-GE), and catabolites (phaseic and dihydrophaseic acid) in flavedo, pulp, bark and xylem of the different genotypes were performed as described by Diretto et al. (2020) with slight modifications. Briefly, frozen tissues were lyophilized and ground to a fine powder, and replicate samples of 200 mg of each genotype were extracted as previously described (Welsch et al., 2008). LC-HRMS was carried out using a Ultimate UHPLC-DAD (Dionex) coupled to a Q-Exactive quadrupole Orbitrap mass spectrometry System (Thermo Fisher Scientific), equipped with a C18 Luna column (150 x 2.0mm, 3µm) as described before (Noronha et al., 2022). Internal standard-based quantification was carried out using the MS data and the quantification software available in the Xcalibur 7.0 software package (Thermo Fisher Scientific, Bremen, Germany). Results are expressed as fold changes with respect to the internal standard.

2.8. Statistical Analysis

At least two biological replicates of each genotype were used for the different experiments of this study and the results are presented as the mean values ± standard deviation. Statistical differences between standard orange cultivars and the corresponding red-fleshed mutants were determined by an unpaired t-test, setting the significance level at $p<0.05$ by XLSTAT software.

3. Results

3.1. Characteristics of the red-fleshed Kirkwood Navel and Ruby Valencia oranges during fruit development and maturation

Kirkwood Navel (K) and Ruby Valencia (R) are two bud independent mutations derived from the standard Palmer Navel and Olinda Valencia, respectively, distinguished by the red colouration of the flesh. However, fruits of K and V showed similar fruit morphology, external appearance, and colouration compared to their original lines (**Figure 2**, **3**).

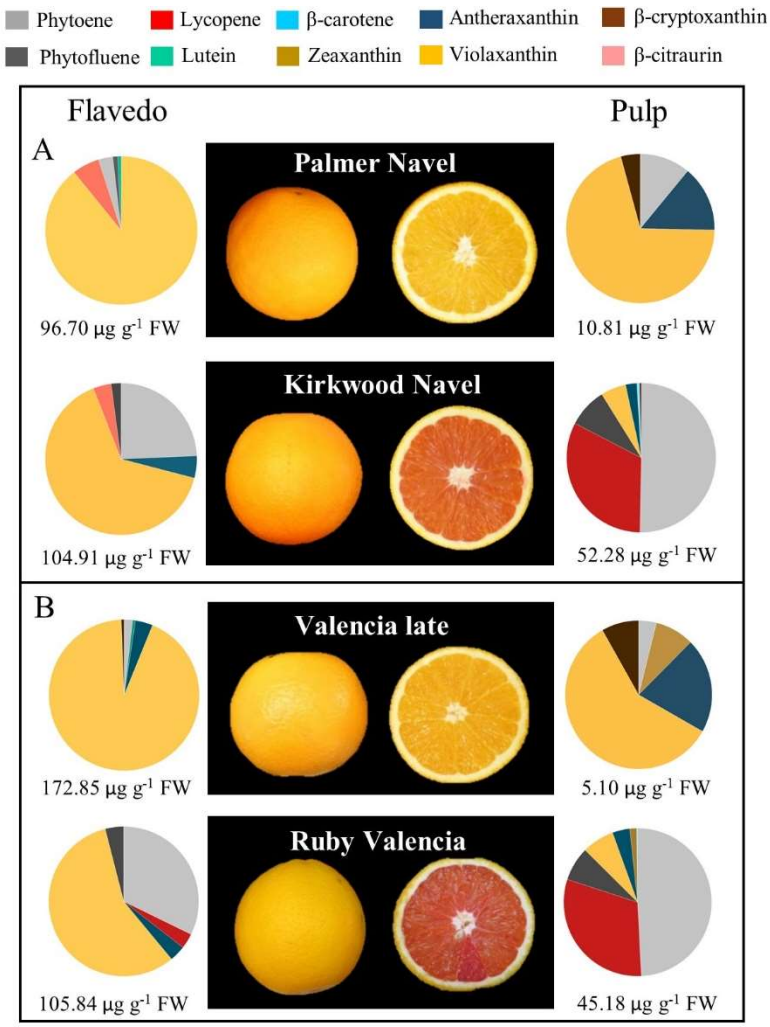


Figure 2. External and internal appearance and carotenoids content and composition of flavedo and pulp of mature fruits of Palmer Navel and Kirkwood Navel **(A)** and Valencia late and Ruby Valencia **(B)** oranges harvested in South Africa. The numbers below indicate total carotenoids concentration (µg g^{-1} FW).

As a first step for the physiological characterization of the altered colouration and biochemical compliment in both mutants, we performed an exploratory analysis of carotenoid composition in mature K and R fruits grown in a commercial orchard in the original country (Citrusdal, Western Cape, South Africa) in the season 2013/2014. The red-fleshed varieties showed an altered carotenoid profile both in the pulp and the flavedo (**Figure 2**). Total carotenoids in the pulp were nearly 5- and 9-times higher in K and R, respectively, than in the corresponding parental. The red pigmentation in the pulp of the mutants was associated with: 1) lycopene accumulation, at concentrations between 14 and 17 μg g^{-1} FW; 2) accumulation of high amounts of 15-*Z*-phytoene (>20 μg g^{-1} FW) and phytofluene (3-5 μg g^{-1} FW), and 3) reduced content of violaxanthin (up to 3 times lower in K than Palmer Navel). Despite the apparent normal orange colour of the flavedo in mutant fruits, total carotenoid content was similar in K and Palmer Navel but higher in Valencia (61%) than in R. Interestingly, the peel of both genotypes contained higher amounts of phytoene and reduced concentration of violaxanthin than the parental genotypes (**Figure 2**).

Further experiments were carried out on trees of K and R, and Foios Navel (N) and Midknight Valencia (V), as representatives of the standard parental varieties, grown in the same experimental orchard under Mediterranean environmental conditions of Valencia (Spain). N and K orange varieties are characterized by mid-season maturity and the period of harvesting would cover between December and February. Instead, Midknight Valencia and Ruby Valencia are late maturing oranges and generally harvested between April and May (Barry et al., 2020). Fruits were harvested from early stages of development to full maturity, covering eight developmental stages, from June to January in K and N, and nine stages, from June to April in R and V. The evolution of peel colouration, determined as the *a/b* Hunter ratio, were similar between fruits of the standard and the red-fleshed orange varieties (**Figure 3A**, **I**, **K**).

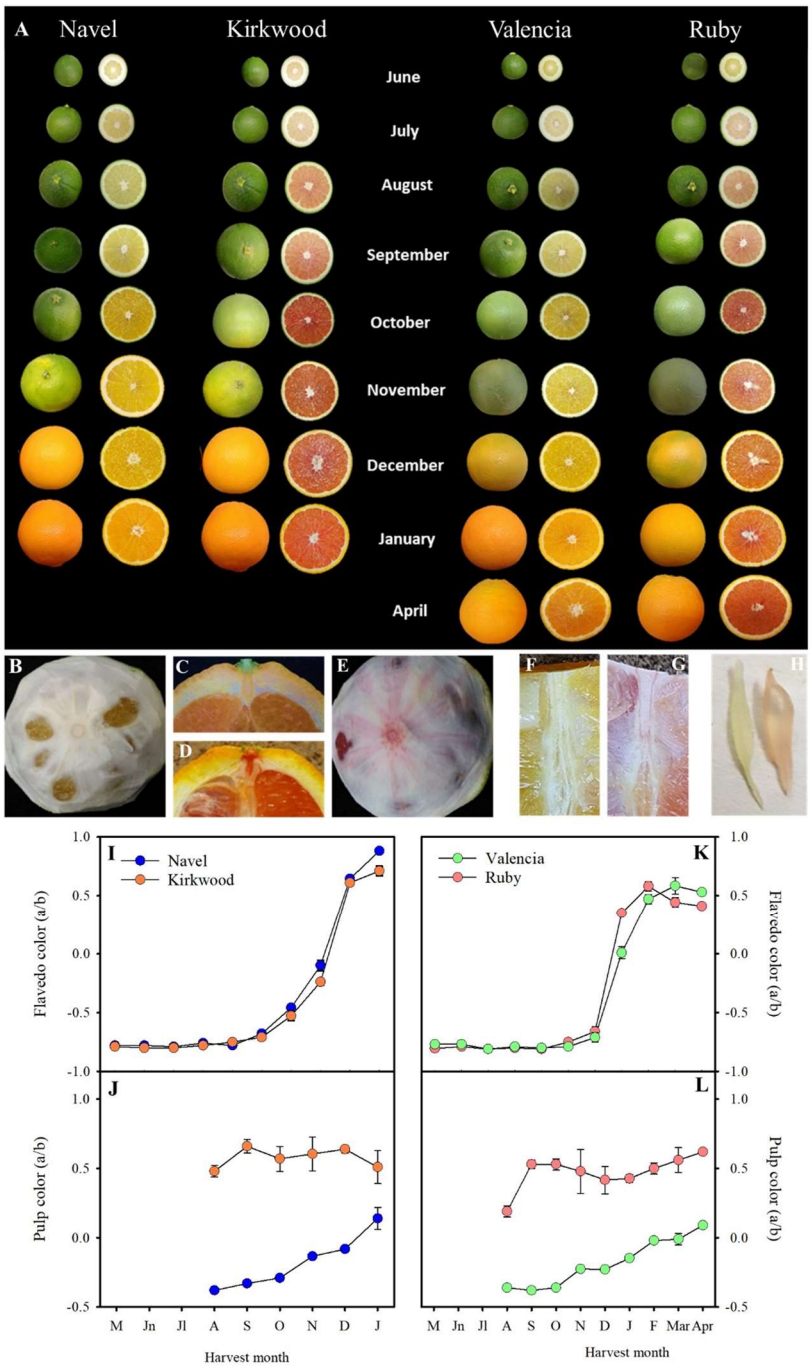


Figure 3. External and internal appearance of Navel, Kirkwood, Valencia and Ruby orange (*Citrus sinensis*) during fruit development and maturation, at the indicated harvest month. Fruits were harvested in the season 2018/2019, from an orchard located in Fundación ANECOOP (Museros, Valencia), Spain **(A)**. Mature fruits of Valencia **(B)** and Ruby **(E)** oranges, in which the external flavedo has been removed to see the color and the veins of the internal albedo layer. Longitudinal sections of the upper side of Valencia **(C)** and Ruby **(D)** oranges showing the notch of the peduncle and calix. Longitudinal section of the pulp of Valencia **(F)** and Ruby **(G)** oranges showing colour of the pulp and the central axis. Juice vesicle of Valencia (left) and Ruby (right) **(H)**. Evolution of the colour of the flavedo **(I** and **K)** and pulp **(J** and **L**) of Navel and Kirkwood, and Valencia and Ruby, respectively, during fruit development and maturation. Data are the means of 30 measurements ± SE.

Mutant fruits did not display red pigmentation in the peel at any stage of maturation. On the contrary, differences in pulp colour between the standard and the red varieties were observed from the early stages of fruit development. From June onwards, the pulp of young fruitlets of both mutants showed a light-pink tint that turned more reddish as fruit developed (September), whereas fruits of N and V displayed greenish-yellow colour (**Figure 3A)**. From October to full maturation, K and R pulp developed a bright-red colouration (**Figure 3A, E**, **G**), while N and V turned to the characteristic yellowish-orange tones of traditional oranges (**Figure 3A**, **B**, **F**). The *a/b* Hunter ratio was higher in the pulp of K and R during the whole fruit development and maturation, exhibiting positive values since the early stages of fruit development (**Figure 3J**, **L**). Moreover, the *a/b* Hunter ratio of K and R mature fruits was approximately 0.5, while N and V fruits displayed values around 0.1, in agreement with the colour exhibited by the corresponding juice vesicles (**Figure 3H**). It is worth to mention that other tissues of K and R, such as the calyx button and axial and central vascular bundle showed reddish pigmentation while the corresponding tissues of N and V fruits remained colourless or yellow-pigmented (**Figure 3B-G**).

Internal maturity index (MI) (ºBrix/acidity) was determined at two ripening stages: at colour-break (October), and at full maturity, January for N and K, and April for V and R fruits. The MI of K was lower than in N at both ripening stages, while R showed similar levels to V (**Supplementary Table 1**, **2**). In general, internal maturation indexes in the red-fleshed mature fruits were virtually identical to the standard Navel and Valencia oranges.

3.2. Carotenoids content and composition in standard Navel and Valencia, and the red-fleshed Kirkwood and Ruby oranges during fruit development and maturation

Carotenoid content and composition in the pulp and flavedo of K and R and the corresponding standard varieties were analysed during fruit development and maturation (**Table 1**, **2** and **Supplementary Table 5**, **6**). Overall, the HPLC-DAD analysis identified twenty-six carotenoids in both fruit tissues (**Supplementary Table 3**).

Differences in carotenoids content and composition in the pulp of the red-fleshed and standard oranges were evident from the early stages of development (June). Indeed, significant accumulation of phytoene and phytofluene occurred in K and R, accounting 90-99% of total carotenoids content, while N and V presented very little or negligible amounts of total carotenoids (**Table 1**, **2**). The concentrations of phytoene and phytofluene substantially

increased during ripening, and in the pulp of mature fruits of K and R reached levels as high as 80 and 148 μg g^{-1} FW, respectively, representing between 80-85% of the total content (**Table 1**, **2**). Moreover, lycopene was detected at early stages in K and R, and its content increased from traces to 5.80 and 9.90 μg g^{-1} FW in mature fruits, respectively. Due to the significant accumulation of carotenes in the pulp of the red-fleshed varieties, the concentration of total carotenoids ranged from 6- to 1,000 times higher than the standard oranges (**Table 1**, **2**).

Table 1. Carotenoid content and composition (μg g^{-1} FW) in the pulp of Navel and Kirkwood Navel at six different stages of development and maturation. A+Z is the sum of antheraxanthin and zeaxanthin. Violaxanthin is the sum of all *E*-violaxanthin, 9-*Z*-violaxanthin and luteoxanthin. Other xanthophylls are the sum of *α*-cryptoxanthin, β-cryptoxanthin, neoxanthin and non-identified xanthophylls (Table S2). Nd, no detected. Traces indicates concentrations lower than 0.05 μg g^{-1}. Data are the mean of three biological replicates ± SD. Asterisks indicate significant differences between Navel and Kirkwood varieties by unpaired Student's t-test ($p<0.05$).

Carotenoids (μg g^{-1} FW)	**Navel**					
	June	**July**	**September**	**October**	**December**	**January**
Phytoene	3.20±0.02*	traces	traces	traces	traces	nd
Phytofluene	0.16±0.08*	nd	nd	nd	nd	nd
ζ-carotene	nd	nd	nd	nd	nd	nd
Neurosporene	nd	nd	nd	nd	nd	nd
Lycopene	nd	nd	nd	nd	nd	nd
δ-carotene	nd	nd	nd	nd	nd	nd
Lutein	0.17±0.02	nd	0.18±0.03	0.07±0.01	0.38±0.03*	0.66±0.19*
β-carotene	nd	nd	nd	nd	nd	nd
A + Z	nd	nd	nd	0.04±0.01	0.88±0.12*	3.46±0.11*
Violaxanthin	0.67±0.09	nd	0.02±0.01	0.77±0.21	4.60±0.22*	9.35±0.83*
Other xanthophylls	0.22±0.09	nd	nd	nd	1.04±0.65	3.35±0.30*
Total carotenoids	4.42±0.42*	traces	0.20±0.02*	0.99±0.32*	6.91±1.25*	16.82±1.91*
Carotenoids (μg g^{-1} FW)	**Kirkwood**					
	June	**July**	**September**	**October**	**December**	**January**
Phytoene	37.55±1.55	45.19±2.15	53.8±4.68	70.91±3.30	79.20±9.62	65.81±4.48
Phytofluene	3.35±0.13	7.8±0.44	9.73±1.14	9.02±0.30	8.63±0.50	14.97±0.34
ζ-carotene	nd	nd	nd	nd	0.12±0.02	0.45±0.12
Neurosporene	0.12±0.02	0.44±0.07	1.01±0.02	1.50±0.16	nd	2.34±0.03
Lycopene	0.19±0.03	0.90±0.09	2.09±0.44	2.50±0.20	5.25±0.26	5.80±0.06
δ-carotene	traces	0.20±0.09	0.28±0.02	0.60±0.20	nd	1.01±0.09
Lutein	nd	nd	nd	nd	0.27±0.02	1.07±0.14
β-carotene	0.15±0.04	nd	0.15±0.02	0.07±0.01	0.34±0.02	0.55±0.21
A + Z	nd	nd	nd	0.08±0.02	1.10±0.03	2.82±0.14
Violaxanthin	0.40±0.15	nd	nd	0.72±0.02	2.62±0.01	3.12±0.14
Other xanthophylls	0.07±0.01	nd	nd	0.15±0.06	0.46±0.11	1.25±0.66
Total carotenoids	41.83±6.41	54.53±8.57	67.06±3.68	85.549±0.99	98.08±0.49	99.19±6.86

Other unusual carotenoids in citrus fruit, such as neurosporene, ζ-carotene and δ-carotene were also detected in variable proportions in the pulp of both mutants, while they were not detected in standard oranges (**Table 1**, **2**). The concentration of these rare carotenoids in K and R pulps increased progressively during maturation until reaching contents of 1-2 μg g^{-1} FW in mature fruits. The presence of low or very low levels of β-carotene (<1 μg g^{-1}) was quantified in mature pulp of K and R, while it was not detected in the standard oranges.

Table 2. Carotenoid content and composition (μg g^{-1} FW) in the pulp of Valencia and Ruby at six different stages of fruit development and maturation. A+Z is the sum of antheraxanthin and zeaxanthin. Violaxanthin is the sum of all E-violaxanthin, 9-Z-violaxanthin and luteoxanthin. Other xanthophylls are the sum of α-cryptoxanthin, β-cryptoxanthin, neoxanthin and non-identified xanthophylls (Table S2). Nd, no detected. Traces indicates concentrations lower than 0.05 μg g-1. Data are the mean of three biological replicates ± SD. Asterisks indicate significant differences between Valencia and Ruby varieties by unpaired Student's t-test ($p<0.05$).

Carotenoids (μg g^{-1} FW)	Valencia					
	June	**July**	**September**	**October**	**January**	**April**
Phytoene	traces	traces	0.03±0.01*	traces	traces	nd
Phytofluene	nd	traces	0.15±0.05*	nd	nd	nd
ζ-carotene	nd	nd	nd	nd	traces	0.09±0.01*
Neurosporene	nd	nd	nd	nd	nd	nd
Lycopene	nd	nd	nd	nd	nd	nd
δ-carotene	nd	nd	nd	nd	nd	nd
Lutein	0.08±0.01*	nd	nd	nd	nd	1.18±0.47
β-carotene	nd	nd	nd	nd	nd	nd
A + Z	nd	nd	nd	0.09±0.01	0.94±0.04*	3.87±0.05*
Violaxanthin	0.50±0.03*	nd	nd	0.40±0.01*	5.18±0.01*	7.59±0.10*
Other xanthophylls	0.18±0.03	nd	nd	0.15±0.17	0.98±0.33	2.81±0.78*
Total carotenoids	0.81±0.18*	traces	0.18±0.01*	0.69±0.13*	7.10±0.89*	15.45±1.91*
Carotenoids (μg g^{-1} FW)	Ruby					
	June	**July**	**September**	**October**	**January**	**April**
Phytoene	17.76±2.45	53.07±3.58	65.7±2.33	72.44±0.59	87.15±6.27	124.24±0.95
Phytofluene	1.21±0.21	4.6±0.7	7.64±1.14	10.94±0.38	14.55±0.37	24.48±0.46
ζ-carotene	nd	nd	0.07±0.03	nd	0.20±0.01	1.24±0.05
Neurosporene	nd	0.18±0.02	0.53±0.03	1.03±0.14	0.63±0.14	2.64±0.01
Lycopene	traces	0.40±0.02	0.17±0.09	1.17±0.21	2.90±0.69	9.95±3.05
δ-carotene	nd	0.05±0.01	nd	nd	0.28±0.09	1.05±0.03
Lutein	0.31±0.01	nd	0.15±0.03	0.29±0.012	0.32±0.41	0.87±0.57
β-carotene	nd	nd	nd	0.38±0.01	0.24±0.05	1.60±0.01
A + Z	nd	nd	nd	0.09±0.01	0.77±0.05	3.52±0.04
Violaxanthin	0.85±0.01	nd	nd	0.12±0.02	2.40±0.22	4.10±0.02
Other xanthophylls	0.18±0.02	nd	nd	nd	0.35±0.21	1.01±0.19
Total carotenoids	20.36±7.58	58.30±3.51	74.26±2.23	86.46±0.21	109.79±3.89	174.70±5.34

Overall, the concentration of carotenes, including lycopene, in the pulp of both red-fleshed varieties increased steadily with ripening and the highest contents were reached at full maturity. The carotenoid profile of standard N and V oranges was characterized by the accumulation of β,β-xanthophylls since October, reaching the highest levels in January and April, respectively. The main carotenoid in mature fruits of both standard oranges was violaxanthin, followed by antheraxanthin and other β,β-xanthophylls. Even tough, β,β-xanthophylls also accumulated in K and R during maturation, their concentrations were between 2- and 3-times lower than in the standard oranges (**Table 1**, **2**).

The flavedo of green fruits (September and October) of the four orange varieties showed the characteristic composition of chloroplastic tissues, being lutein, the predominant carotenoid followed by minor concentrations of β,β-xanthophylls (violaxanthin, neoxanthin, zeaxanthin and anteraxanthin) and α- and β-carotene (**Supplementary Table 5**, **6**). In all varieties, an increase of β,β-xanthophylls and phytoene occurred during ripening concomitantly with a decrease of lutein and α- and β-carotene. The main difference in flavedo between the red and the standard varieties was the concentration of phytoene, which was between 2- and 20-times higher in K and R (**Supplementary Table 5**, **6**). On the other hand, the content of violaxanthin in December and January in the flavedo of K and R was between 30% and 50% lower than in the corresponding standard oranges, respectively.

3.3. Plastid ultrastructure in the juice vesicles of Navel and Kirkwood oranges

To obtain a more thorough insight into the alteration in carotenoid complement of mutant fruits, the plastids ultrastructure of juice vesicles cells from the N and K immature (**Figure 4A**, **C**) and mature (**Figure 4B**, **D**) fruits were examined by transmission electron microscopy (TEM). Differences in the ultrastructure of plastids were identified between both genotypes and ripening stages (**Figure 4A-D**). Plastids of immature fruits of standard N oranges presented a globular-type structure with plastoglobuli (**Figure 4A**), while chromoplasts of mature fruits were characterized by the increase in the number and size of plastoglobuli (**Figure 4B**). However, plastids of immature K fruits showed the presence of internal tubular membranes, starch granules and a lower number or absence of plastoglobuli (**Figure 4C**). In addition, juice vesicles of mature mutant fruits contained chromoplasts with different morphology and organization than those of standard oranges: reduced number of

plastoglobuli, long structures compatible with lycopene crystals, and a high number of round electron-lucent vesicles (**Figure 4D**). Thus, the changes in carotenoid content and composition in the red-fleshed orange mutants are also associated with alterations in plastid ultrastructure.

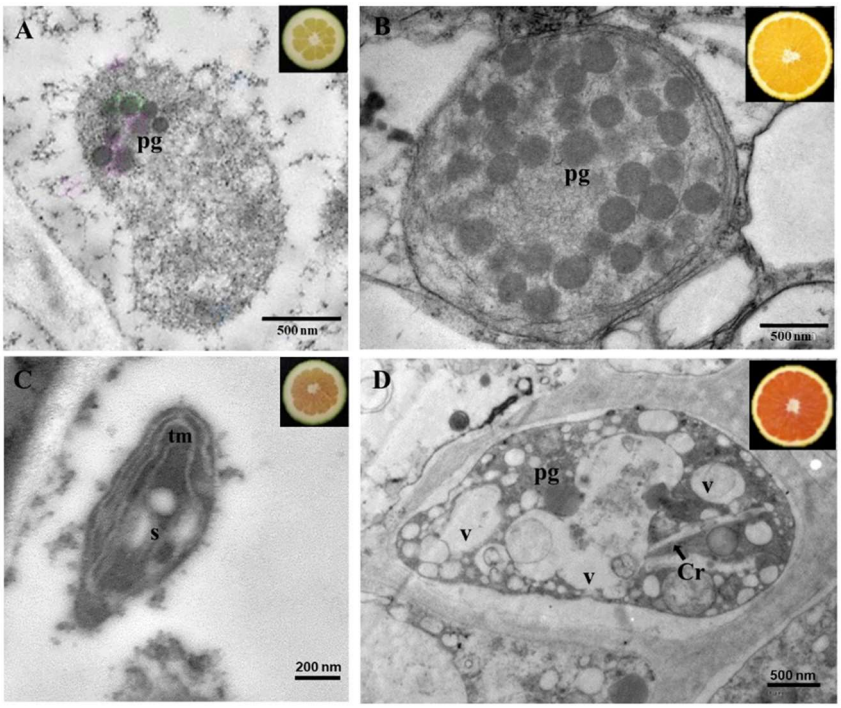


Figure 4. Transmission electron microscopy pictures of chromoplasts of the pulp of immature **(A)** and mature **(B)** fruits of Navel, and immature **(C)** and mature **(D)** fruits of Kirkwood. Pg, plastoglobuli; tm, tubular membranes; V; vesicles; S, starch; Cr, crystals of lycopene.

3.4. Expression of genes involved in carotenoid metabolism in standard Navel and Valencia, and the red-fleshed Kirkwood and Ruby oranges during fruit development and maturation

The transcriptional profiling of 26 genes related to carotenoid metabolism and accumulation was analysed in the pulp and flavedo of the mutants K and R, and the corresponding standard varieties N and V during fruit development and maturation (**Figure 5**, **6** and **Supplementary Figure 2**, **3)**. Most of the genes showed a similar expression pattern between the standard oranges and their respective red-fleshed varieties, even though some variety- or ripening-specific differences were observed (**Figure 5, 6**). Transcripts of *β-CHX2* were not detected in any of the samples analysed, and *PSY2*, *ε-LCY* and *CCD4b* were only detected in flavedo.

In general, in the four varieties similar time–course gene expression patterns during development and ripening were found. The genes of the carotenoid precursors *DXS1, HDR* and *HDS* experienced a transient up-regulation in the pulp during fruit development or

maturation, while expression of *DXS2*, *GGPS1* and *GGPS3* was sustained or decreased (**Figure 5, 6**). Notably, the expression of *PSY*, *PDS*, *Z-ISO*, *ZDS*, *β-LCY2 (a* and *b* alleles*)* and *β-CHX*, enzymes involved in the serial conversion of phytoene to β,β-xanthophylls, were up-regulated during ripening. The maximum level of expression for these genes was reached approximately in November and January, depending on the orange group (Navel or Valencia) (**Figure 5, 6**), coinciding with the maximum increment in the accumulation of β,β-xanthophylls.

Transcript levels of *β-LCY1* did not substantially change during ripening. The pulp of both mutants showed significantly higher expression of *DXS1* than the standard varieties in June (early stage of fruit development), however, these differences were not sustained during ripening. The expression of other carotenoid biosynthetic genes in the pulp of K and R revealed a delay in their up-regulation compared to the standard varieties (**Figure 5, 6**). In October, K fruits showed lower relative expression of *HDR*, *GGPS1*, *GGPS3*, *PSY*, *PDS*, *ZDS*, *β-LCY1*, *β-LCY2* than N fruits, but in November only *β-LCY1*, *β-LCY2* and *β-CHX* genes were reduced in K compared to N (**Figure 5**). Similarly, the expression of *PDS*, *ZDS*, *Z-ISO*, *β-LCY1*, *β-LCY2* and *β-CHX* genes was also lower in R compared to V in October and November (**Figure 6**). For most of the carotenoid biosynthetic genes, transcript levels decreased at later stages of ripening (**Figure 5, 6**).

Regarding the genes encoding for 9-*Z*-epoxycarotenoid dioxygenases (*NCED1* and *2*), which are involved in the catabolism of 9-*Z*-xanthophylls and the production of ABA, a relative high expression was detected at immature stages (June-July) that decreased progressively until the beginning of maturation, to increase again between November and January (**Figure 5, 6**). Interestingly, in June, both K and R showed significantly lower expression of *NCED1* than N and V oranges, respectively. Some differences were observed in the expression of *NCED1* and *2* genes at other ripening stages between the red and the blond oranges, but without a consistent trend. Additionally, *CCD1*, which also encodes for a carotenoid cleavage dioxygenase enzyme, showed a transient up-regulation with a maximum expression in November or December. To elucidate whether the massive accumulation of carotenoids in the red-fleshed mutants may be associated with alterations in proteins related to carotenoid storage, genes involved in carotenoid-sequestering structures *(FIBs)*, chaperone activity (*HSP21)* and esterification of xanthophylls *(PYPs)* were examined.

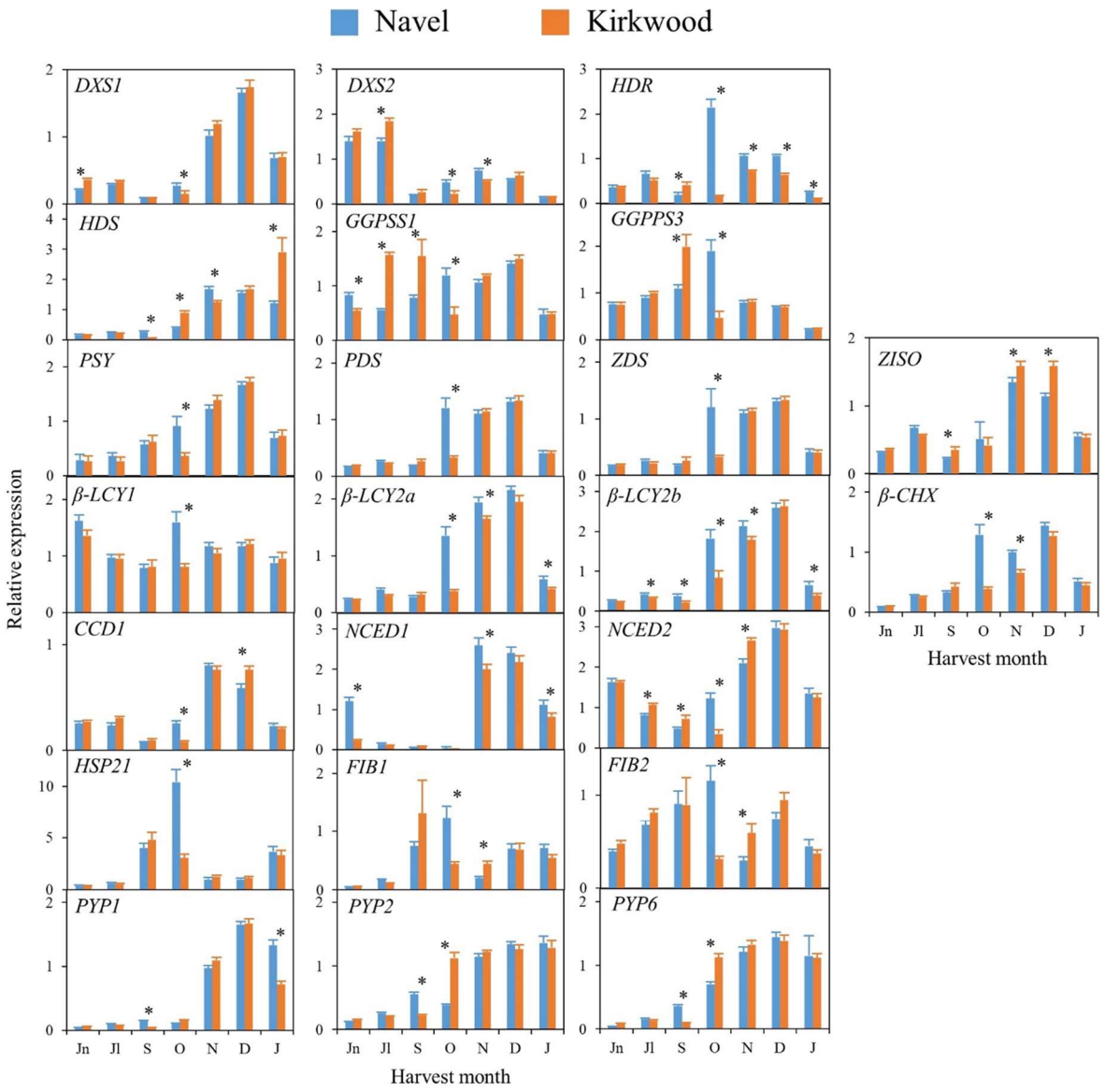


Figure 5. Relative expression levels of genes of carotenoids precursors (*DXS1*, *DXS2*, *HDR*, *HDS* and *GGPPS1* and *GGPPS3*), carotenes and xanthophylls biosynthesis (*PSY, PDS, Z-ISO, ZDS, β-LCY1, β-LCY2a, β-LCY2b, β-CHX*), carotenoids catabolism (*CCD1b*), ABA biosynthesis (*NCED1* and *NCED2*), carotenoid-associated protein (*HSP21*, *FIB1* and *FIB2*) and xanthophyll esterification (*PYP1, 2* and *6*) in the pulp of Navel and Kirkwood during fruit development and maturation. Jn, June; Jl, July; S, September; O, October; N, November; D, December; J, January. Data are the mean of three biological replicates ± SD. Asterisks indicate significant differences between Navel and Kirkwood varieties by unpaired Student's t-test ($p < 0.05$).

The expression profiles of *FIB* genes did not follow a definite trend, but in general, their levels of expression were down-regulated along maturation. The expression of *HSP21* experienced a sudden increase concomitantly with the shift of external colour from green to yellow (October), showing a major induction in N than in K.

This work examined for the first time the expression of *PYP1, 2* and *6* genes, the putative enzymes responsible for xanthophylls fatty acid esterification during fruit ripening, in the red-fleshed oranges. In general, *PYPs* genes exhibited an up-regulation pattern during ripening but higher levels of *PYP2* and *PYP6* transcripts were detected in both red-fleshed varieties in October. Nevertheless, minor differences in the expression of *PYPs* were observed between varieties at later stages of ripening (**Figure 5, 6**).

The expression of 26 genes related to carotenoid metabolism and accumulation was also analysed in flavedo of the four orange genotypes. As a general observation, in the four varieties the relative transcript levels for all the genes was between 2 and 50-times higher in the flavedo than in the pulp. The transcripts of *PSY2*, *ε-LCY* and *CCD4b* were only detected in flavedo. Similarly to the expression profiling described for the pulp, most of the genes evaluated in the flavedo displayed a comparable expression pattern in the standard than in the red-fleshed varieties. However, some differences were identified for certain genes and ripening stages: genes of carotenoids precursors *DXS1*, *DXS2* and *GGPS3* were down-regulated during ripening. In contrast, *HDR*, *HDS* and *GGPS1*, and most of the carotenoid biosynthetic genes (*PSY, PDS, ZDS, Z-ISO*, *β-LCY2a/b* and *β-CHX*) were progressively up-regulated during maturation (**Supplementary Figure 2**, **3**). The expression levels of *ε-LCY* and *β-LCY1* genes were maintained relatively constant during maturation. In Navel oranges, the genes involved in carotenoid catabolism, *NCEDs*, *CCD1* and *CCD4b*, were up-regulated, whereas in Valencia oranges displayed a fluctuating pattern. Finally, *FIBs*, *HSP21* and *PYPs* genes were up-regulated in the flavedo of all varieties and, interestingly, their expression was substantially higher in the flavedo of mature fruits of K than N (**Supplementary Figure 2**).

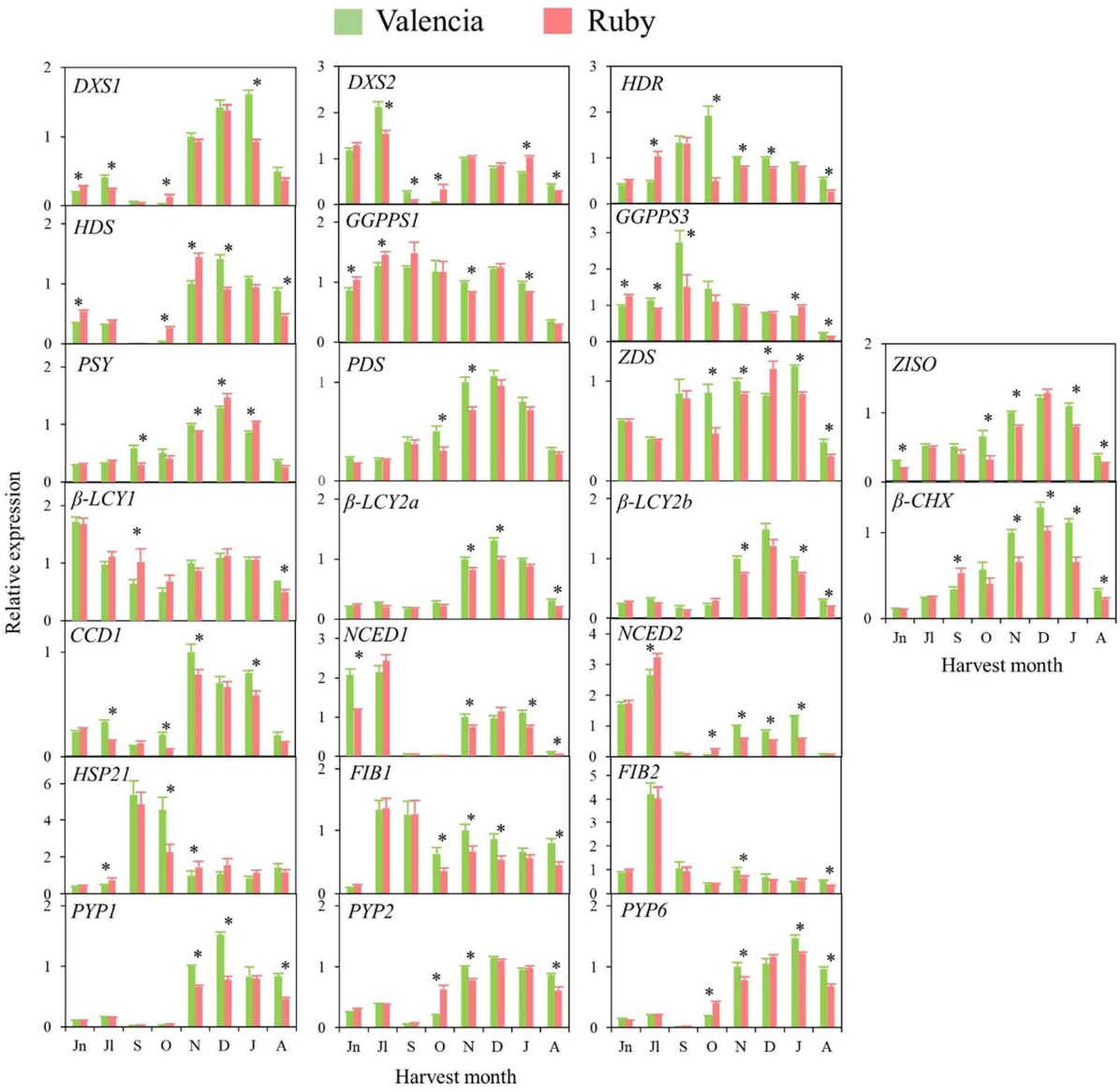


Figure 6. Relative expression levels of genes of carotenoids precursors (*DXS1*, *DXS2*, *HDR*, *HDS* and *GGPPS1* and *GGPPS3*), carotenes and xanthophylls biosynthesis (*PSY*, *PDS*, *Z-ISO*, *ZDS*, *β-LCY1*, *β-LCY2a*, *β-LCY2b*, *β-CHX*), carotenoids catabolism (*CCD1b*), ABA biosynthesis (*NCED1* and *NCED2*), carotenoid-associated protein (*HSP21*, *FIB1* and *FIB2*) and xanthophyll esterification (*PYP1, 2* and *6*) in the pulp of Valencia and Ruby during fruit development and maturation. Jn, June; Jl, July; S, September; O, October; N, November; D, December; J, January; A, April. Data are the mean of three biological replicates ± SD. Asterisks indicate significant differences between Valencia and Ruby varieties by unpaired Student's t-test ($p < 0.05$).

3.5. Sequences of ε-LCY, β-LCY1 and β-LCY2 in standard Navel and Valencia, and the red-fleshed Kirkwood and Ruby oranges

The large amounts of carotenes upstream lycopene in the pulp of K and R fruits compared with the standard N and V envisage alterations in the cyclization of lycopene from the very early stages of fruit development. Alterations in the sequences of *ε-LCY*, *β-LCY1* and/or *β-*

LCY2 leading to non-functional proteins could be, indeed, a primary cause for lycopene accumulation in the red-fleshed oranges. To explore this hypothesis, the full-length coding sequences of *ε-LCY*, *β-LCY1* and/or *β-LCY2* of the red-fleshed varieties were examined for possible mutations. For each gene, two alleles, *a* and *b*, were identified: *β-LCY1*a (OP441052), *β-LCY1*b (OP441053), *β-LCY2a (*OP441054*), β-LCY2b (*OP441055*), ε-LCYa* (OP441056) and *ε-LCYb* (OP441057) (**Supplementary Figure 4** and **Supplementary Table 7**), which agrees with the heterozygosity of the sweet orange genome (Wu et al., 2014). The alleles *a* and *b* of the *ε-LCY*, *β-LCY1*, *β-LCY2* genes differed in 5, 18 and 27 SNPs that resulting in 5, 10 and 21 amino acid changes, respectively (**Supplementary Table 7**). The sequence analyses of alleles *a* and *b* for each gene and variety revealed no differences between the standard and the red-fleshed genotypes.

3.6. ABA and its catabolites in standard Navel and Valencia, and the red-fleshed Kirkwood and Ruby oranges during fruit development and maturation

The levels of abscisic acid (ABA), of its sugar-conjugated form ABA-glucosyl ester (ABA-GE), its catabolites, phaseic acid (PA) and dihydrophaseic acid (DPA), were determined in the pulp of the four orange genotypes at four stages of fruit development and ripening by LC-HRMS analysis (**Figure 7**).

ABA and ABA-GE levels increased in the standard varieties throughout fruit maturation showing the maximum accumulation in mature fruits. However, in the pulp of K and R the relative levels of ABA and ABA-GE remained constant during ripening. Therefore, the relative levels of ABA and ABA-GE were between 2 and 3-times higher in the pulp of standard oranges compared to their respective mutants at colour break and mature stage. The levels of PA and DPA in the red-fleshed and standard oranges followed a similar pattern during ripening with minor and non-consistent differences detected between varieties or ripening stages (**Figure 7**).

In flavedo, minor differences were detected between the red-fleshed and standard varieties. K contained lower levels of ABA, ABA-GE and DA than N fruits in October, while R and V fruits did not show significant differences in ABA or its catabolites (**Supplementary Table 8**, **9**).

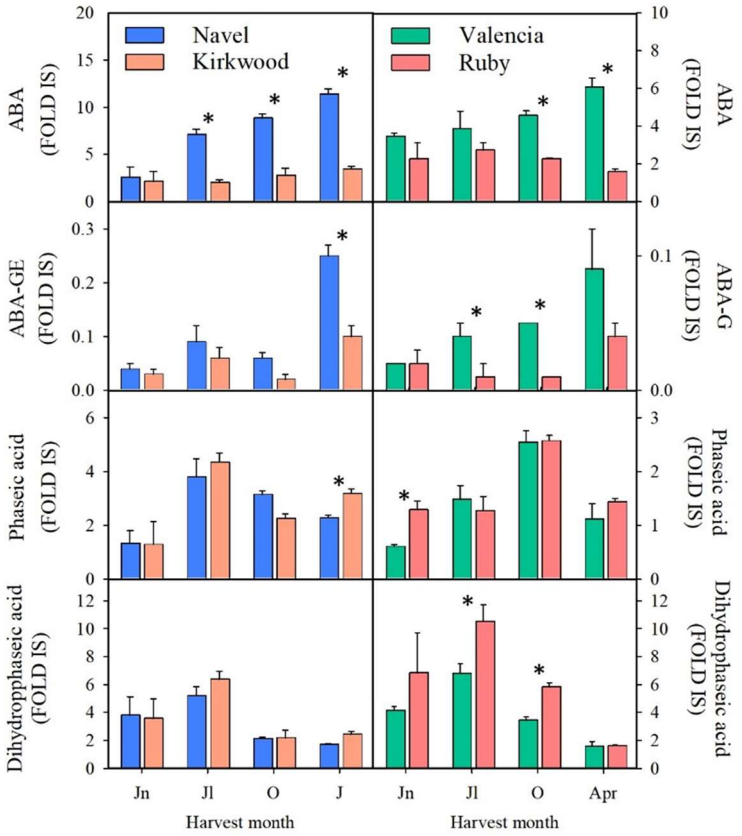


Figure 7. Content of abscisic acid (ABA), abscisic acid glucose ester (ABA-GE), phaseic acid and dihydrophaseic acid (Fold Internal Standard) in the pulp of Navel and Kirkwood (left panels) and Valencia and Ruby (right panels) during fruit development and maturation. Jn, June; Jl, July; O, October; J, January; Apr, April. Data are the mean of three biological replicates ± SD. Asterisks indicate significant differences between Kirkwood and Ruby and their respective standard varieties by unpaired Student's t-test ($p < 0.05$).

3.7. Chlorophylls and carotenoids composition in leaves of Navel and Valencia and the red-fleshed mutants Kirkwood and Ruby

Chlorophyll and carotenoid composition analyses in leaves revealed noticeable differences between the red-fleshed and standard orange varieties. (**Table 3**). More specifically, in the leaves of both mutants, the content of chlorophyll 'a' and 'b' were higher than in the standard varieties, resulting in total chlorophylls being 20-30% higher. Leaves of K and R presented larger total carotenoid concentrations than the corresponding N and V. Total carotenoids in K and R leaves accounted 317.19 μg g^{-1} FW and 306.67 μg g^{-1} FW, respectively, compared with 251.84 μg g^{-1} FW and 232.45 μg g^{-1} FW of N and V, respectively.

Lutein was the main carotenoid in the leaves of all varieties, followed by violaxanthin, other β,β-xanthophylls, and α-carotene and β-carotene. K and R leaves contained elevated concentrations of α-carotene and lutein although not statistically significant for lutein in K and

for α-carotene in R. Nevertheless, the most remarkable feature regarding carotenoid composition in leaves of both mutants was the large accumulation of phytoene, with an 8- and 6-fold increment with respect to N and V, respectively (**Table 3**).

Table 3. Chlorophylls and carotenoids content and composition (μg g^{-1} FW) in leaves of Navel, Kirkwood Navel, Valencia and Ruby varieties. The amount of violaxanthin represents the sum of all-*E*-violaxanthin, 9-*Z*-violaxanthin isomers. Other β,β-xanthophylls are the sum of antheraxanthin, zeaxanthin and neoxanthin. Data are the mean of three biological replicates ± SD. Asterisks indicate significant differences between Kirkwood and Ruby and their respective standard varieties by unpaired Student's t-test ($p<0.05$).

Chlorophylls (μg g^{-1})	**Navel**	**Kirkwood**	**Valencia**	**Ruby**
Chlorophyll a	1116.65 ± 15.38*	1557.93 ± 60.74	1447.22 ± 7.00*	1773.95 ± 220.28
Chlorophyll b	413.29 ± 1.65*	426.03 ± 5.19	456.83 ± 1.33*	593.70 ± 1.65
Total chlorophylls	1529.95 ± 17.03*	1983.97 ± 65.93	1904.05 ± 5.66*	2367.66 ± 17.03
Carotenoids (μg g^{-1})				
Phytoene	3.43 ± 0.28*	25.84 ± 4.97	3.09 ± 0.57*	17.59 ± 0.59
Lutein	140.19 ± 8.23	151.60 ± 8.51	124.27 ± 12.23*	164.44 ± 14.30
α-carotene	23.09 ± 3.78*	38.10 ± 5.31	19.12 ± 2.88	23.03 ± 2.82
β-carotene	17.27 ± 3.76	21.56 ± 4.72	23.18 ± 6.65	19.68 ± 2.36
Violaxanthin	46.63 ± 3.76	56.67 ± 3.61	36.29 ± 2.24	42.99 ± 3.56
Other β,β-xanthophylls	21.23 ± 2.53	23.42 ± 1.90	26.50 ± 0.40*	39.52 ± 2.83
Total carotenoids	251.84 ± 21.78*	317.19 ± 29.09	232.45 ± 5.91*	306.67 ± 30.25

Lutein was the main carotenoid in the leaves of all varieties, followed by violaxanthin, other β,β-xanthophylls, and α-carotene and β-carotene. K and R leaves contained elevated concentrations of α-carotene and lutein although not statistically significant for lutein in K and for α-carotene in R. Nevertheless, the most remarkable feature regarding carotenoid composition in leaves of both mutants was the large accumulation of phytoene, with an 8- and 6-fold increment with respect to N and V, respectively (**Table 3**).

3.8. Carotenoid composition in stems of Valencia and the red-fleshed mutant Ruby

A remarkable field observation that was made in the case of the stems of the Ruby and Kirkwood trees under South African growing conditions regarded an unusually reddish colouration at the inner side of the bark, specifically the layers of the phloem/cambium, between the bark and xylem/wood tissue (a trait that was not observed under Spanish growing conditions) (**Figure 8A-D**). This distinct coloration could be easily distinguished by peeling the bark from the branches, which was more noticeable in older shoots and branches. The

coloration continued in the whole tree until the graft union, whereafter the normal pale colour associated with citrus xylem was visible. By contrast, the same tissue in standard trees had an absence of any pigmentation and showed the characteristic green-yellow colouration of normal citrus branches (**Figure 8E-H**).

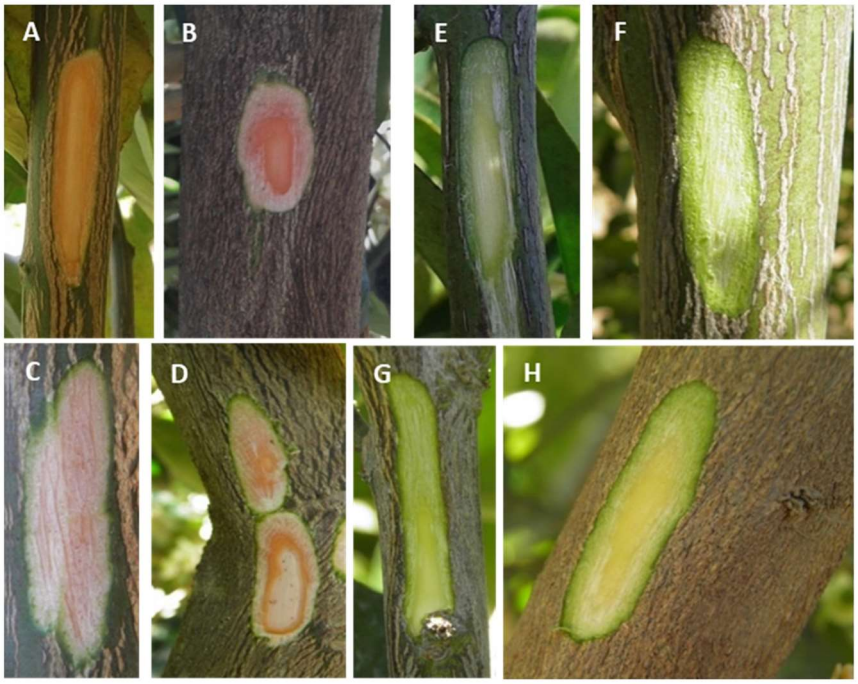
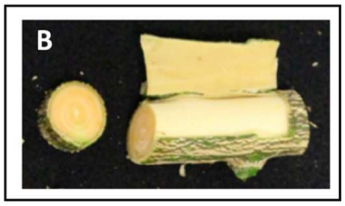


Figure 8. Representative pictures of young stems of Kirkwood **(A, B)**, Ruby **(C, D)** Navel **(E, F)** and Valencia **(G, H)** orange trees grown in South Africa. A small thin layer of the external bark was longitudinally removed to see the colouration of the inner bark and wood.

Since this unusual red pigmentation of the inner layer of the stems of citrus trees has not been previously reported, we inferred that it might be related to the accumulation of lycopene in these mutants. Therefore, the carotenoid composition was analysed in bark and wood tissues of the standard Valencia orange and its red mutant R from trees growing in Citrusdal (South Africa) (**Table 4**). The bark (containing the phloem/cambium) and xylem (wood) tissues were separated and analysed independently (**Table 4**).

Table 3. Chlorophylls and carotenoids content and composition (μg g^{-1} FW) in leaves of Navel, Kirkwood Navel, Valencia and Ruby varieties. The amount of violaxanthin represents the sum of all-*E*-violaxanthin, 9-*Z*-violaxanthin isomers. Other β,β-xanthophylls are the sum of antheraxanthin, zeaxanthin and neoxanthin. Data are the mean of three biological replicates ± SD. Asterisks indicate significant differences between Kirkwood and Ruby and their respective standard varieties by unpaired Student's t-test ($p<0.05$).



Carotenoids (μg g^{-1} DW)	Valencia		Ruby	
	Bark	**Wood**	**Bark**	**Wood**
Phytoene	5.45 ± 1.81*	1.16 ± 0.05*	389.45 ± 20.58	139.01 ± 32.51
Phytofluene	nd	nd	38.35 ± 1.38	5.10 ± 3.31
α-carotene	2.01 ± 1.00	nd	4.27 ± 1.00	nd
Lutein	104.99 ± 4.14*	1.09 ± 0.11	56.57 ± 3.78	nd
β-carotene	4.93 ± 1.20	nd	4.30 ± 0.64	nd
Lycopene	nd	nd	0.65 ± 0.08	traces
Antheraxanthin	4.72 ± 2.67	nd	nd	nd
Violaxanthin	31.56 ± 1.56*	0.10 ± 0.10	11.46 ± 0.20	nd
Neoxanthin	23.16 ± 0.86*	nd	11.66 ± 3.81	nd
Total carotenoids	176.82 ± 8.63*	2.35 ± 0.04*	516.70 ± 28.29	146.29 ± 35.76
ABA and ABA-related metabolites				
ABA	0.17 ± 0.04*	0.16 ± 0.01*	0.03 ± 0.01	0.03 ± 0.01
ABA-GE	nd	nd	0.04 ± 0.01	nd
DA	19.60 ± 1.54*	0.16 ± 0.02	4.83 ± 0.26	0.15 ± 0.01
PA	1.80 ± 0.14*	0.02 ± 0.01	0.16 ± 0.01	0.03 ± 0.01

In both genotypes, the concentration of carotenoids in the bark was higher than in the xylem tissue, and the differences between tissues were greater in the standard Valencia (74-times higher in bark) than in R (3.5-fold higher). The carotenoid content in bark and xylem of R was between 3 and 62 times higher than in the respective tissues of V. Moreover, both tissues of the R accumulated large concentrations of phytoene and significant amounts of phytofluene compared to V. Interestingly, low but still detectable amounts (<1 μg g^{-1} DW) of lycopene were quantified in the bark, while trace levels were detected in the xylem. As in the fruit pulp, a significantly lower concentration of xanthophylls was found in the bark of R compared to that

of V. Interestingly, the bark and xylem of the red-fleshed variety also contained 20% less ABA and of DA and PA than those of the standard V (**Table 4**).

This exceptional trait in branch and shoot tissue of the red-fleshed orange varieties led us to hypothesize that other lycopene-accumulating citrus species could also exhibit lycopene accumulation in these tissues. To explore this hypothesis, branches of three grapefruit (*Citrus paradisi*) varieties with different capacity to accumulate lycopene in the pulp and cultivated under similar environmental and nutritional conditions (IVIA, Valencia, Spain) were peeled off and examined. The Marsh grapefruit, which contains very low concentration of carotenoids in the pulp (Gross, 1987; Alquezar et al., 2013; Lado et al., 2015), did not show any distinctive colouration in the phloem/cambium. Nevertheless, Rio Red and Star Ruby, grapefruit varieties which accumulate lycopene in the pulp, exhibited a noteworthy reddish colouration in these tissues **(Supplementary Figure 5**). Altogether, these observations suggest that the metabolic alteration leading to lycopene accumulation affects not only the flesh of the fruit (oranges and grapefruit), but also other plant tissues (as xylem, calyx or vascular bundles).

4. Discussion

Accumulating lycopene in the pulp of citrus fruits is an unusual feature highly attractive to producers and consumers. Carotenoid metabolism in red-fleshed fruits of several citrus species, including grapefruit, pummelo, lemon and sweet orange, has been largely studied but the specific molecular mechanisms leading to lycopene accumulation in the pulp of sweet oranges has not yet been elucidated (Tadeo et al., 2020). The availability of two novel red-fleshed sweet orange mutants belonging to different genetic backgrounds (K to the Navel group and R to the Valencia group) allows for a reliable characterization of the carotenoid metabolism, to identify the molecular basis of the alteration leading to this particular phenotype. These red-fleshed mutants K and R were originally identified in South Africa (**Figure 2**) and were grafted and propagated in trees located in experimental orchards in Spain, retaining their stability as to their distinctive phenotypic alterations. This allows us to perform, under identical agronomic and climatic Mediterranean conditions, a comparative biochemical and molecular study of the carotenogenesis with standard Navel and Valencia genotypes during fruit development and maturation (**Figure 4**).

The red colouration of the pulp is the most remarkable phenotypic characteristic of K and R oranges. Other parameters analysed related to fruit development and maturation, as shape, external colour or internal maturity index (ºBrix or acidity) were very similar to that of their corresponding standard varieties, indicating that the colour of the flesh is the major phenotypic alteration in these fruits (**Figure 3**; **Supplementary Table 1**, **2**).

Comparison of the carotenoid content and composition in both red-fleshed mutants revealed a similar exceptional profiling with respect to the standard varieties. In the pulp of K and R oranges, indeed, more than 80% of total carotenoids correspond to linear carotenes, with large amounts of phytoene and phytofluene, and moderate content of lycopene, which provides their characteristic red pigmentation (**Figure 2**, **3**). This unique carotenoid profile of mature K and V oranges resembles that previously reported for the red-fleshed orange Cara Cara (Xu et al., 2006; Liu et al., 2007; Alquézar et al., 2008; Rodrigo et al., 2015; Lu et al., 2017; Liu et al., 2021). However, this altered carotenoid profile is different to that of the red Hong Anliu mutant, derived from the Chinese Anliu sweet orange, which accumulates lycopene in the pulp without increasing other upstream carotenes (Liu et al., 2007; Xu et al., 2009), suggesting that the basis of Hong Anliu alteration is different to that occurring in other red orange genotypes.

The early reddish-pink shade and accumulation of carotenes (including lycopene) in the pulp of K and R does not seem to be a ripening-related event. The mutation is manifested from the initial stages of carotenogenesis and occurs much earlier than the upsurge of carotenoids during maturation (**Table 1, 2**). This early pigmentation in the pulp of the mutants has also been observed in Cara Cara (Alquézar et al., 2008; Lu et al., 2017), pink lemon (Lana et al., 2020) and some red pummelos (Yan et al., 2018; Promkaew et al. 2020). However, in other red-fleshed genotypes, as Hong Anliu orange (Liu et al., 2007), red grapefruits (Alquezar et al., 2013) and Red Guanxi pummelo (Liu et al., 2016), lycopene accumulates during fruit ripening but not at the early stages of development. It is worth noting that in the pulp of grapefruits (*C. paradisi*), pummelos (*C. maxima*/*grandis*) and lemons (*C. limon*), the carotenogenesis is different to that of orange-coloured varieties (oranges and mandarins), and these fruits accumulate lower carotenoid content with a virtual absence of β,β-xanthophylls (Tadeo et al., 2020). Thus, the differential pattern of carotenes accumulation suggests that the alteration in carotenoid

metabolism leading to lycopene accumulation and the red pulp may differ among citrus species and varieties.

The carotenoid profiling between the standard and the red-fleshed oranges revealed that, besides the presence of lycopene in the pulp, a remarkable feature is the extremely high concentration of phytoene, reaching values of 80-125 μg g^{-1} FW in both flavedo and pulp tissues of mature fruits (**Table 1**, **2**; **Supplementary Table 5**, **6**). The phytoene concentrations in the red-fleshed varieties are among the highest ever-reported in *Citrus* (Lado et al., 2015; Rodrigo et al., 2019) and other fruit and vegetable (Meléndez-Martinez et al., 2015). Phytoene and phytofluene are highly bioavailable and may be involved in health-promoting effects such as skin protection, antioxidants and cancer prevention (Engelman et al., 2011; Mapelli-Brahm and Meléndez-Martínez, 2021). Regarding human consumption, the uptake of a single K or R orange would provide between 10-15 mg of phytoene, which is superior to the daily intake for most of the major carotenoids detected in humans (Mapelli-Brahm and Meléndez-Martínez, 2021). Therefore, these red-fleshed oranges are a very valuable source of colourless carotenes, in addition to lycopene, for human consumption and to develop novel food/feed as well as cosmetics, enriched in these carotenes.

Other interesting feature of the K and R carotenoid profile was the accumulation of ε-carotene and neurosporene in the pulp (**Table 1**). These carotenes are intermediate products of the desaturation reactions from phytofluene to lycopene (**Figure 1**) and are not usually detected in the pulp of citrus (Rodrigo et al., 2013b). The accumulation of ε-carotene and neurosporene has been reported in other citrus mutants with a partial blockage at initial steps of the carotenoid biosynthetic pathway (Alquézar et al., 2009, 2013; Rodrigo et al., 2019). In the context of these results, the accumulation of δ-carotene in the red-fleshed oranges is also of particular significance. The accumulation of this intermediate product in the synthesis of ε-carotene (**Figure 1**), with only a ε-ring at one end, suggests a partial impairment in the β-ring cyclization in the pulp of these genotypes.

The pulp of the red mutants during maturation accumulated 2 to 3-times less violaxanthin than the standard varieties (**Tables 1**, **2**). This xanthophyll is the main end-product of the carotenoid biosynthetic pathway in sweet oranges (**Figure 1**) (Kato et al., 2004; Rodrigo et al., 2004; Tadeo et al., 2020). However, there is not a clear consensus on whether the accumulation

of lycopene in red-fleshed mutants is associated with a reduced concentration of β,β-xanthophylls, since conflicting results have been reported in Hong Anliu and Cara Cara (Liu et al., 2007, 2021; Alquezar et al., 2008; Lado et al., 2015; Lu et al., 2017). The reduction of violaxanthin in the pulp of K and R oranges was accompanied by significant less ABA and its glycosylated form ABA-GE (**Figure 7**). A decrease in the ABA content in the pulp of red compared to standard varieties has been previously explained by a lower expression of *NCED1* and *NCED2* genes, the main enzyme regulating synthesis of ABA (Xu et al., 2009; Pan et al., 2012; Alquézar et al., 2013; Liu et al., 2021). However, it is likely that in the K and R mutants the reduced content of 9-Z-violaxanthin, the ABA precursor, may be limiting for the synthesis of this hormone rather than or in addition to, the expression of *NCEDs* (**Figure 5**, **6**). It has been recently suggested that the pulp of citrus fruit is an active site of ABA synthesis, where the hormone is rapidly metabolized towards the conjugated product ABA-GE (Liu et al., 2021). Our results in the pulp of K and R showed that the reduction in ABA-GE levels paralleled the decrease in ABA during ripening (**Figure 7**), in line with those of Cara Cara and Hong Anliu oranges (Liu et al. 2021). In summary, the reduction in ABA and its conjugated ABA-GE is a distinctive characteristic in red-fleshed citrus, suggesting a partial blockage in the carotenoid biosynthesis pathway limiting the flow to downstream metabolites. This limitation in ABA and ABA-GE synthesis appears not to be deleterious for fruit development and ripening, as most of the physiological processes analysed in the pulp of these fruit were not substantially altered in respect to the standard oranges.

The atypical accumulation of carotenoids in the K and V mutants was associated with altered morphology of the chromoplasts ultrastructure, which contained tubular membranes and lycopene crystalloid-like structures not observed in standard sweet oranges and an unusual distribution of plastoglobuli (**Figure 4**). Other studies have reported similar differences in the ultrastructure of chromoplasts from red-fleshed orange and grapefruit during fruit maturation (Lado et al., 2015; Lu et al., 2017). These changes in plastidial structures might not be the reason for lycopene accumulation and could more be likely the consequence of the anomalous carotenoid accumulation, which requires an alternative sub-cellular organization and organelle structure (Nogueira et al., 2013; Zeng et al., 2015; Lu et al., 2017). Furthermore, the chromoplasts of the pulp of K mature fruits showed the presence of round translucent vesicles (**Figure 4**) resembling those previously described in Pinalate orange

mutant, likely containing large amounts of colourless carotenes (Lado et al., 2015). It is then possible that these vesicles may serve as storage structures for phytoene/phytofluene in the red-fleshed mutants

The analysis of flavedo and leaves of the red-fleshed varieties revealed that the alterations in the carotenoid composition of these tissues are different to the pulp and may provide clues about the molecular basis of this trait. The carotenoid biosynthesis in flavedo of citrus fruit is regulated differently and independently compared to the pulp (up to 10-times higher in mature oranges) (**Supplementary Table 5**, **6**) (Tadeo et al., 2020). Carotenoid composition in the flavedo and leaves of other red-fleshed oranges has been scarcely studied (Liu et al., 2007; Alquezar et al., 2008), and in this study we found that, unlike the pulp, the levels of violaxanthin, ABA and ABA-GE in the flavedo of mature K and R fruits were similar to those of the standard varieties (**Supplementary Table 5-9**). Interestingly, the leaves of these varieties, as in the flavedo, presented an elevated content of phytoene (**Table 3**). These results indicate that the carotenoid profile is also altered in flavedo and leaves of K and R genotypes, but not to such extend as in the pulp.

The carotenoid profile and the high content of linear carotenes in the pulp of the K and R mutants resembled those found in Cara Cara fruits (Alquezar et al., 2008; Lu et al., 2017; De Ancos et al. 2020; Liu et al., 2021). In Cara Cara, it has been suggested that these alterations may be related to increased levels of carotenoid precursors or to an enhanced PSY activity (Alquezar et al., 2008). Nevertheless, subsequent studies using inhibitors of PDS (NFZ) and lycopene cyclase (CPTA) demonstrated that PSY activity is not higher in Cara Cara compared to standard Navel oranges (Lu et al., 2017). The transcriptional analysis of the carotenoid precursors genes in K and R oranges showed arbitrary differences that generally were not consistently maintained during the whole development and ripening (**Figure 5**, **6**). Therefore, a transcriptional enhancement of the MEP pathway genes appears not to be the primary basis for the carotenoid alteration in K and R oranges. Some studies proposed that low transcript level of *β-LCY2* gene or mutations in amino acids critical for the *β-LCY2* activity as the molecular mechanisms of lycopene accumulation in Red Anliu orange (Lu et al., 2016) and red grapefruits (Alquezar et al., 2009, 2013). Similarly, a reduction in *LCYs* gene expression was proposed in pink lemon and red pummelos (Lana et al., 2020; Promkaew et al., 2020; Tatmala et al., 2020). Nonetheless, neither key differences in the expression of *β-LCYs* nor mutations in

their sequences were associated with lycopene accumulation in Cara Cara (Alquézar et al., 2008; Lu et al., 2017). In other plants, different mechanistic alterations have been proposed to be responsible for lycopene accumulation, i.e., mutations in the *β-LCY2* gene that originate reduced enzyme activity or, alternatively, post-translational modifications of β-LCY enzymes could be responsible for a deficient cyclization of lycopene (Blas et al., 2010; Devitt et al., 2010). In red watermelon varieties, the levels of β-LCY protein correlated negatively with lycopene accumulation (Zhang et al., 2020). The expression of the β-*LCY2a/b* genes was significantly lower in the pulp of K and R during maturation, which may favour the accumulation of upstream carotenes, including lycopene. However, transcript levels of *β-LCY2a/b*, and also of *β-LCY1* and *ε-LCY*, do not show consistent differences between the red and the standard oranges at the early stages of fruit development to substantiate the profound alterations in the carotenoid composition already observed at these physiological stages (**Figure 5**, **6**; **Supplementary Figure 2**, **3**). Moreover, we also explored potential mutations in the *ε-LCY*, *β-LCY1* and *β-LCY2* gene sequence that may jeopardize the cyclization of lycopene. As expected, due to the heterozygosity of sweet oranges, two alleles were identified for each *LCY* gene. Nevertheless, there was no difference in the sequence for any *LCY* gene between the red-fleshed and the blond oranges (**Supplementary Figure 4)**. Therefore, it seems unlikely that alterations in the transcriptional profiling of *LCYs* genes or mutations in their coding sequences are responsible for the unusual carotenoid accumulation in the K and R oranges. It would be plausible to suggest the existence of other mechanisms, still unknown, associated with an accurate lycopene cyclase activity or post-transcriptional modifications of *LCY* genes. Further investigations would be necessary to confirm these hypotheses.

The transcriptional analysis of other carotenoid biosynthetic genes in the red-fleshed and standard oranges only showed differences in the expression of a few genes, which did not explain the differences in the carotenoid content and composition (**Figure 5**, **6**; **Supplementary Figure 2**, **3**). Lu et al. (2017) suggested that potential alterations in the carotenoid degradation capacity could explain the increase in carotenoids content in Cara Cara fruits. To explore this possibility, we analysed the expression of several carotenoid cleavage dioxygenases genes (*CCD1*, *CCD4b* and *NCEDs*) involved in the catabolism of carotenoids (**Figure 5**, **6**; **Supplementary Figure 2**, **3**). Our data indicate that the red-fleshed genotypes do not show regular differences in transcript levels of these genes. Furthermore, the lower accumulation of

xanthophylls and ABA detected in the pulp of K and R are not consistent with impaired carotenoid catabolism.

Carotenoid synthesis in fruit occurs concomitantly with the differentiation of chloroplasts into chromoplasts, leading to the development of sink structures to store newly produced carotenoids (Sun et al., 2018). Recently, we found overexpression of *FIB1*, *FIB2* and *HSP21* in the pulp of the pink-fleshed lemon, suggesting an enhanced carotenoid accumulation and storage capacity (Lana et al., 2020). The expression levels in the pulp of K and V (**Figure 5**, **6**) do not support the involvement of fibrillins and the *HPS21* chaperone in the unusual accumulation of carotenes in the red-fleshed oranges. Another critical process for the massive accumulation of xanthophylls in chromoplastic tissues is their esterification with fatty acids (Rodrigo and Zacarias, 2019). The esterification of xanthophylls is a ripening-regulated event in citrus fruits that is tightly linked to the stability and accumulation of xanthophylls in chromoplastic tissues (Ma et al., 2017; Zacarias-Garcia et al., 2020). To explore if this process might be altered in the red-fleshed mutants, we examined for the first time the expression of three *PYP*/*XAT* genes likely responsible for the esterification of xanthophylls in fruits (Zacarias-Garcia et al., 2020) in K and R oranges. The expression of *PYP* genes in pulp and flavedo of the four genotypes assessed in this work showed an up-regulated pattern during ripening, and more importantly, no differences between the red and the blond oranges were observed. Taken together, we might conclude that the differential and usual accumulation of carotenoids in the red-fleshed K and V orange is not related to alterations in their capability for storage in specialized organelles or for esterification.

One of the most striking phenotypic features of the red-fleshed mutants is the reddish-pink colour of the calyx and its vascular axe, the vascular bundles of the albedo (**Figure 3B**-**G**), and the bark, particularly, the phloem layer of the shoots/branch (**Figure 8**). The woody tissue of the stem (comprising the cambium, the xylem and the pith) in the mutants also showed a pink/red shade, suggesting an unusual carotenoid accumulation in respect to standard orange stems. The bark of standard branches is an active carotenoid-accumulating site with high concentrations of lutein and lower levels of β,β-xanthophylls. However, despite the wood being almost devoid of carotenoids, the bark and wood accumulate similar amounts of ABA, indicating that the pathway is operative in both tissues, although the bark accumulates larger carotenoids (more than 80-times higher than the wood) (**Table 4**). Interestingly, the carotenoid

content in the bark and wood of the mutant branches was remarkably high, mainly by increments in phytoene (70 and 120-times, respectively), phytofluene, and the presence of lycopene, which explains the reddish colouration of these tissues. By contrast, the metabolites downstream to lycopene were substantially reduced in the mutant, i.e., xanthophylls (2-3-fold reduction) and ABA and its catabolites (5-fold reduction). To our knowledge, this is the first time that the carotenoid content and composition, and ABA, its stored form (ABA-GE) and catabolites have been reported in *Citrus* stems (**Table 4**). Kuromori et al. (2014) demonstrated that phloem cells in vascular tissue of Arabidopsis are an active site of ABA synthesis that can be transported to other target cells. These observations revealed that the alteration in the carotenoid composition in these mutants is not restricted to fruit tissues, but manifests in other tissues undergoing active carotenoid and ABA synthesis. Considering that the bark of standard orange trees is very active for the synthesis of carotenoids and ABA, we hypothesize that a blockage or bottleneck in the lycopene cyclization step in the pathway would induce accumulation of upstream carotenes, lycopene and also a downstream reduction in ABA and its catabolites. On the other hand, the observation that the vascular bundles of the central axis of the fruit and the phloem of the branches in grapefruit similarly exhibited red colouration to their ability to accumulate lycopene in the pulp (**Supplementary Figure 5**), corroborates our hypothesis, suggesting that this may be a trait associated with the red-fleshed genotype, not exclusively in oranges, but also in other *Citrus* sp.

In summary, gene expression analysis of the carotenoid metabolism, including genes of carotenoid precursors, biosynthesis, catabolism and storage, during fruit development and ripening did not reveal conspicuous alterations in the K and R oranges that might explain their anomalous carotenoid composition in the pulp in comparison with the standard genotypes. Biochemical data of carotenoid composition in pulp and stems, particularly the increase of upstream carotenes (phytoene, phytofluene) and lycopene, as well as δ-carotene, and the reduced content of downstream products such as violaxanthin and ABA, firmly suggests impairment at the lycopene β-cyclization step in these mutants. The mutation is also manifested in the leaves and flavedo of the red-fleshed oranges, with the most evident trait being the higher levels of colourless carotenes. Therefore, the mutation is manifested in all the carotenogenic tissues/organs of the mutants, but with different consequences since this pathway is distinctly regulated in each tissue. In this context, it is important to highlight that

in flavedo, the relative expression levels of virtually all carotenoid biosynthetic genes, including *β-LCYs*, is approximately 10-fold higher than in pulp. This fact results in an elevated capacity of this tissue to transform lycopene into β-carotene, which may partially alleviate the bottleneck and prevent lycopene accumulation in this tissue. Therefore, in other less carotenogenic tissues, i.e., pulp or stems, the limited cyclization of lycopene would have a more profound effect rendering extremely unbalanced carotenoids composition. Future studies, including the involvement of omics approaches, will be required to unravel the genetic basis of this intriguing reddish phenotype.

Conflict of Interest

The authors declare no conflicts of interest.

Author contributions

Conceptualization, P.J.C, M.J.R. and L.Z.; methodology, J.Z-G, M.J.R. and L.Z.; experimental work, formal analysis and data curation, J.Z-G and G.D, writing—original draft preparation, J.Z-G; writing—review, J.Z-G, P.J.C, G.D, M.J.R. and L.Z.; supervision and funding acquisition, M.J.R. and L.Z. All authors have read and agreed to the published version of the manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ fpls.2022.1034204/full#supplementary-material.

References

Alquézar, B., Rodrigo, M. J., and Zacarías, L. (2008). Regulation of carotenoid biosynthesis during fruit maturation in the red-fleshed orange mutant Cara Cara. *Phytochemistry* 69, 1997-2007. doi:10.1016/j.phytochem.2008.04.020.

Alquézar, B., Rodrigo, M. J., Lado, J., and Zacarías, L. (2013). A comparative physiological and transcriptional study of carotenoid biosynthesis in white and red grapefruit (*Citrus paradisi* Macf.). *Tree Genet. Genomes* 9, 1257-1269, doi:10.1111/ppl.12332.

Alquézar, B., Zacarías, L., and Rodrigo, M. J. (2009). Molecular and functional characterization of a novel chromoplast-specific lycopene β-cyclase from Citrus and its relation to lycopene accumulation. *J. Exp. Bot.* 60, 1783-1797. doi:10.1093/jxb/erp048.

Amengual, J. (2019). Bioactive properties of carotenoids in human health. *Nutrients*, 11, 2388. doi:10.3390/nu11102388.

Barry, G. H., Caruso, M., and Gmitter, F. (2020). "Commercial scion varieties", in The Genus *Citrus*, ed. M. Talon, M. Caruso and F. Gmitter (Cambridge, UK: Woodhead Publishing), 83-104.

Blas, A. L., Ming, R., Liu, Z., Veatch, O. J., Paull, R. E., Moore, P. H., et al. (2010). Cloning of the papaya chromoplast-specific lycopene beta-cyclase, CpCYC-b, controlling fruit flesh color reveals conserved microsynteny and a recombination hot spot. *Plant Physiol.* 152(4), 2013-22. doi:10.1104/pp.109.152298.

Böhm, V., Lietz, G., Olmedilla-Alonso, B., Phelan, D., Reboul, E., Bánati, D., et al. (2021). From carotenoid intake to carotenoid blood and tissue concentrations - implications for dietary intake recommendations. *Nutr Rev*. 79(5), 544-573. doi: 10.1093/nutrit/nuaa008.

De Ancos, B., Rodrigo, M. J., Sánchez-Moreno, C., Cano, M. P., and Zacarías, L. (2020). Effect of high-pressure processing applied as pre-treatment on carotenoids, flavonoids and vitamin C in juice of the sweet oranges 'Navel' and the red-fleshed 'Cara Cara'. *Food Res Int.* 132, 109105. doi.org/10.1016/j.foodres.2020.109105.

Devitt, L. C., Fanning, K., Dietzgen, R. G., and Holton, T. A. (2010). Isolation and functional characterization of a lycopene beta-cyclase gene that controls fruit colour of papaya (*Carica papaya* L.). *J. Exp. Bot.* 61(1), 33-9. doi:10.1093/jxb/erp284.

Diretto, G., Frusciante, S., Fabbri, C., Schauer, N., Busta, L., Wang, Z., et al. (2020). Manipulation of β-carotene levels in tomato fruits results in increased ABA content and extended shelf life. *Plant Biotechnol. J.* 18(5), 185-1199. doi: 10.1111/pbi.13283.

Eggersdorfer, M., and Wyss, A. (2018). Carotenoids in human nutrition and health. *Arch. Biochem. Biophy.* 652, 18-26. doi.org/10.1016/j.abb.2018.06.001.

Engelmann, N. J., Clinton, S. K. and Erdman, J. W. (2011) Nutritional aspects of phytoene and phytofluene, carotenoid precursors to lycopene. *ASN* 2, 51-61. doi:10.3945/an.110.000075.

Gross, J. (1987). Carotenoids: Pigments in Fruits. London, UK: London Academic Press.

Ikoma, Y., Matsumoto, H., and Kato, M., (2016). Diversity in the carotenoid profiles and the expression of genes related to carotenoid accumulation among citrus genotypes. *Breed. Sci.* 66, 139-147. doi:10.1270/jsbbs.66.139.

Kato, M. (2012). Mechanism of carotenoids accumulation in Citrus fruit. *J. Jpn. Soc. Hortic. Sci.* 81(3), 219-233. doi:10.1104/pp.103.031104.

Kato, M., Ikoma, Y., Matsumoto, H., Sugiura, M., Hyodo, H., and Yano, M. (2004). Accumulation of carotenoids and expression of carotenoid biosynthetic genes during maturation in citrus fruit. *Plant Physiol.* 134(2), 824-37. doi: 10.1104/pp.103.031104.

Kato, M., Matsumoto, H., Ikoma, Y., Okuda, H., and Yano, M. (2006). The role of carotenoid cleavage dioxygenases in the regulation of carotenoid profiles during maturation in citrus fruit. *J. Exp. Bot.* 57(10), 2153-2164. doi.org/10.1093/jxb/erj172.

Kuromori, T., Sugimoto, E., and Shinozaki, K. (2014). Intertissue signal transfer of abscisic acid from vascular cells to guard cells. *Plant Physiol.* 164(4), 1587-92. doi: 10.1104/pp.114.235556.

Lado, J., Cronje, P., Alquézar, B., Page, A., Manzi, M., Gómez-Cadenas, A., et al. (2015). Fruit shading enhances peel color, carotenes accumulation and chromoplast differentiation in red grapefruit. *Physiol. Plant.* 154(4), 469-84. doi: 10.1111/ppl.12332.

Lado, J., Zacarias, J., Rodrigo, M. J., and Zacarías, L. (2020). "Visualization of carotenoid-storage structures in fruits by transmission electron microscopy", in Plant and Food Carotenoids: Methods and Protocols, ed. M. Rodríguez-Concepción and R. Welsch (New York, U. S. A: Springer Science+Business Media, LLC, part of Springer Nature), 235-244.

Lana, G., Zacarias-Garcia, J., Distefano, G., Gentile, A., Rodrigo, M.J., and Zacarias, L. (2020). Transcriptional analysis of carotenoids accumulation and metabolism in a pink-fleshed lemon mutant. *Genes* 11(11), 1294. doi: 10.3390/genes11111294.

Liu, C., Zhang, H., He, M., Liu, X., Chen, S., He, Z., et al. (2021). Lycopene accumulation in Cara Cara red-flesh Navel orange is correlated with weak abscisic acid catabolism. *J. Agric. Food Chem*. 69(29), 8236-8246. doi: 10.1021/acs.jafc.1c03766.

Liu, Q., Xu, J., Liu, Y., Zhao, X., Deng, X., Guo, L., et al. (2007). A novel bud mutation that confers abnormal patterns of lycopene accumulation in sweet orange fruit (*Citrus sinensis* L. Osbeck). *J. Exp. Bot.* 58, 4161-4171. doi:10.1093/jxb/erm273.

Liu, W., Ye, Q., Jin, X., Han, F., Huang, X., Cai, S., et al. (2016). A spontaneous bud mutant that causes lycopene and β-carotene accumulation in the juice sacs of the parental Guanxi pummelo fruits (*Citrus grandis* (L.) Osbeck). *Sci. Hortic*. 198, 379-384. doi.org/10.1016/j.scienta.2015.09.050.

Lu, P. J., Wang, C.Y., Yin, T. T., Zhong, S. L., Grierson, D., Chen, K. S., et al. (2017). Cytological and molecular characterization of carotenoid accumulation in normal and high-lycopene mutant oranges. *Sci. Rep.* 7, 1-15. doi:10.1038/s41598-017-00898-y.

Lu, S., Zhang, Y., Zheng, X., Zhu, K., Xu, Q., and Deng, X. (2016). Molecular characterization, critical amino acid identification, and promoter analysis of a lycopene β-cyclase gene from citrus. *Tree Genet. Genomes* 12, 106. doi.org/10.1007/s11295-016-1066-z.

Lux, P. E., Carle, R., Zacarías, L., Rodrigo, M.-J., Schweiggert, R. M., and Steingass, C. B. (2019). Genuine carotenoid profiles in sweet orange [*Citrus sinensis* (L.) Osbeck cv. Navel] peel and pulp at different maturity stages. *J. Agric. Food Chem.* 67(47), 13164-13175. doi.org/10.1021/acs.jafc.9b06098.

Ma, G., Zhang, L., Iida, K., Madono, Y., Yungyuen, W., Yahata, M., et al. (2017). Identification and quantitative analysis of β-cryptoxanthin and β-citraurin esters in Satsuma mandarin fruit during the ripening process. *Food Chem.* 234, 356-364. doi.org/10.1016/j.foodchem.2017.05.015

Ma, G., Zhang, L., Matsuta, A., Matsutani, K., Yamawaki, K., Yahata, M., et al. (2013). Enzymatic formation of β-citraurin from β-cryptoxanthin and zeaxanthin by carotenoid

cleavage dioxygenase4 in the flavedo of citrus fruit. *Plant Physiol.* 163(2), 682-695. doi.org/10.1104/pp.113.223297.

Ma, G., Zhang, L., Sugiura, M., and Kato, M. (2020). "Citrus and health," in The Genus *Citrus*, ed. M. Talón, M. Caruso and F. Gmitter (Cambridge, UK: Woodhead Publishing), 495-511.

Mapelli-Brahm, P., and Meléndez-Martínez, A. J. (2021). The colourless carotenoids phytoene and phytofluene: sources, consumption, bioavailability and health effects. *Curr. Opin. Food Sci.* 41, 201-209. doi.org/10.1016/j. cofs.2021.04.013.

Mariutti, L.R.B., and Mercadante, A.Z. (2018). Carotenoid esters analysis and occurrence: What do we know so far? *Arch. Biochem. Biophys*. 648, 36-43. doi: 10.1016/j.abb.2018.04.005.

Meléndez-Martínez, A. J., Böhm, V., Iren, G., Borge, A., Cano, M. P., Fikselová, M., et al. (2021). Carotenoids: considerations for their use in functional foods, nutraceuticals, nutricosmetics, supplements, botanicals, and novel foods in the context of sustainability, circular economy, and climate change. *Annu. Rev. Food Sci. Technol.* 12, 433-460. doi.org/10.1146/annurev-food-062220-013218.

Meléndez-Martínez, A. J., Mapelli-Brahm, P., Benítez-González, A., and Stinco, C. M. (2015). A comprehensive review on the colorless carotenoids phytoene and phytofluene. *Arch. Biochem. Biophys*. 15(572), 188-200. doi: 10.1016/j.abb.2015.01.003.

Mendes, A. F. S., Chen, C., Gmitter, F. G., Moore, G. A., and Costa, M. G. C. (2011). Expression and phylogenetic analysis of two new lycopene β-cyclases from *Citrus paradisi*. *Physiol. Plant.* 141, 1-10. doi:10.1111/j.1399-3054.2010.01415.x.

Moise, A.R., Al-Babili, S., and Wurtzel, E.T. (2014). Mechanistic aspects of carotenoid biosynthesis. *Chem. Rev.* 114, 164−193. doi: 10.1021/cr400106y.

Monselise, S. P., and Halvey, A. H. (1961). Detection of lycopene in pink orange fruit. *Science* 133, 1478. doi:10.1126/science.133.3463.1478.

Müller, L., Fröhlich, K., and Böhm, V. (2011) Comparative antioxidant activities of carotenoids measured by ferric reducing antioxidant power (FRAP) ABTS bleaching assay (αTEAC) DPPH assay and peroxyl radical scavenging assay. *Food Chem.* 129, 139-148. doi:10.1016/j.foodchem.2011.04.045

Nogueira, M., Mora, L., Enfissi, E.M., Bramley, P.M., and Fraser, P.D. (2013). Subchromoplast sequestration of carotenoids affects regulatory mechanisms in tomato lines expressing different carotenoid gene combinations. *Plant Cell.* 25(11), 4560-79. doi: 10.1105/tpc.113.116210.

Noronha, H., Silva, A., Silva, T., Frusciante, S., Diretto, G., and Gerós, H. (2022). VviRafS5 is a raffinose synthase involved in cold acclimation in grapevine woody tissues. *Front. Plant Sci*. 12:754537. doi: 10.3389/fpls.2021.754537.

Pan, Z., Zeng, Y., An, J., Ye, J., Xu, Q., and Deng, X. (2012). An integrative analysis of transcriptome and proteome provides new insights into carotenoid biosynthesis and regulation in sweet orange fruits. *J Proteomics*. 75(9), 2670-84. doi: 10.1016/j.jprot.2012.03.016.

Pfaffl, M. W., Horgan, G. W., and Dempfel, L. (2002). Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR (2002). *Oxford Univ. Press* 30, 9-36. doi:10.1093/nar/30.9.e36.

Promkaew, P., Pongprasert, N., Wongs-Aree, C., Kaewsuksaeng, S., Opio, P., Kondo, S., et al. (2020). Carotenoids accumulation and carotenoids biosynthesis gene expression during

fruit development in pulp of Tubtim-Siam pummelo fruit. *Sci. Hortic*. 260, 108870. doi:10.1016/j.scienta.2019.108870.

Rajendran, P., Nandakumar, N., Rengarajan, T., Palaniswami, R., Gnanadhas, E. N., and Lakshminarasaiah, U. (2014). Antioxidants and human diseases. *Clin. Chim. Acta* 436, 332-347. doi:10.1016/j.cca.2014.06.004.

Rao, A., and Rao, L. G. (2007). Carotenoids and human health. *Pharmacol. Res.* 55, 207-216. doi:10.1016/j.phrs.2007.01.012.

Rodrigo, M. J., Alquézar, B., Alós, E., Lado, J., and Zacarias, L. (2013b). Biochemical bases and molecular regulation of pigmentation in the peel of Citrus fruit. *Sci. Hortic.* 163, 46-62. doi:10.1016/j.scienta.2013.08.014.

Rodrigo, M. J., and Zacarías, L. (2019). "Biosynthesis and esterification of carotenoids during fruit ripening", in Carotenoid esters in foods: physical, chemical and biological properties, ed. A. Z. Mercadante (London, UK: The Royal Society of Chemistry), 137-159.

Rodrigo, M. J., Cilla, A., Barberá, R., and Zacarías, L. (2015). Carotenoid bioaccessibility in pulp and fresh juice from carotenoid-rich sweet oranges and mandarins. *Food Funct.* 6, 1950-1959. doi:10.1039/c5fo00258c.

Rodrigo, M. J., Lado, J., Alós, E., Alquézar, B., Dery, O., Hirschberg, J., et al. (2019). A mutant allele of ζ-carotene isomerase (Z-ISO) is associated with the yellow pigmentation of the "Pinalate" sweet orange mutant and reveals new insights into its role in fruit carotenogenesis. *BMC Plant Biol*. 19(1), 465. doi: 10.1186/s12870-019-2078-2.

Rodrigo, M. J., Marcos, J. F., and Zacarías, L. (2004). Biochemical and molecular analysis of carotenoid biosynthesis in flavedo of orange (*Citrus sinensis* L.) during fruit development and maturation. *J. Agric. Food Chem.* 52, 6724-6731. doi: 10.1021/jf049607f.

Rodrigo, M.J., Alquézar, B., Alós, E., Medina, V., Carmona, L., Bruno, M., et al. (2013a). A novel carotenoid cleavage activity involved in the biosynthesis of Citrus fruit-specific apocarotenoid pigments. *J. Exp. Bot.* 64, 4461-4478. doi:10.1093/jxb/ert260.

Rodríguez-Concepcion, M., Avalos, J., Bonet, M. L., Boronat, A., Gomez-Gomez, L., Hornero-Mendez, D., et al. (2018). A global perspective on carotenoids: Metabolism, biotechnology, and benefits for nutrition and health. *Prog. Lipid Res.* 70, 62-93. doi: 10.1016/j.plipres.2018.04.004.

Saunt, J. (2000). Citrus Varieties of the World. Norwich, England: Sinclair International Ltd.

Schweiggert, R. M., and Carle, R. (2017). Carotenoid deposition in plant and animal foods and its impact on bioavailability. *Crit. Rev. Food Sci. Nutr.* 57(9), 1807-1830. doi: 10.1080/10408398.2015.1012756.

Simkin, A. J., Gaffé, J., Alcaraz, J. P., Carde, J. P., Bramley, P. M. Fraser, P. D. et al. (2007). Fibrillin influence on plastid ultrastructure and pigment content in tomato fruit. *Phytochemistry*, 68, 1545-1556. doi: 10.1016/j.phytochem.2007.03.014.

Stahl, W., and Sies, H. (2003). Antioxidant activity of carotenoids. *Mol. Aspects Med.* 24, 345-351. doi:10.1016/s0098-2997(03)00030-x.

Stewart, I., and Wheaton, T. A. (1972). Carotenoids in *Citrus*: their accumulation induced by ethylene. *J. Agric. Food Chem.* 20, 448-449, doi:10.1021/jf60180a024.

Sun, T., Yuan, H., Cao, H., Yazdani, M., Tadmor, Y., and Li, L. (2018). Carotenoid metabolism in plants: the role of plastids. *Mol. Plant* 11, 58-74. doi: 10.1016/j.molp.2017.09.010.

Tadeo, F. R., Terol, J., Rodrigo, M. J., Licciardelo, C., and Sadka, A. (2020). "Fruit growth and development", in The Genus *Citrus*, ed. M. Talón, M. Caruso and F. Gmitter (Cambridge, UK: Woodhead Publishing), 245-289.

Talón, M., Caruso, M., and Gmitter, F. (2020). The Genus Citrus. Cambridge, UK: Woodhead Publishing.

Tatmala, N., Ma, G., Zhang, L., Kato, M., and Kaewsuksaeng, S. (2020). Characterization of carotenoid accumulation and carotenogenic gene expression during fruit ripening in red colored pulp of 'siam red ruby' pumelo (*Citrus grandis*) cultivated in Thailand. *Hortic. J.* 89, 237-243. doi:10.2503/hortj.UTD-147.

Welsch, R., Wust, F., Bar, C., Al-Babili, S. and Beyer, P. (2008) A third phytoene synthase is devoted to abiotic stress-induced abscisic acid formation in rice and defines functional diversification of phytoene synthase genes. *Plant Physiol.* 147, 367– 380. doi.org/10.1104/pp.108.117028.

Wu, G., Prochnik, S., Jenkins, J., Salse, J., Hellsten, U., Murat, F., et al. (2014). Sequencing of diverse mandarin, pummelo and orange genomes reveals complex history of admixture during citrus domestication. *Nat. Biotechnol.* 32, 656–662. doi.org/10.1038/nbt.2906.

Xu, C. J., Fraser, P. D., Wang, W. J., and Bramley, P. M. (2006). Differences in the carotenoid content of standard citrus and lycopene-accumulating mutants. *J. Agric. Food. Chem.* 54, 5474-5481. doi:10.1021/jf060702t.

Xu, Q., Yu, K., Zhu, A., Te, J., Liu, Q., Zhang, J., et al. (2009). Comparative transcripts profiling reveals new insight into molecular regulating lycopene in a sweet orange (*Citrus sinensis*) red-flesh mutant. *BMC Genomics* 10:540. doi:10.1186/1471-2164-10-540.

Yan, F., Shi, M., He, Z., Wu, L., Xu, X., He, M., et al. (2018). Largely different carotenogenesis in two pummelo fruits with different flesh colors. *PLoS ONE* 13, e0200320. doi.org/10.1371/journal.pone.0200320

Ye, J. L., Zhu, A. D., Tao, N. G., Xu, Q., Xu, J., and Deng, X. X. (2010. Comprehensive analysis of expressed sequence tags from the pulp of the red mutant 'Cara Cara' Navel orange (*Citrus sinensis* Osbeck). *J. Integr. Plant Biol.* 52(10), 856-67. doi: 10.1111/j.1744-7909.2010.00952.x.

Yu, K., Xu, Q., Da, X., Guo, F., Ding, Y., and Deng, X. (2012) Transcriptome changes during fruit development and ripening of sweet orange (*Citrus sinensis*). *BMC Genom.* 13:10. https://doi.org/10.1186/1471-2164-13-10.

Zacarías-García. J., Lux, P.E., Carle, R., Schweiggert, R.M., Steingass, C.B., Zacarías, L., et al. (2021). Characterization of the Pale Yellow Petal/Xanthophyll Esterase gene family in citrus as candidates for carotenoid esterification in fruits. *Food Chem.* 342:128322. doi: 10.1016/j.foodchem.2020.128322.

Zeng. Y., Du. J., Wang. L., Pan, Z., Xu, Q., Xiao, S., et al. (2015). A comprehensive analysis of chromoplast differentiation reveals complex protein changes associated with plastoglobule biogenesis and remodeling of protein systems in sweet orange flesh. *Plant Physiol.* 168(4), 1648-65. doi: 10.1104/pp.15.00645.

Zhang, H., Chen, J., Peng, Z., Shi, M., Liu, X., Wen, H., et al. (2021). Integrated transcriptomic and metabolomic analysis reveals a transcriptional regulation network for the biosynthesis of carotenoids and flavonoids in 'Cara Cara' Navel orange. *BMC Plant Biol.* 21(1), 29. doi:10.1186/s12870-020-02808-3.

Zhang, J., Sun, H., Guo, S., Ren, Y., Li, M., Wang, J., et al. (2020). Decreased protein abundance of lycopene β-cyclase contributes to red flesh in domesticated watermelon. *Plant Physiol.* 183(3), 1171-1183. doi:10.1104/pp.19.01409.

Zhang, L., Ma, G., Shirai, Y., Kato, M., Yamawaki, K., Ikoma, Y. et al. (2012). Expression and functional analysis of two lycopene β-cyclases from citrus fruits. *Planta* 236(4):1315-25. doi: 10.1007/s00425-012-1690-2.

Zhang, Y., Liu, Y., Liu, F., Zheng, X., Xie, Z., Ye, J., et al. (2019). Investigation of chromoplast ultrastructure and tissue-specific accumulation of carotenoids in citrus flesh. *Sci. Hortic.* 256, 108547. doi:org/10.1016/j. scienta.2019.108547.

3.2. CAPÍTULO 2

Bioactive compounds, nutritional quality and antioxidant capacity of the red-fleshed Kirkwood Navel and Ruby Valencia oranges

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Abstract

Kirkwood Navel and Ruby Valencia are two spontaneous bud mutations of the ordinary Washington Navel and Valencia late oranges characterized by the red coloration of their flesh. The purpose of this study was to analyze the physiological features, internal fruit quality, contents of relevant bioactive compounds and antioxidant capacity in the pulps of the redfleshed fruits compared with the ordinary oranges during late development and maturation. In general, the content of sugars, organic acids, vitamin C, tocopherols, total phenolics and flavonoids, the hydrophilic antioxidant capacity and their changes during maturation were similar in the red-fleshed oranges and in the corresponding blond oranges. However, the mature Ruby fruits contained lower concentrations of sugars, malic and succinic acid and higher levels of citric acid than the ordinary Valencia. The major difference between the pulps of the Kirkwood and Ruby oranges and those of the ordinary oranges was the higher lipophilic antioxidant capacity and SOAC (singlet oxygen absorption capacity) of the former. Together, the high and unique content and composition of carotenoids in Kirkwood and Ruby may contribute to an enhanced antioxidant capacity without any detrimental effects on other fruit-quality attributes, making these varieties good sources of phytochemicals for the fresh fruit and juice processing citrus industries.

Keywords: red-fleshed oranges; maturation; nutritional quality; bioactive compounds; antioxidant

1. Introduction

Citrus is one of the most important commercial fruit crops worldwide in terms of production, market trade and consumption. The demand for and acceptance of citrus fruits for fresh consumption or juice production is highly variable across different national and international markets, depending on the consumer choices and preferences but, in general, it is mainly based on the external appearance, organoleptic properties, nutritional quality and health-related benefits [1].

The consumption of citrus fruits is associated with anti-inflammatory effects, obesity control, a decrease in the incidence of cardiovascular diseases and a reduction in the risk of certain cancers [2–4]. Therefore, the current concern over the prevention of health problems through nutrition is leading to intensive studies of the nutritional composition and antioxidant properties of citrus fruits. In this sense, oxidative stress plays a pivotal role in the development of human diseases and evidence indicates that the several beneficial effects of citrus metabolites in human health are associated with their antioxidant activity [5,6].

The content and composition of nutrients and bioactive and antioxidant metabolites in citrus fruits vary widely among the different species and varieties, tissues and maturation stages, influencing their quality, nutritional value and health-promoting effects [7]. Orange fruits (*Citrus sinensis*) are relevant sources of various nutrients and phytochemicals, including carbohydrates, protein, lipids, vitamins, mineral elements, phenolics, limonoids and carotenoids among others [4,7,8]. Sugars are the main nutrients of the fruits of most citrus species and, with organic acids, have a strong impact on the characteristic flavour of the fruit, playing a crucial role in its organoleptic properties and consumer acceptance [1,3]. Carotenoids are fat-soluble compounds responsible for the coloration of the peels and pulps of the fruits of most citrus species and varieties, and their content is a key issue in their commercial acceptability [9]. The diversity of pigmentation in citrus fruits is mainly due to the genetic and environmental variability in the content and composition of carotenoids [10]. Citrus phenolics have been widely characterized in orange fruits, and phenolic acids and flavonoids are the largest group of naturally occurring phenolic compounds; they are present as both free state (aglycones) and glycosyl derivatives [4]. The flavonoid composition in fruits of different citrus species has been studied in depth; it has been shown that their content may

fluctuate during fruit development and maturation [11,12]. Flavonoids, in general, display an ample range of biological and health-related activities, such as their anti-inflammatory and protective role against chronic diseases, including cardiovascular diseases and diabetes [12,13].

Citrus fruits are also important sources of vitamins [7,8]. For example, citrus fruits are recognized worldwide as some of the main sources of vitamin C (ascorbic acid). Ascorbic acid is the most abundant vitamin in orange fruits and, as many authors suggest, the major antioxidant in Citrus [13,14]. Although the accumulation of L-ascorbic acid in the pulp of citrus fruits is, in general, moderate in comparison with other fruits, such as kiwi or strawberry, the large consumption of fresh oranges and orange juice made from this crop make it a major source of vitamin C in human nutrition [15]. On the other hand, tocopherols are plant isoprenoids belonging to the chemical family of tocochromanols. With the exception of tocotrienols, they are the only natural compounds that exhibit vitamin E activity in animal cells, with α-tocopherol being the most potent vitamin E form [16,17]. The target and synergistic activity of these phytochemicals and others determine the antioxidant capacity of citrus fruits and, therefore, most of their health-related properties [6]. Several recent reviews gather the latest insights into the biological effects of complex mixtures of phytochemicals from food matrices [18–20]. More specifically, several studies proposed different mechanisms of action or interactions between plant antioxidants. For instance, it has been demonstrated that vitamin C is able to regenerate oxidized vitamin E and carotenoids [21,22]. Additional evidence suggested that the combination of flavonoids (rutin) and carotenoids (lycopene and lutein) leads to synergistic effects that prevent low-density lipoprotein oxidation [23].

The accumulation of the carotene lycopene in the fruit pulp confers a particular bright reddish coloration, but this is an unusual feature in the genus Citrus, restricted to a few varieties and mutants [9]. The red-fleshed Hong Anliu and Cara oranges, identified in China and Venezuela, respectively, have been intensively investigated during last decade. Several studies focused on the analysis of their carotenoid composition [24–27], accumulation of vitamins and bioactive compounds [28,29], antioxidant activity and cytoprotective effects [30–32] and alterations in other metabolic pathways related to fruit quality in comparison with standard varieties [33–37].

The availability of new red-fleshed orange varieties is of special relevance to the provision of novel products with distinctive value for local and international markets, but also to understand the biochemical basis of lycopene accumulation in the pulps of citrus fruits and whether this feature may alter other parameters of fruit quality. We recently reported the characterization of carotenoid metabolism during development and maturation in two new red-fleshed orange mutants: Kirkwood Navel (referred in this work as Kirkwood or K) and Ruby Valencia (Ruby or R). These spontaneous bud mutations are derived from the ordinary Navel and Valencia late oranges, respectively, and are already being commercially propagated in order to check their adaptation to Mediterranean growing and environmental conditions [38]. The pulps of both mutants contain higher amounts of total carotenoids than the ordinary Navel and Valencia oranges from the early stages of fruit development and, moreover, accumulate large concentrations of phytoene and phytofluene, moderate amounts of lycopene, low levels of β-carotene and minor concentrations of violaxanthin compared to the ordinary varieties [38]. Hence, the appealing coloration and the high concentration of carotenes make these new orange varieties rich sources of carotenoids for consumption and novel food products with potential benefits for health.

The objective of this work was to conduct a comparative analysis of the chemical composition, nutritional quality and antioxidant capacity in the fruits of the red-fleshed varieties, Kirkwood and Ruby, and the corresponding standard varieties, Navel and Valencia, respectively, in order to provide reliable data on their fruit-quality characteristics under edaphoclimatic Mediterranean conditions. We report, for the first time, the evolution of internal quality-related parameters, such as sugars and organic acids, from the late stages of fruit development to full maturation, as well as the accumulation of vitamins and other bioactive compounds, such as ascorbic acid, tocopherols, total phenolics and flavonoids. Furthermore, the relevance of these changes is complemented by the analysis of the carotenoid composition in the fruit pulp at two maturity stages and the assessment of the antioxidant capacity by different in vitro assays.

2. Materials and methods

2.1. Plant Material

Fruits of the two ordinary sweet orange (Citrus sinensis) genotypes, Washington Navel (cv. Foios, N) and Valencia late (cv. Midknight, V) and those of the red-fleshed varieties, K and R, were cultivated at the Fundación ANECOOP (Museros, Valencia, Spain). Washington Navel oranges are characterized by precocious maturity and bear large seedless fruits. They are the main orange group produced for fresh consumption. On the other hand, Valencia late oranges belong to the Blancas botanical group; they are characterized by the absence of navel in their fruits, late maturity and the absence of a bitter flavour in their juices [39]. Fruits of each genotype were harvested from adult trees grafted under Citrange Carrizo rootstock (Citrus sinensis x Poncirus trifoliata), grown in the same orchard and under the same agronomical and environmental conditions [14]. N and K fruits were harvested at five stages during the following months: August (immature green), October (green–breaker), November (breaker), December (mature) and January (fully mature) and those of V and R were harvested at six stages: August (immature green), October (mature green), December (green–breaker January (breaker), March (mature) and April (fully mature) (**Figure 1**).

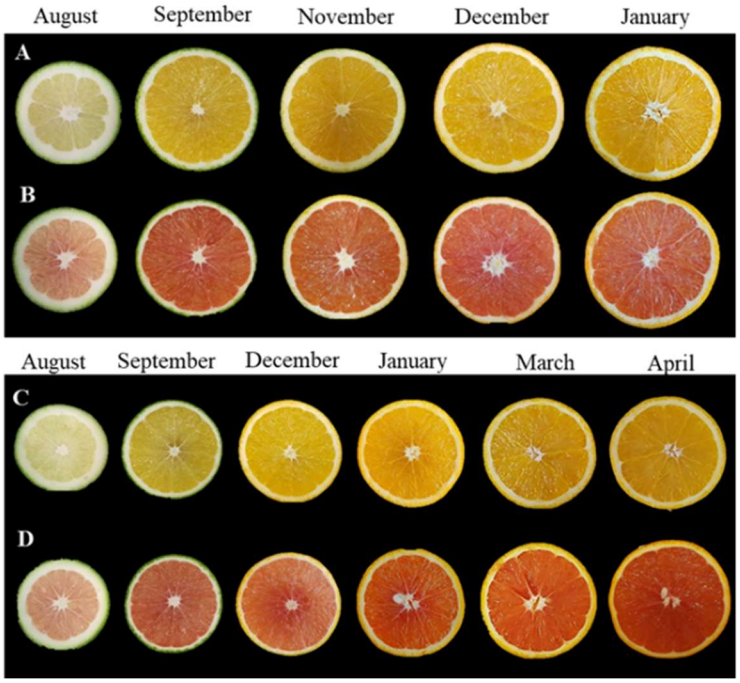

Figure 1. Internal appearance of the Navel (A), Kirkwood (B), Valencia (C) and Ruby (D) orange fruits used in this study.

At each selected stage, at least 50 fruits per variety were harvested and delivered to the laboratory. Fruits were picked up from the external canopy; they were of similar size and free of external defects. Color of the flavedo and pulp was measured and small pieces (around 1 cm3) of the pulp containing juice vesicles without segment membranes were excised and frozen in liquid nitrogen, ground to fine powder and stored at −80 °C until analysis. Juice was extracted with a domestic electric hand squeezer (Citromatic MPZ22, Braun, Barcelona, Spain), filtered through a metal filter (0.8 mm pore size), frozen in liquid nitrogen and stored at −20 °C until analysis. Fruit samples were harvested during two consecutive seasons (2018–2019; 2019–2020) and data reported here are representative of one season unless otherwise indicated.

2.2. Fruit Weight, Size, Color Index and Internal Maturity Determination

At least 20 fruits were individually weighed and height and diameter were measured for each variety and harvest date. Color of the flavedo and pulp was measured using a CR-400 Minolta chromameter on three different positions and is expressed as the a/b Hunter ratio. Color measurements were not determined in fruits harvested in August since the pulp diameter was too small to obtain a reliable determination in three different positions. Data of color index for each cultivar are the means ± SD of at least 10 fruits.

Total soluble solids (TSS) and total titratable acidity (TA) of the juice were determined using a digital refractometer PAL-BX/ACID1 (ATAGO, Tokyo, Japan). TSS is expressed as °Brix and TA as mg citric acid/100 mL of juice; maturity index (MI) was calculated as the TSS/TA ratio. Determination of internal maturity was analyzed in fruits of the four varieties at the different harvest times, except for those harvested in August.

2.3. Sugar and Organic Acid Determination

Sugars and organic acids were determined essentially as described by Pérez-Través et al. [40]. Pulp tissue (2 g) was homogenized with 2 mL of Milli-Q water and then centrifuged for 10 min at 3000 g at 4 °C. The supernatants were diluted 3-fold and filtered through a 0.22 micrometer nylon filter (Symta, Madrid, Spain). A liquid chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a refraction index detector was used. For glucose, fructose, citric acid, malic acid, quinic acid and succinic acid, a HyperREZTM XP Carbohydrate H+ 8 micrometer column (Thermo Fisher Scientific) was used, protected by a HyperREZTM XP Carbohydrate (Thermo Fisher Scientific) guard column. The conditions

used in the analysis were as follows: eluent, H2SO4 1.5 mM; flux, 0.6 mL/min; and oven temperature, 50 °C. For sucrose determination, a Pb column was used (Hi-Plex Pb, 300 × 7.7 mm, Agilent Technologies (Santa Clara, CA, USA); the conditions used in this analysis were: eluent, Milli-Q water; flux, 0.6 mL/min; and the column temperature was set at 50 °C. Samples were extracted twice and results are the mean of two replicates (mean ± SD).

2.4. L-Ascorbic Acid Determination

L-Ascorbic acid from the pulp of the fruit was extracted and determined essentially as described in Alòs et al. [41], using a Waters ACQ Arc SysCore HPLC equipped with a DAD, Empower 3 software and an Ultrabase C18 column.

2.5. Tocopherol Determination

Tocopherols in the pulp were extracted and quantified essentially as described in Rey et al. [16], using a Waters HPLC system (Acquity® Arc™, Waters, Barcelona, Spain) coupled with a fluorescence detector (2475 FLR Detector, Waters, Barcelona, Spain) and a YMC C30 column (150 × 4.6 mm, 3 μm) (Teknokroma, Barcelona, Spain). Identification and quantification of the different tocopherols was achieved by comparison with the retention times and peak areas of authentic standards of δ-, γ- and α-tocopherol (Sigma-Aldrich, Barcelona, Spain), and the concentrations expressed as μg/g of fresh weight. Samples were extracted twice and results are the mean of two replicates (mean ± SD).

2.6. Carotenoid Determination

Carotenoids were extracted and analyzed in the pulps of mature fruits of the four varieties (December and January for Navel oranges, and March and April for Valencia oranges) as described by Rodrigo et al. [26], using a Waters a liquid chromatography system (HPLC) equipped with a 600E pump, a photodiode array detector (DAD), model 2998 and Empower3 software (Waters, Barcelona, Spain). A C30 carotenoid column (250 × 4.6 mm, 5 μm) was coupled to a C30 guard column (20 × 4.0 mm, 5 μm) (YMC, Teknokroma, Spain). The carotenoids were identified by absorbance spectra and retention time; peaks were integrated at their individual maximal wavelength and their contents were calculated using the appropriate calibration curves of lycopene (Extrasynthese) for lycopene, neurosporene and δ-carotene, lutein (Sigma), β-carotene (Sigma), β-cryptoxanthin (Extra-synthese), zeaxanthin (Extrasynthese), anteraxanthin (CaroteNature) for anteraxanthin and mutatoxanthin and

violaxanthin (CaroteNature) for violaxanthin isomers and luteoxanthin. Phytoene, phytofluene and ζ-carotene were previously purified by thin-layer chromatography from carotenoid extracts of Pinalate orange fruits [26]. The spectroscopic characteristics of all carotenoids detected in the pulps of Navel, Kirkwood, Valencia and Ruby oranges are shown in Table S1.

2.7. Analysis of Total Phenolics and Flavonoids

Phenolic content was determined by the method of Folin–Ciocalteu, as described by Singleton and Rossi [42], with slight modifications. Briefly, 0.5 g of pulp tissue were extracted with 5 mL of MeOH using a homogenizer (Polytron, Eschbach, Germany). The extract was centrifuged for 10 min at 3000 g at 4 °C and the supernatant used to estimate the phenolic content. A 50-microliter aliquot of the extract was mixed with 500 μL of solution (Na2CO3 2% and NaOH 0.1 M) and incubated for 15 min at room temperature in darkness. Next, 50 μL of Folin–Ciocalteu reagent were added (diluted 50% with purified water) and incubated for 30 min. The absorbance was measured at 724 nm in a UV/Vis microplate spectrophotometer (Multiskan FC, Thermo Scientific, Waltham, Massachusetts, USA) and compared with a standard curve of gallic acid (Sigma-Aldrich, Barcelona, Spain). Results were expressed as mg of gallic acid equivalents (GAE) per 100 g of fresh weight. Determinations were performed per triplicate in each extract.

Total flavonoids were determined in the same supernatant used to analyze total phenolics following the method described by Lafuente et al. [43]. Two supernatant aliquots of 100 μL were diluted with 400 μL of Milli-Q water and 30 μL of 5% NaNO2. After 5 min of incubation at room temperature, 30 μL of 10% AlCl3 were added and the reaction was stopped by adding 200 μL of 1 N NaOH. Flavonoid contents were determined by UV/Vis microplate spectrophotometer comparing the absorbance at 350 nm with a standard curve of hesperidin (Sigma-Aldrich, Barcelona, Spain). Results were expressed as mg of hesperidin equivalents (HesE) per 100 g of fresh weight. Determinations were performed per triplicate in each extract.

2.8. Flavonoid Determination

Flavonoids were extracted and purified from pulp tissue as follows. Briefly, pulp tissue (0.5 g) was extracted with 5 mL of Milli-Q water centrifuged at 3000 g for 30 min at 4 °C. Next, the extract was subsequently filtered through a 0.45-micrometer PVDF filter (13 mm diameter,

Análisis Vínicos, Tomelloso, Ciudad Real, Spain). Flavonoid content was determined by HPLC-DAD. Separation of flavonoids was carried out with a XBridgeTM BEH C18 (4.6 × 150-millimeter, 2.-micrometer Column XP), maintained at 40 °C, and a secondary gradient elution with water 0.1% TFA (solvent A) and acetonitrile 0.1% TFA (solvent B) at a flow rate of 1 mL/min as follows. From 0 to 5 min 100% A, 5 to 30 min 95% A and 5% B, 30 to 33 min 50% A and 50% B and from 33 to 37 min 100% B. Wavelength was registered from 220 to 550 nm and flavonoids were quantified at 280 nm. Identification and quantification of the different flavonoids was achieved by comparison with the retention times and peak areas of authentic standards of hesperidin, narirutin, naringin, eriocitrin, dydimin and rutin (Sigma-Aldrich, Barcelona, Spain). Concentrations are expressed as mg/100 g of fresh weight. Samples were extracted twice and the results are the mean of two replicates (mean ± SD).

2.9. Determination of the Antioxidant Capacity

The hydrophilic antioxidant capacity (HAC) was determined by the DPPH free-radical assay (2,2-diphenyl-1-picrylhydrazyl), as described by Rey et al. [44]. The assay was replicated twice and a curve of ascorbic acid was used as a standard. Results were expressed as mg of ascorbic acid equivalents (AsAE) per 100 g of fresh weight. DPPH scavenging capacity was expressed as inhibition percentages by Formula (1):

% DPPH scavenging capacity = [(517 nmAcontrol – 517 nmAsample)/ 517 nmAcontrol] × 100 (1)

The HAC was also determined in the same supernatant used in DPPH assay by using FRAP (ferric reducing antioxidant power) assay, as described by Benzie and Strain [45], with some modifications. First, a reagent was prepared with 10 mM TPTZ (2,4,6-tripyridy-Striazine, Sigma), 20 mM FeCl3.6H2O and 300 mM acetate buffer, pH 3.6, at 1:1:10, v:v:v and incubated in a bath at 37 °C for 10 min. In a 96-well plate 10 μL of each sample were mixed with 290 μL of FRAP reagent and incubated at 37 °C in darkness for 30 min. Thereafter, the absorbance change of the mixture was determined in a UV/Vis microplate spectrophotometer (Multiskan FC, Thermo Scientific, Madrid, Spain) at 595 nm. The assay was replicated twice and a curve of ascorbic acid was used as a standard. Results were expressed as mg of ascorbic acid equivalents (AsAE) per 100 g of fresh weight.

The ABTS (2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) assay was used to determine antioxidant capacity in comparison to a standard antioxidant, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma, Madrid, Spain). Total antioxidant capacity was quantified as described by Legua et al. [46], with slight modifications, which enabled us to determine antioxidant capacity due to both hydrophilic and lipophilic compounds in the same extraction. Absorbance of the extracts was determined with a UV/Vis microplate spectrophotometer (SPECTROstar® Omega, BMG Labtech, Offenburg, Germany) at 730 nm. A calibration curve was drawn with Trolox (Sigma, Madrid, Spain) and results are expressed as Trolox equivalent antioxidant capacity (TEAC) per 100 g of fresh weight.

2.10. Analysis of Singlet Oxygen Absorption Capacity (SOAC)

Analysis of SOAC in the pulp of the fruits was performed as previously described by Rey et al. [44]. The capacity of the extract to scavenge the 1O2 radical was determined using endoperoxide (EP, Invitrotech, Kyoto, Japan) as a singlet oxygen generator and 2,5-diphenyl-3,4-benzofuran (DPBF, Sigma–Aldrich, Barcelona, Spain) as an UV-Vis absorption probe in a 96-well quartz glass microplate (Hellma, Müllheim, Germany). Changes in absorbance at 413 nm were monitored for 90 min at 35 °C using a UV-Vis spectrophotometer microplate reader; α-Tocopherol (Sigma–Aldrich, Barcelona, Spain) was used as a blank standard. The SOAC value was calculated by Formula (2):

$$(t1/2 \text{ sample} - t1/2 \text{ blank}) / (t1/2\ \alpha\text{-toc} - t1/2 \text{ blank}) \times ([\alpha\text{-toc, g L}^{-1}] / [\text{sample, g L}^{-1}]) \quad (2)$$

Each sample was analyzed in two independent assays and samples were run in triplicate in each plate.

2.11. Statistical and Principal Component Analysis (PCA)

Results are presented as the mean of two biological replicates ± standard deviation (SD). Differences between the red-fleshed and the corresponding ordinary variety for each harvest date were analyzed by t-test ($p < 0.05$) using XLSTAT software 2019.3.2 (Addinsoft, Paris, France). The correlations between bioactive compounds and antioxidant capacity measured by different assays were examined by Pearson's correlation ($p < 0.05$). Two kinds of principal component analysis (PCA) were built. The first PCA was set up using data for vitamin C, tocopherols, total phenolics, total flavonoids, DPPH, FRAP, ABTS-H, ABTS-L by XLSTAT software. The second PCA was built with the same variables and levels of carotenoids. The

data for total carotenoids for this second PCA analysis were obtained from our previous work [38], using the same biological samples.

3. Results and Discussion

The red-fleshed oranges, Kirkwood Navel (K) (https://www.biogold-em.com/assets/va---spec-sheet---kirkwood-red-navel-(print).pdf) (accessed on 23 September 2022) and Ruby Valencia (R) (https://www.biogold-em.com/assets/va---spec-sheet---ruby-valencia-(print).pdf) (accessed on 23 September 2022), originated from spontaneous bud mutations of the blond-colored Palmer Navel and Olinda Valencia oranges, respectively, grown in commercial orchards. These varieties were developed under agronomical and environmental Mediterranean conditions, maintaining the distinctive color singularities and carotenoid complements in the pulp [34]. These mutants are characterized by their accumulation of large concentrations of the colorless carotenes, phytoene and phytofluene, considerable amounts of lycopene, low levels of β-carotene and reduced amounts of violaxanthin, the major carotenoid in orange fruits, compared to their ordinary varieties N and V, respectively. In the current study, we report a comprehensive analysis of the content of relevant fruit-quality components and the antioxidant capacity of these new red-fleshed varieties in comparison to the corresponding ordinary oranges.

3.1. Physiological Parameters, Color and Internal Quality in the Red-Fleshed Kirkwood Navel and Ruby Valencia, and the Ordinary Navel and Valencia Oranges

The fruits growth, measured as changes in diameter and height, increased markedly from August to October in the four genotypes, corresponding to the cell-enlargement period (stage II) of citrus fruit development, in which the increase in pulp size is responsible for most of the growth [47]. From October to January, for N and K, and from October to April, for V and R, the rate of fruit growth was reduced and most of the maturation-related events occurred during this period (stage III). During this period, no differences in fruit size were detected between the two red-fleshed genotypes and the corresponding ordinary oranges (Tables 1 and 2). These results indicate that final fruit size at harvest is similar for each mutant with respect to its standard variety, which is line with observations in other red-fleshed oranges [25,27].

The main feature of K and R mutants is the inner coloration of the fruit. The internal appearance of the Navel, Kirkwood, Valencia and Ruby orange fruits is shown in Figure 1.

The a/b Hunter ratio revealed that the colors of the pulps of the K and R fruits were completely different compared to their respective standard lines (Tables 1 and 2). In the fruits of the N and V, the a/b values were negative and slightly positive, corresponding to green–yellow shades, but in both mutants, the color of pulp showed considerably higher positive values, indicating a red coloration of the pulp. These differences in color parameters were patent from the early stages of development, indicating that the alteration in pulp pigmentation was not linked to the maturation process, as has been previously suggested [38].

Table 1. Changes in fruit diameter and height, peel and pulp color and internal quality parameters of Navel and the red-fleshed Kirkwood oranges during development and maturation.

Parameter	Navel				
	Aug	**Oct**	**Nov**	**Dec**	**Jan**
Diameter (cm)	4.63 ± 0.14	7.27 ± 0.16	7.18 ± 0.14	7.39 ± 0.27	7.70 ± 0.19
Height (cm)	4.58 ± 0.15	6.81 ± 0.18	6.81 ± 0.15	7.20 ± 0.27*	7.55 ± 0.21
Peel color (a/b)	-	-0.72 ± 0.02	0.49 ± 0.04	0.66 ± 0.04	0.76 ± 0.03
Pulp color (a/b)	-	-0.24 ± 0.01*	-0.12 ± 0.07*	-0.06 ± 0.01*	0.00 ± 0.01*
TSS (°Brix)	-	10.65 ± 0.07*	11.55 ± 0.07	12.55 ± 0.21	12.75 ± 12.8
TA (mg CA/100 mL)	-	1.28 ± 0.01*	1.01 ± 0.04	0.86 ± 0.05	0.84 ± 0.02
MI (TSS/TA)	-	8.32 ± 0.06*	11.47 ± 0.52*	14.59 ± 0.03	15.11 ± 0.44
Parameter	Kirkwood				
	Aug	**Oct**	**Nov**	**Dec**	**Jan**
Diameter (cm)	4.76 ± 0.14	7.00 ± 0.24	6.84 ± 0.11	7.11 ± 0.14	7.54 ± 0.18
Height (cm)	4.66 ± 0.19	6.43 ± 0.12	6.49 ± 0.11	6.53 ± 0.18	7.16 ± 0.11
Peel color (a/b)	-	-0.72 ± 0.02	0.37 ± 0.02	0.57 ± 0.03	0.77 ± 0.02
Pulp color (a/b)	-	0.66 ± 0.07	0.41 ± 0.05	0.46 ± 0.05	0.54 ± 0.03
TSS (°Brix)	-	10.15 ± 0.07	11.45 ± 0.35	12.55 ± 0.11	12.80 ± 0.01
TA (mg CA/100 mL)	-	1.46 ± 0.01	1.14 ± 0.02	0.87 ± 0.03	0.83 ± 0.04
MI (TSS/TA)	-	6.95 ± 0.01	9.98 ± 0.21	14.42 ± 0.10	15.29 ± 0.16

Asterisks indicate significant differences between the red-fleshed orange K and the N orange for the same harvest month ($p \leq 0.05$, *t*-test). TSS: total soluble solids; TA: titratable acidity; CA: citric acid; MI: maturity index.

Moreover, in the fruits of both mutants, the a/b values decreased during maturation; this was probably related to dilution caused by the expansion of the pulp (Tables 1 and 2). The color index of the peel changed from negative to positive, corresponding to the typical transformation from green to yellow or orange to yellow, in all varieties.

Thus, the external colorations of the mutant fruits were visually similar to those of the respective ordinary genotypes (Tables 1 and 2). These results were in good agreement with those of other orange mutants, in which the red-fleshed phenotype is not associated with alterations in the coloration of the peel [24,25,27,38].

Table 2. Changes in fruit diameter and height, peel and pulp color, and internal-quality parameters of Valencia and the red-fleshed Ruby oranges during development and maturation.

Parameters	Valencia					
	Aug	**Oct**	**Dec**	**Jan**	**Mar**	**Apr**
Diameter (cm)	4.28 ± 0.09	6.18 ± 0.09*	6.44 ± 0.08*	6.59 ± 0.17	6.91 ± 0.11	6.95 ± 0.13
Height (cm)	4.24 ± 0.10 *	6.10 ± 0.09	6.25 ± 0.12	6.46 ± 0.11	6.81 ± 0.09	7.08 ± 0.14
Peel color (a/b)	-	-0.75 ± 0.01	0.39 ± 0.03*	0.66 ± 0.03	0.46 ± 0.02	0.66 ± 0.04
Pulp color (a/b)	-	-0.34 ± 0.01*	-0.16 ± 0.01*	-0.07 ± 0.01*	0.00 ± 0.01*	0.06 ± 0.01*
TSS (°Brix)	-	7.80 ± 0.02	8.66 ± 0.05	9.02 ± 0.03*	10.70 ± 0.21	11.50 ± 0.10
TA (mg CA/100 mL)	-	1.92 ± 0.09	1.44 ± 0.17	1.24 ± 0.03	1.10 ± 0.06	0.99 ± 0.07
MI (TSS/TA)	-	4.06 ± 0.08*	6.01 ± 0.21	7.27 ± 0.15	9.72 ± 0.16	11.60 ± 0.87
Parameters	Ruby					
	Aug	**Oct**	**Dec**	**Jan**	**Mar**	**Apr**
Diameter (cm)	4.13 ± 0.11	5.73 ± 0.09	5.83 ± 0.27	6.32 ± 0.08	6.52 ± 0.09	6.51 ± 0.10
Height (cm)	4.15 ± 0.11	5.83 ± 0.25	6.03 ± 0.26	6.11 ± 0.11	6.32 ± 0.10	6.59 ± 0.11
Peel color (a/b)	-	-0.79 ± 0.01	0.16 ± 0.03	0.59 ± 0.02	0.49 ± 0.03	0.67 ± 0.02
Pulp color (a/b)	-	0.72 ± 0.06	0.55 ± 0.05	0.55 ± 0.03	0.65 ± 0.03	0.60 ± 0.03
TSS (°Brix)	-	7.85 ± 0.07	9.05 ± 0.11	9.80 ± 0.03	10.86 ± 0.05	11.95 ± 0.20
TA (mg CA/100 mL)	-	1.75 ± 0.09	1.59 ± 0.06	1.50 ± 0.08	1.30 ± 0.17	1.05 ± 0.04
MI (TSS/TA)	-	4.48 ± 0.08	5.69 ± 0.30	6.53 ± 0.34	8.35 ± 1.10	11.38 ± 0.54

Asterisks indicate significant differences between the red-fleshed orange K and the N orange for the same harvest month ($p \leq 0.05$, *t*-test). TSS: total soluble solids; TA: titratable acidity; CA: citric acid; MI: maturity index.

The analysis of the internal quality parameters (°Brix and total acidity) revealed only minor differences between the K and N fruits at the initial maturity stages (October and November) (Table 1). In general, the internal maturation progressed at a slower rate in the fruits of the Valencia group (V and R, Table 2) than in those of the Navel group, in accordance with the late harvesting of the Valencia genotypes. No significant differences in the juice content of the mature fruits were recorded between the red-fleshed and the corresponding blond oranges (data not shown). Together, these results indicate that the evolution of the internal quality parameters in the red-fleshed K and R genotypes was similar to that of the ordinary varieties. Other studies with the red-fleshed variety, Cara Cara, showed lower ºBrix and maturity indices than the ordinary Navel variety [29,30], but whether these discrepancies may be related to differences in the growing or environmental conditions or to the genotype remains to be ascertained [48]. Our results indicate that under the conditions of our study, the reddish inner

coloration is the main distinctive trait between the K and V fruits and the corresponding traditional varieties, whereas the external appearance, size and maturity are similar.

3.2. Changes in Sugars and Organic Acids in the Red-Fleshed Kirkwood Navel and Ruby Valencia and in the Ordinary Navel and Valencia Oranges

The analysis of the main sugars in the pulps of the four genotypes revealed a progressive accumulation during development and maturation (Figure 2). The concentrations of sucrose, fructose and glucose in the Navel oranges ranged from 28–46 g/l 14–25 g/l and 17–30 g/l, respectively, whereas in the Valencia group, they were 8–46 g/l, 8–27 g/l and 9–21 g/l. The concentrations of sugars detected in these varieties were in accordance with previous reports for fruits of the Navel and Valencia varieties [35,48–50]. It is well known that fruit sweetness depends not only on the content of individual sugars, but also on the ratio between the main sugars [49]. In the present study, sucrose was the dominant sugar in the fruits of the four orange varieties and the ratio of sucrose, fructose and glucose was approximately 2:1:1, consistent with previous results in other orange varieties [1,49,50]. No significant differences in the sugar content were observed between the N and K fruits in most of the stages of development analyzed (Figure 2). By contrast, the V fruits contained slightly larger concentrations of sugars compared to the R fruits in August, October and December. More importantly, mature V fruits (April) accumulated 15% more sucrose and 30% more glucose and fructose than those of R (Figure 2E,F). Kafkas et al. [35] and Brasili et al. [29] also found that mature Navel oranges had higher levels of glucose and fructose, but lower levels of sucrose compared to the red-fleshed variety, Cara Cara.

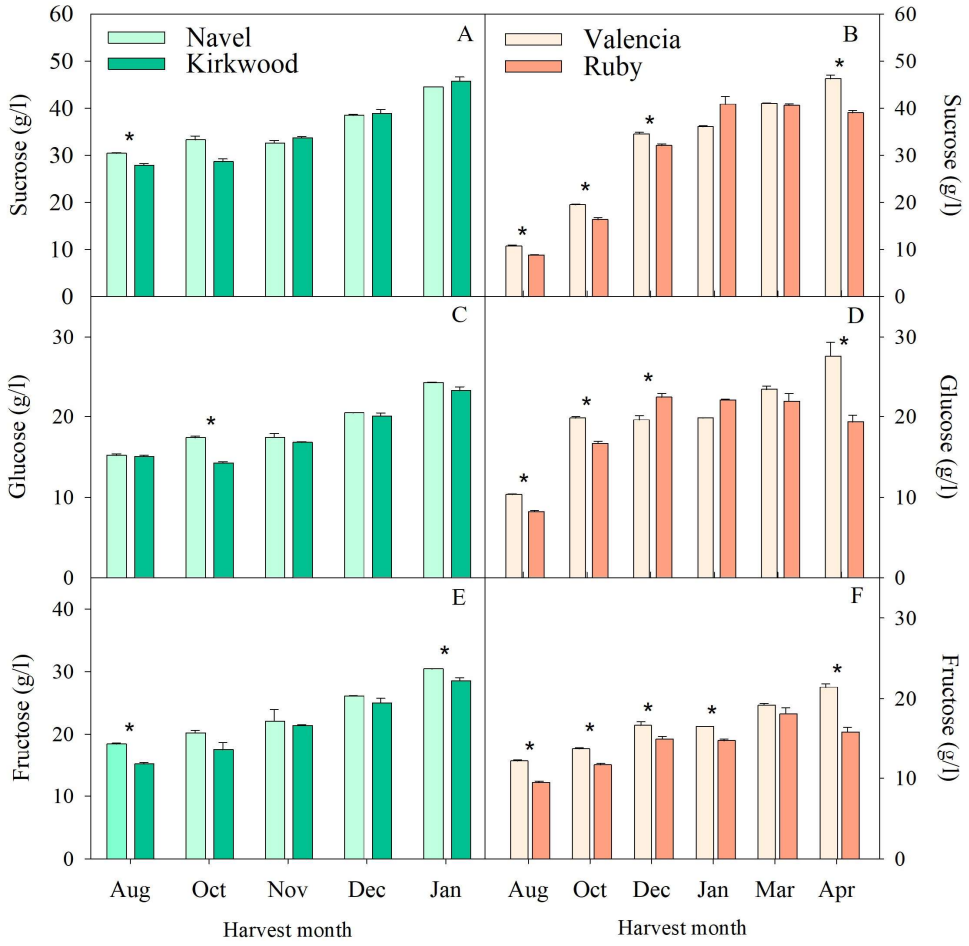

Figure 2. Concentrations of sucrose (A,B), glucose (C,D) and fructose (E,F) in the pulps of Navel and Kirkwood (left panel) and Valencia and Ruby (right panel) oranges during late fruit development and maturation. Results are the mean of two biological replicates ± SD. Asterisks indicate significant differences between the red-fleshed K and R and the respective ordinary oranges, N and V, for each harvest month by t-test ($p \leq 0.05$).

In this study, we detected four major organic acids in the pulps of the four varieties evaluated (Figure 3). Citric acid was the most abundant organic acid, followed by quinic, succinic and malic acid. The concentrations of the citric and succinic acid decreased in all the varieties during maturation. The malic acid remained relatively stable during maturation, whereas the quinic acid increased progressively during maturation in the N and K and remained with minor fluctuations in the V and R (Figure 3G,H). The concentrations of malic acid in the V and R fruits decreased from August to December and then experienced a gradual increase until full maturity (Figure 3D). The pattern of organic acid accumulation in this study was relatively similar to that generally observed in fruits of other varieties [48,50].

In the orange fruits, the free acids increased rapidly in stage II of the fruits development and then declined in concentration due to the dilution caused by the increase in size and water content of the pulp [47], according to our results. In the two red-fleshed mutants, the accumulation of organic acids appears to have been differentially affected, whereas in the K, only occasional differences were detected and in the mature fruits, the final acid concentration was similar to that of the N oranges (Figure 3A). The concentration of citric acid in the R was between 20 and 30% higher than in the ordinary V during the whole development and maturation period (Figure 3B). In this mutant, by contrast, the malate and succinate were lower in several stages of maturation (Figure 3E,F). Nonetheless, these alterations may not have exerted a critical influence on the final taste and flavor or on the internal maturity index, probably due to the equilibrium with the other metabolites contributing to this property [48]. The accumulation of sugars and organic acids during development and maturation has been studied in detail in fruits of the red-fleshed orange, Hong Anliu [24,36]. This mutant displayed a genuine profile of sugars and organic acids, with a higher accumulation of sugars and a reduction of around fourfold in citric acid compared to the Anliu orange [36].

A comparative gene expression analysis indicated the downregulation of the transcription of the genes relevant to citric acid accumulation, those related to electron transport to the TCA cycle or to the proton pump involved in the transport of citrate to the vacuole [51,52]. The fact that the two red-fleshed mutants analyzed in this work did not have as severe a reduction in their citrate contents as the Hong Anliu mutant indicates that changes in these compounds may not necessarily be linked to the abnormal pigmentation of the mutants but, rather, that they are more likely the result of other pleiotropic effects on this mutant.

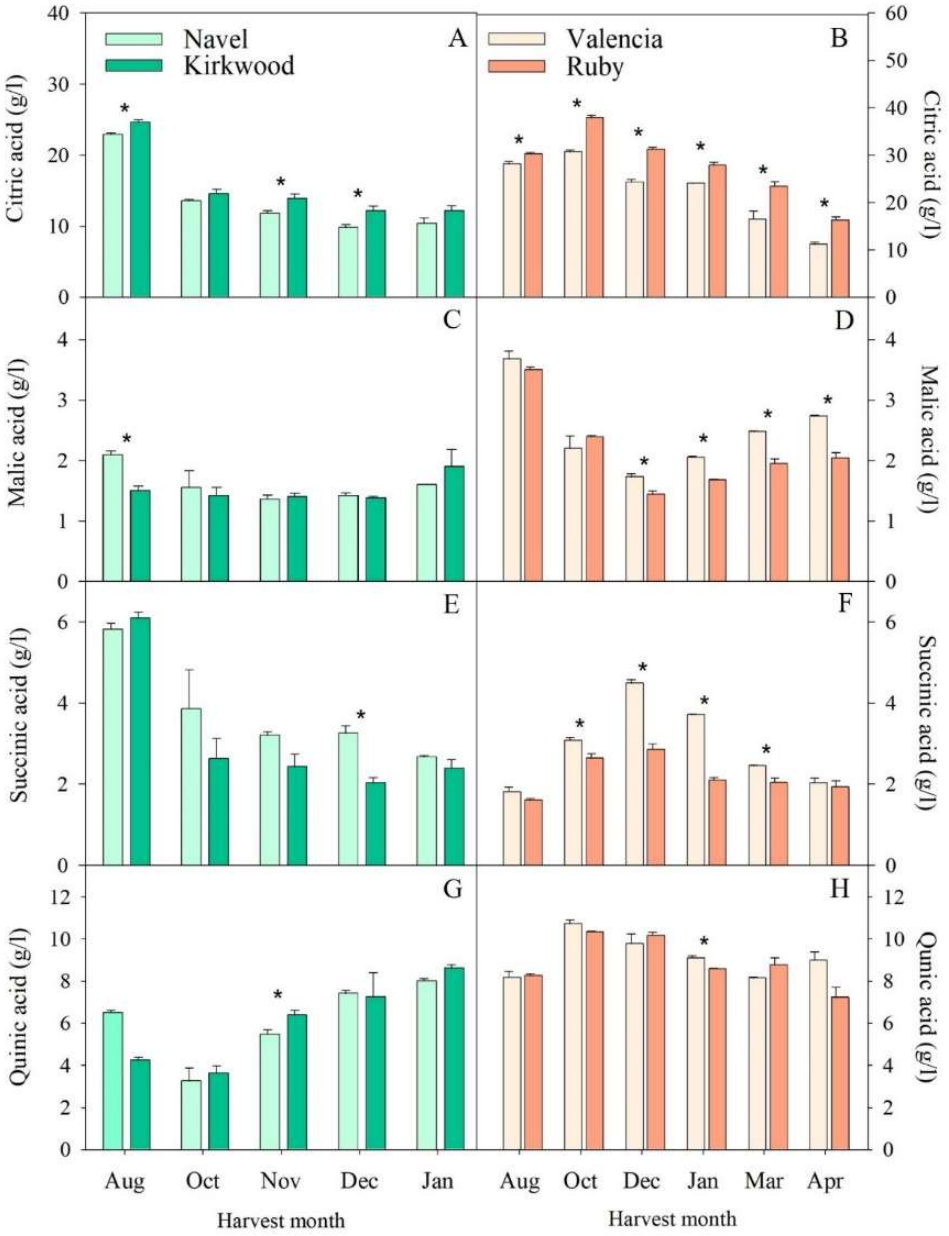

Figure 3. Concentrations of citric acid (A,B), malic acid (C,D), succinic acid (E,F) and quinic acid (G,H) in the pulps of Navel and Kirkwood (left panel) and Valencia and Ruby (right panel) oranges during late fruit development and maturation. Results are the mean of two biological replicates ± SD. Asterisks indicate significant differences between the red-fleshed K and R and the respective ordinary oranges, N and V, for each harvest month by t-test ($p \leq 0.05$).

3.3. Accumulation of Ascorbic Acid, Tocopherols, Phenolic and Flavonoids Compounds in the Red-Fleshed Kirkwood Navel and Ruby Valencia and the Ordinary Navel and Valencia Oranges

The concentrations of ascorbic acid and tocopherols, as well as those of phenolics and flavonoids, in the pulps of the red-fleshed oranges, K and R, and the respective blond genotype, were also analyzed during development and maturation. The orange fruit is recognized as a significant source of vitamin C. The concentrations of ascorbic acid in the pulps

of Navel and Valencia oranges were relatively similar and, in general, declined moderately during maturation (Figure 4A,B).

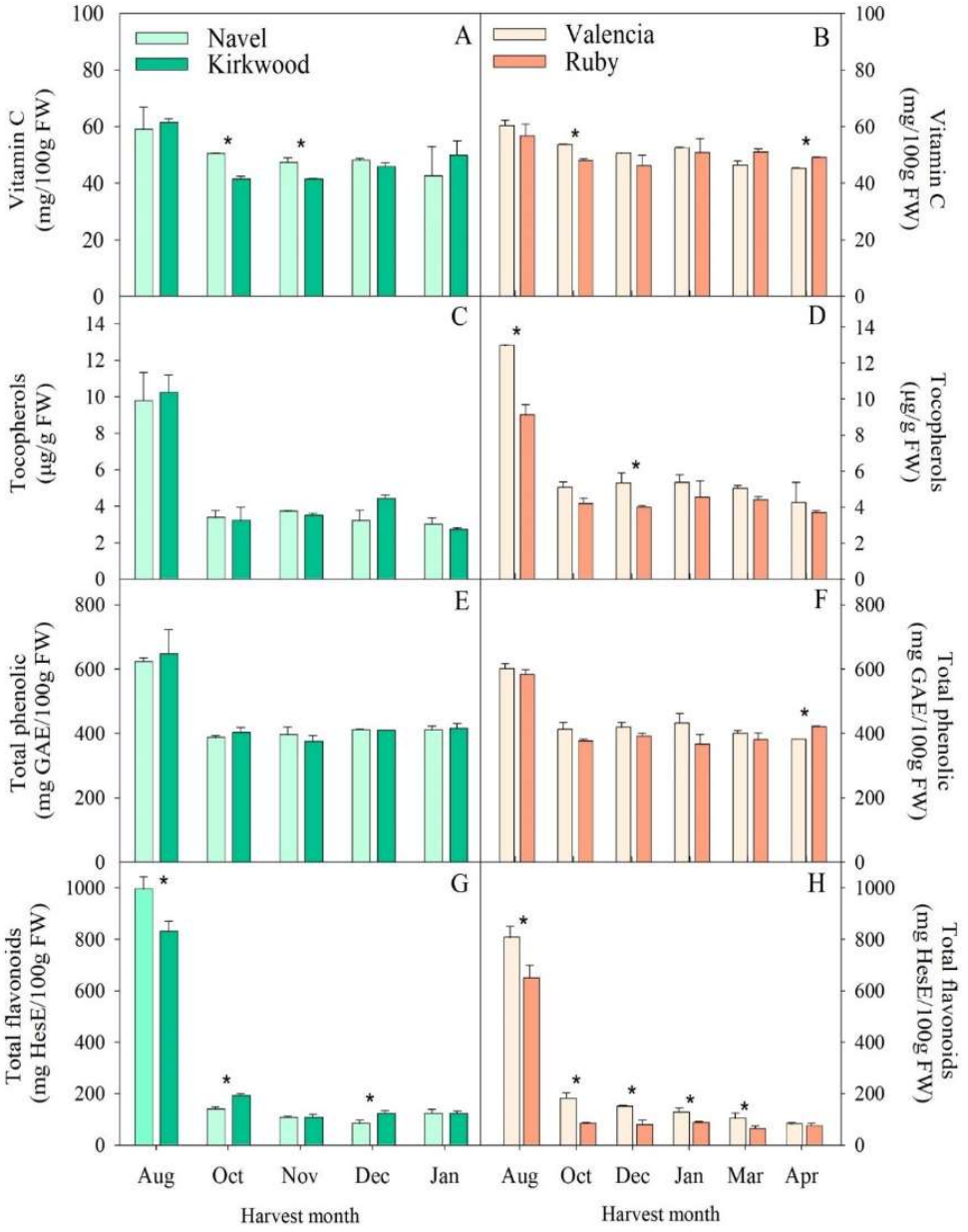

Figure 4. Evolution of vitamin C (A,B), tocopherols (C,D), total phenolic (E,F) and total flavo-noid (G, H) contents in the pulps of Navel and Kirkwood (left panel) and Valencia and Ruby (right panel) oranges during late fruit development and maturation. Results are the mean of two biological replicates ± SD. Asterisks indicate significant differences between the red-fleshed K and R and the respective ordinary oranges, N and V, for each harvest month by t-test ($p \leq 0.05$).

These results are consistent with those found in other citrus varieties, in which the changes in this vitamin were mainly variety-dependent and related to the maturation stage of the fruit [14,41]. However, other studies indicated that Cara Cara fruits contained lower vitamin C content than ordinary Navel oranges [30,35], pointing out that differences in the content of this

vitamin in the red-fleshed mutants appear to be genotype-dependant but are not associated with its distinctive coloration.

Information about the tocopherol content in citrus fruits is very limited, and the regulation of tocopherols accumulation in fruits of different citrus species have only been investigated in recent studies [16,53,54]. The α-Tocopherol is the predominant form of to-copherol (around 99% of the total) in the pulps of the four varieties at all harvest stages, and trace amounts of γ-tocopherol were detected only in some samples (data not shown). In the current study, we found the maximum tocopherol concentration in the pulps of the immature fruits in August (10–13 μg/g FW); the values were similar in the four varieties analyzed. After this period, the total tocopherol experienced a sharp decline (54–69%) in October, after which it remained stable during the whole maturation process and without relevant differences between the blond and the red-fleshed oranges (Figure 4C,D). To our knowledge, this is the first study reporting the accumulation of tocopherols in red-fleshed orange varieties, which is in accordance with the pattern of changes reported in the pulps of other citrus species [54].

The analysis of the total phenolics and flavonoids in the pulps of the four orange varieties revealed that the immature fruits (August) contained the highest concentrations, which that thereafter (Figure 4). Previous studies have also reported a decrease in flavonoid concentrations during fruit development and the maturation of orange fruits [11,28]. These changes have been related to the oxidation of polyphenols by the polyphenol oxidase during maturation [55]. Brasili et al. [29] and De Ancos et al. [30], using the Folin–Ciocalteu assay, found similar total phenolic contents in the juices of ordinary Navel and Cara oranges. Nonetheless, other studies reported higher phenolic levels in the pulp of Cara than in that of ordinary Navel [31]. These discrepancies indicate that the growing and environmental conditions of the samples are important sources of the variability of these bioactive compounds [48].

Although the pattern of flavonoid accumulation during maturation was similar to that of the total phenolics, it is interesting that the immature fruits of the two red-fleshed oranges had lower flavonoid levels than those of the ordinary oranges (Figure 4G,H). Moreover, the concentration of these compounds in R remained below that of the ordinary V over most of the maturation period. These results are consistent with those obtained by Chen et al. [28], in

which the contents of hesperidin and narirutin, the major flavonoids in orange fruits, were higher in the blond-fleshed Seike Navel and Anliu oranges than in the red-fleshed Cara Cara and Red Anliu, respectively. Whether these differences in total flavonoids may be associated with the pigmentation of the red-fleshed varieties should be further investigated.

To ascertain the quantitative and qualitative differences in flavonoids between the ordinary and the red-fleshed oranges, we analyzed the composition of the main flavonoids in the pulps of the mature fruits harvested in January for the Navel varieties and in April for those of the Valencia (Table 3). Five flavanones (hesperidin, dydimin, narirutin, naringin and eriocitrin) and one flavonol (rutin) were identified in all the samples. The major flavonoid in all the varieties was hesperidin, followed by narirutin. These results agreed with those reported in the literature [28,30,56,57]. However, the relative amount and proportion for each flavonoid is very variable, depending on the variety, maturity state, origin and climatic conditions [7]. In this study, the flavonoid contents varied slightly between the V and R fruits. The V contained higher amounts of rutin and naringin, but lower amounts of narirutin, than the R oranges (Table 3). No significant differences were detected between the R and N oranges and, in general, the total flavonoid contents were similar between the red-fleshed and the corresponding ordinary varieties (Table 3). It should be noted that other studies reported inconsistent differences in flavonoid contents between blond oranges and the red-fleshed oranges, Hong Anliu or Cara Cara [28,30]. Thus, it is likely that differences in the growing and climatic conditions, rootstock, harvest time and other variables have a pivotal influence on the contents of these bioactives. Since the fruits used in the current study were grown in the same orchard and harvested under identical conditions, our results are more likely to reflect likely genotype differences than environment-related influences.

Table 3. Contents of individual and total flavonoids (mg/100g FW) in the pulps of mature fruits, Navel and Valencia, and the corresponding red-fleshed oranges, Kirkwood and Ruby Valencia, during development and maturation. Fruits of Navel and Kirkwood were harvested in January and those of Valencia and Ruby in April.

Flavonoid	**Navel**	**Kirkwood**	**Valencia**	**Ruby**
Rutin	2.23 ± 0.17	2.69 ± 0.60	2.21 ± 0.02*	2.04 ± 0.02
Eriocitrin	0.98 ± 0.09	1.08 ± 0.13	0.84 ± 0.12	0.76 ± 0.01
Narirutin	7.58 ± 1.05	9.08 ± 1.90	3.76 ± 0.15*	4.45 ± 0.07
Naringin	0.28 ± 0.03	0.30 ±0.07	0.63 ± 0.05*	0.45 ± 0.02
Hesperidin	46.05 ± 9.02	60.11 ± 4.72	53.93 ± 5.72	53.48 ± 1.35
Dydimin	0.85 ± 0.13	1.17 ± 0.26	0.46 ± 0.12	0.72 ± 0.07
Total	57.96 ± 10.43	74.43 ± 7.41	61.84 ± 6.17	61.90 ± 1.52

Results are the mean of two biological replicates ± SD. Asterisks indicate significant differences between the red-fleshed oranges, K and R and the ordinary varieties, N and V, respectively ($p \leq 0.05$, t-test).

3.4. Hydrophilic and Lipophilic Antioxidant Capacity in the Red-Fleshed Kirkwood Navel and Ruby Valencia and the Ordinary Navel and Valencia Oranges

One of the limitations in the interpretation of the antioxidant capacity of citrus fruits is the determination of the relative contribution of lipophilic compounds, such as carotenoids and tocopherols, to the antioxidant capacity, since most studies focus on the antioxidant contribution of hydrophilic compounds, such as vitamin C and flavonoids [14,58]. In this work, we assayed the evolution of the HAC and LAC in the four varieties during fruit development and maturation. The analysis of the HAC assessed by DPPH and FRAP assays showed a maximum antioxidant capacity in August, which declined progressively throughout the fruits' growth and maturation in the four varieties (Figure 5A–D). However, the ABTS assay of the hydrophilic extracts revealed an increasing antioxidant capacity during the fruits' maturation (Figure 5E,F). This discrepancy in the hydrophilic antioxidant capacity may indicate differences in the main compounds contributing to the activity in the different assays and in their changes during maturation. In fruits of other citrus species, variable patterns in the hydrophilic antioxidant capacity have been reported, such as a progressive increment in Yuzu (*Citrus junos*) [59], or a slight decrease in the fruits of lemon [55] and oranges [60].

Our results revealed that the contents of vitamin C positively correlated with the DPPH ($r^2 = 0.79$) and FRAP ($r^2 = 0.67$). Moreover, the total phenolic and flavonoid contents displayed a good positive correlation with the DPPH ($r^2 = 0.86$ and $r^2 = 0.89$, respectively) and FRAP

activity (r^2 = 0.65 and r^2 = 0.72, respectively) (Table S2). These results indicate that vitamin C and polyphenols are major contributors to the HAC in all the genotypes evaluated, which is in agreement with previous observations [14,30,61]. However, the ABTS-H did not correlate significantly with either the vitamin C or the polyphenols (Table S2). This result might indicate that the scavenging capacity of the ABTS•+ of the hydrophilic fraction is likely due to individual phenolics/flavonoids rather than the total concentration [14,61]. The comparison of the HAC values by the different assays revealed that, in general, there were negligible differences between the red-fleshed and the ordinary genotypes. The V fruits presented 15–20% more HAC by ABTS than the R in the fruits harvested in August, December and April (Figure 5F). These results are consistent with the higher amount of total flavonoids detected in the pulp of the V in the several stages of development (Figure 4H). Several studies reported higher antioxidant capacity in the pulp and juice of ordinary Navel than those of Cara Cara; this was probably related to the higher levels of vitamin C [29,30,56]. In previous studies, we described higher HAC in Ruby oranges than in the blond Valencia, even though the vitamin C concentrations were similar [14]. Therefore, it is likely that the large variability in the composition of the bioactive compounds in the different orange varieties, which are strongly influenced by fruit maturity and environmental and agronomical growing conditions, may account for the discrepancies in the analysis of the antioxidant capacity among the different studies [6,56].

On the other hand, the analysis of the LAC, measured by ABTS (ABTS-L), revealed the highest values in the four varieties in immature fruits (August), which dropped sharply in October, after which they remained relatively constant or experienced a slight increase during maturation (Figure 5). Interestingly, the lipophilic ABTS activity was higher in several stages of fruit maturity in the red-fleshed fruits of K and R in comparison with the corresponding blond oranges (Figure 5G,H). As far as we know, this is the first study to investigate the LAC in the pulps of citrus fruits during the whole process of maturity.

The ABTS-L showed a significant positive Pearson's correlation with the concentration of tocopherols (r^2 = 0.85) (Table S2). Tocopherols are efficient ROS scavengers and, therefore, their abundance in citrus fruit can play an important role in the total antioxidant capacity of different species and varieties [54]. Our Pearson's analysis also showed a good positive correlation coefficient with phenolics (r^2 = 0.87) and flavonoids (r^2 = 0.85). Several reports have

obtained an efficient extraction of phenolics and flavonoids from orange tissues using ethyl acetate as a solvent [62,63]. Since, in the current study, ethyl acetate was the solvent used to extract the lipophilic compounds to test the LAC by ABTS assay, it is reasonable to assume a certain contribution of these compounds to this activity. More importantly, we recently reported that the reddish coloration of the pulps of K and R fruits is due to the accumulation of the carotene, lycopene, which is known to have significant antioxidant capacity [64,65], as well as to high amounts of the colorless carotenes, phytoene and phytofluene [38]. Since carotenoids make a significant contribution to the antioxidant capacity in the lipophilic fractions [58,61,66], it is likely that the higher lipophilic capacity found in the mature fruits of the red-fleshed oranges may be due to their specific carotenoid content and composition [14].

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Overall, these results indicate that the pulp extracts of citrus fruits can effectively scavenge different types of free radicals under in vitro conditions. The variability of the assays performed suggests that multiple mechanisms and different bioactive compounds may contribute to their antioxidant capacity.

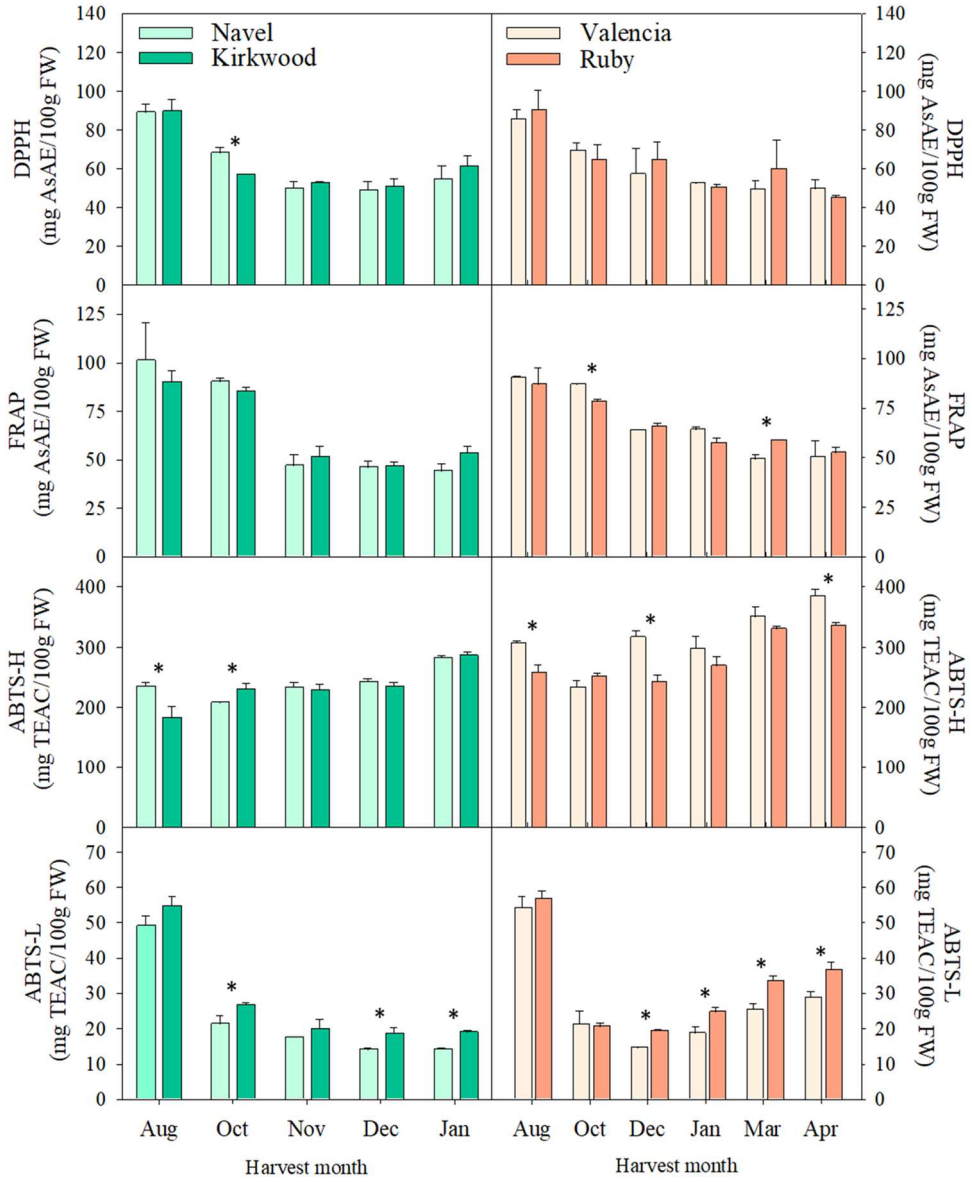

Figure 5. Evolution of the antioxidant capacity, evaluated by DPPH (A,B), FRAP (C,D), ABTS-H (E,F) and ABTS-L (G,H) assays, in the pulps of Navel and Kirkwood (left panel) and Valencia and Ruby (right panel) oranges during late fruit development and maturation. Results are the mean of two biological replicates ± SD. Asterisks indicate significant differences between the red-fleshed K and R and the respective ordinary oranges, N and V, for each harvest month by t-test ($p \leq 0.05$).

For instance, Liu et al. [67] reported that the combination of lycopene + vitamin E + vitamin C showed the highest synergy in the DPPH assay, suggesting that the mechanisms of this mixture are associated with the transfer of electrons from the carotenoid to the α-tocopheroxyl radical to regenerate tocopherol, and from vitamin C to the resulting carotenoid radical cation to regenerate the carotenoids.

Although the classical antioxidant methods used in the present study are based on different chemical mechanisms, all the citrus varieties studied in this work followed a similar trend and exhibited relatively similar values in terms of total antioxidant capacity. Therefore, the powerful antioxidant properties attributed to orange fruits are the result of the combination of phytochemicals and synergistic mechanisms [57]. Stahl et al. [22] found that mixtures of carotenoids are more effective than single compounds in preventing oxidative damage in multilamellar liposomes. Additionally, Chen et al. [68] demonstrated that combinations of carotenoids (lycopene and lutein) with flavonoids enhanced antioxidant capacity in cell cultures compared with individual components.

3.5. Singlet Oxygen Absorption Capacity (SOAC) and Its Relationship with Carotenoid Concentrations in the Red-Fleshed Kirkwood Navel and Ruby Valencia and the Ordinary Navel and Valencia Oranges

Singlet oxygen ($^1O_2$) is a reactive oxygen species (ROS) generated in biological systems, which reacts with many biological targets, inducing the degradation of cellular components. Carotenoids are efficient quenchers of 1O2 in biological systems [69]. The antioxidant activity of carotenoids depends on the number of conjugated double bonds present in their chemical structure. Thus, it is widely accepted that lycopene is the carotenoid with the greatest antioxidant activity, since it features the largest number of conjugated double bonds [70].In this work, we used the SOAC assay [69] to further analyze the contribution of carotenoids to the antioxidant capacity of the red-fleshed varieties. The SOAC was determined in the pulps of mature fruits harvested in December and January for the N and K oranges and in March and April for the V and R oranges. The K and R fruits a showed 40–50% higher SOAC capacity than the N and V fruits for the two maturation stages analyzed (Figure 6).

The carotenoid contents and compositions in the pulps of these samples were analyzed to ascertain their potential relationships with the differences in SOAC capacity. The total carotenoids were 12 and 18 times higher in the mature K and V fruits than in the N and V, respectively (Table S3 and S4). The carotenoid profiles of the ordinary blond oranges mainly composed violaxanthin as the major carotenoid, followed by lower amounts of luteoxanthin, anteraxanthin, β-cryptoxanthin, lutein and linear carotenes.

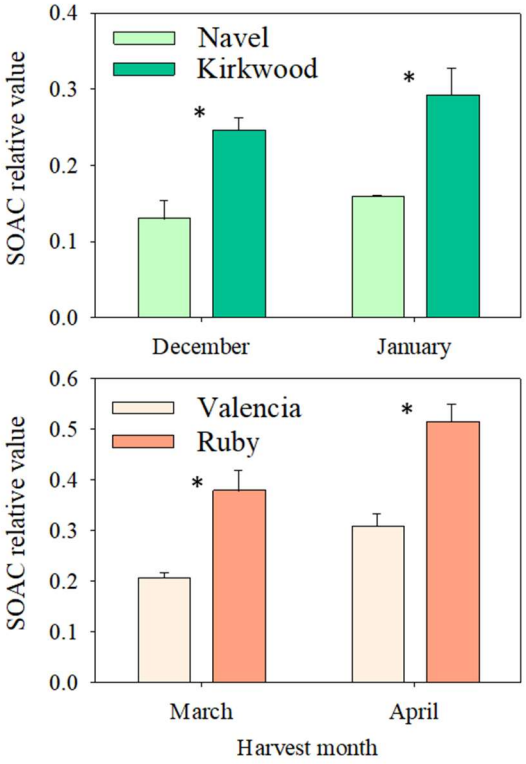

Figure 6. Singlet oxygen absorption capacity (SOAC) in the pulps of Navel and Kirkwood oranges (upper panel) harvested in December and January and Valencia and Ruby oranges (lower panel) harvested in March and April. Results are the mean of two biological replicates ± SD. Asterisks indicate significant differences between the red-fleshed K and R and the respective ordinary orange varieties, N and V, for each harvest month by t-test ($p \leq 0.05$).

The pulps of the red-fleshed oranges contained large amounts of phytoene and phytofluene, moderate concentrations of lycopene (7–9 μg/g FW) and approximately 40–50% lower amounts of violaxanthin than the ordinary varieties. In both red-fleshed oranges, low concentrations of β-carotene, with recognized pro-vitamin A activity, were detected, but not in the ordinary oranges (Tables S3 and S4). The carotenoid compositions in the R and K and the higher content of most of the carotenoids in the R than in the K were in accordance with our previous results [38].

The correlation analysis between the SOAC and the content of total carotenoids, phytoene+phytofluene and lycopene, integrating data from the four varieties and the two harvest dates, revealed a strong positive correlation with the variables (Table S5). These results indicate that the higher SOAC in the red-fleshed oranges was likely due to the difference in the content of these carotenoids. It has been proposed that lycopene is the most effective

scavenger of singlet oxygen, followed by phytofluene and phytoene, by both electron transfer and free-radical deactivation [65]. Therefore, it is reasonable to assume that, together with lycopene, the unusually high levels of colorless carotenes contribute to a higher level of $^1O_2$-quenching activity in the red-fleshed oranges.

3.6. Multivariate Analysis (PCA) of Bioactive Compounds, Sugars, Organic Acids and Antioxidant Capacity in the Red-Fleshed Kirkwood Navel and Ruby Valencia and the Ordinary Navel and Valencia Oranges during Fruit Development and Maturation

A principal component analysis (PCA) was carried out to explore the relationship between the contents of the different compounds (sucrose, glucose, fructose, citric acid, malic acid, succinic acid, quinic acid, vitamin C, tocopherols, total phenolics and total flavonoids) and the antioxidant activity (DPPH, FRAP, ABTS-H and ABTS-L) (Figure 7A,B). In the first analysis, the two principal components (PCs) explained 89.82% and 85.27% of the total variance, for the Navel and Valencia oranges, respectively (Figure 7A,B). The samples were separated by the maturity stage of the fruits, indicating that this was the main factor in their variability.

In the second PCA analysis, including total carotenoids [38], the samples of the red-fleshed varieties were well separated from the blond oranges (Figure 7C,D). Interestingly, the PC1 explained 64.92% (Figure 7C) and 58.03% (Figure 7D) of the variance, separating the samples according to the stage of harvest, whereas the PC3 accounted for 7.11% (Figure 7C) and 10.76% (Figure 7D) of the variance, discriminating the samples by their carotenoid contents. Together, these results indicate that the concentrations of the different compounds analyzed and their respective antioxidant capacities may discriminate the stage of maturity of the different orange varieties (both red and blond oranges), but the discrimination between the red and the blond oranges was achieved through the carotenoid content.

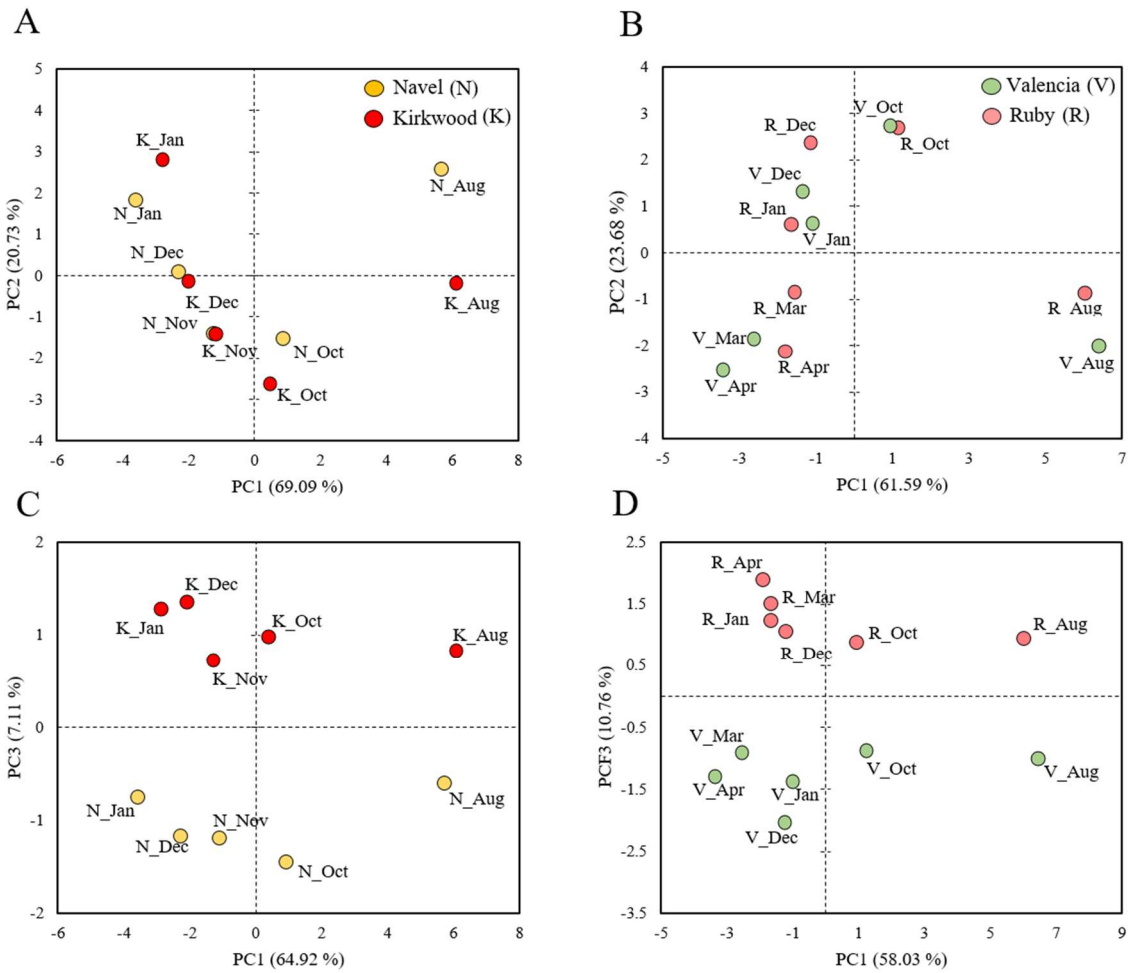

Figure 7. Principal component analysis (PCA) of the variables analyzed (vitamin C, tocopherols, total phenolics, total flavonoids, DPPH, FRAP, ABTS-H and ABTS-L) in Navel and Kirkwood oranges (A), and Valencia and Ruby oranges (B). Principal component analysis (PCA) of the variables analyzed and total carotenoid contents adapted from Zacarías-García et al. [38], in Navel and Kirkwood oranges (C), and Valencia and Ruby oranges (D). Aug: August; Oct: October; Nov: November; Dec: December; Jan: January; Mar: March; Apr; April.

4. Conclusions

The analysis of the quality attributes, bioactive compounds and antioxidant activities from the last stages of fruit development to full maturity revealed that the new red-fleshed varieties of sweet orange, Kirkwood Navel and Ruby Valencia, displayed, under Mediterranean growing conditions, a pattern and timing of maturation and a harvesting date similar to those of the ordinary Navel and Valencia varieties. No relevant differences were detected in the accumulation of sugars, organic acids, phenolics, flavonoids, vitamin C or tocopherols between the red Kirkwood and the blond Navel. However, the Ruby Valencia fruits had lower levels of sugars, malic acid and succinic acid and higher levels of citric acid than the ordinary Valencia fruits. The main feature of both red-fleshed oranges was the presence of higher lipophilic antioxidant capacity (LAC) and singlet oxygen absorption capacity (SOAC) than in the ordinary oranges, which was probably due to their lycopene content and, in particular, to their high levels of phytoene and phytofluene. Taken together, the results suggest that these novel red-fleshed orange varieties offer new products to the fresh-citrus market and juice-processing industries, with improved properties primarily derived from their carotenoid content and composition, as well as their lipophilic and singlet oxygen antioxidant capacity, without any detrimental effects on their other fruit-quality attributes. Further experiments with cell cultures, in vivo systems or clinical trials would be appropriate to evaluate the additional health-related benefits of these red-fleshed orange fruits.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox11101905/s1. Table S1: Spectroscopic characteristics of the main carotenoids identified in the chromatograms of the pulp extracts of Navel, Kirkwood, Valencia and Ruby orange varieties; Table S2: Pearson's correlation coefficients (r2) among vitamin C, tocopherols, total phenolics, total flavonoids, DPPH, FRAP, ABTS-H and ABT-L; Table S3: Carotenoid contents and compositions (μg/g) in the pulps of Navel and Kirkwood fruits harvested in December and January. ND: non-detected; Table S4: Carotenoid contents and compositions (μg/g) in the pulps of Valencia late and Ruby fruits harvested in March and April. ND: non-detected; Table S5: Pearson's correlation coefficients (r2) among total carotenoids (TC), phytoene + phytofluene content (PE+PF), lycopene (LYC) and singlet oxygen absorption capacity (SOAC) evaluated in the pulps of Navel and Kirkwood fruits harvested in December and January and in the pulps of Valencia and Ruby fruits harvested in March and April.

Author Contributions: Conceptualization, M.-J.R. and L.Z.; methodology, J.Z.-G.; experimental work, J.Z.-G. and L.P.-T.; formal analysis and data curation, J.Z.-G.; writing—original draft prep-aration, J.Z.-G.; writing—review and editing, J.-V.G., M.-J.R. and L.Z.; supervision and funding acquisition, M.-J.R. and L.Z. All authors have read and agreed to the published version of the manuscript.

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References

1. Ma, G.; Zhang, L.; Sugiura, M.; Kato, M. Citrus and health. In The Genus *Citrus*; Talón, M., Caruso, M., Gmitter, F., Eds.; Woodhead Publishing: Cambridge, UK, 2020; pp. 495–511.
2. Lv, X.; Zhao, S.; Ning, Z.; Zeng, H.; Shu, Y.; Tao, O.; Xiao, C.; Lu, C. Liu, Y. Citrus fruits as a treasure trove of active natural metabolites that potentially provide benefits for human health. *Chem. Cent. J.* **2015**, *9*, 68.
3. Rampersaud, G.C.; Valim, M.F. 100% citrus juice: Nutritional contribution, dietary benefits, and association with anthropometric measures. *Crit. Rev. Food. Sci. Nutr.* **2017**, *57*, 129–140.
4. Saini, R.K.; Ranjit, A.; Sharma, K.; Prasad, P.; Shang, X.; Gowda, K.G.M.; Keum, Y.S. Bioactive compounds of Citrus fruits: A review of composition and health benefits of carotenoids, flavonoids, limonoids, and terpenes. *Antioxidants* **2022**, *11*, 239.
5. Rajendran, P.; Nandakumar, N.; Rengarajan, T.; Palaniswami, R.; Gnanadhas, E.N.; Lakshminarasaiah, U.; Gopas, J.; Nishigaki, I. Antioxidants and human diseases. *Clin. Chim. Acta* **2014**, *436*, 332–334.
6. Zou, Z.; Xi, W.; Hu, Y.; Nie, C.; Zhiquin, Z. Antioxidant activity of Citrus fruits. *Food Chem.* **2016**, *196*, 885–896.
7. Lu, X.; Zhao, C.; Shi, H.; Liao, Y.; Xu, F.; Du, H.; Xiao, H.; Zheng, J. Nutrients and bioactives in citrus fruits: Different citrus varieties, fruit parts, and growth stages. *Crit. Rev. Food Sci. Nutr.* **2021**, *5*, 1–24.
8. Roussos, P.A. Oranges (*Citrus sinensis* L. Osbeck). In *Nutritional Composition of Fruit Cultivars*; Simmods, M.S.J., Preedy, V.R., Eds.; Academic Press: Cambridge, MA, USA, 2016; pp. 469–496.
9. Tadeo, F.R.; Terol, J.; Rodrigo, M.J.; Licciardelo, C.; Sadka, A. Fruit growth and development. In *The Genus Citrus*; Talón, M., Caruso, M., Gmitter, F., Eds.; Woodhead Publishing: Cambridge, UK, 2020; pp. 495–508.
10. Rodrigo, M.J.; Alquézar, B.; Alòs, E.; Lado, J.; Zacarías, L. Biochemical bases and molecular regulation of pigmentation in the peel of Citrus fruit. *Sci. Hortic.* **2013**, *163*, 46–62.
11. Multari, S.; Licciardello, C.; Caruso, M.; Martens, S. Monitoring the changes in phenolic compounds and carotenoids occurring during fruit development in the tissues of four citrus fruits. *Food Res. Int.* **2020**, *134*, 109228.
12. Zhao, C.; Wang, F.; Lian, Y.; Xiao, H.; Zheng, J. Biosynthesis of citrus flavonoids and their health effects. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 566–583.
13. Martí, N.; Mena, P.; Cánovas, J.A.; Micol, V.; Saura, D. Vitamin C and the role of citrus juices as functional food. *Nat. Prod. Commun.* **2009**, *4*, 677–700.
14. Zacarías-García, J.; Rey, F.; Gil, J.V.; Rodrigo, M.J.; Zacarías, L. Antioxidant capacity in fruit of Citrus cultivars with marked differences in pulp coloration: Contribution of carotenoids and vitamin C. *Food Sci. Technol. Int.* **2021**, *27*, 210–222.
15. Fenech, M.; Amaya, I.; Valpuesta, V.; Botella, M.A. Vitamin C Content in Fruits: Biosynthesis and Regulation. *Front. Plant Sci.* **2019**, *9*, 2006.
16. Rey, F.; Rodrigo, M.J.; Zacarias, L. Accumulation of tocopherols and transcriptional regulation of their biosynthesis during cold storage of mandarin fruit. *Postharvest Biol. Technol.* **2021**, *180*, 111594.

17. Traber, M.G.; Sies, H. Vitamin E in humans: Demand and delivery. *Annu. Rev. Nutr.* **1996**, *16*, 321–347.
18. van Breda, S.G.; de Kok, T.M. Smart combinations of bioactive compounds in fruits and vegetables may guide new strategies for personalized prevention of chronic diseases. *Mol. Nutr. Food Res.* **2018**, *62*, 1700597.
19. Natarajan, T.D.; Ramasamy, J.R.; Palanisamy, K. Nutraceutical potentials of synergic foods: A systematic review. *J. Ethn. Food* **2019**, *6*, 27.
20. Zhanga, L.; Virgous, C.; Si, H. Synergistic anti-inflammatory effects and mechanisms of combined phytochemicals. *J. Nutr. Biochem.* **2019**, *69*, 19–30.
21. Niki, E.; Noguchi, N.; Tsuchihashi, H.; Gotoh, N. Interaction among vitamin C, vitamin E, and β-carotene. *Am. J. Clin. Nutr.* **1995**, *62*, 1322S–1326S.
22. Stahl, W.; Junghans, A.; de Boer, B.; Driomina, E.S.; Briviba, K.; Sies, H. Carotenoid mixtures protect multilamellar liposomes against oxidative damage: Synergistic effects of lycopene and lutein. *FEBS Lett.* **1998**, *427*, 305–308.
23. Milde, J.; Elstner, E.F.; Grassmann, J. Synergistic effects of phenolics and carotenoids on human low-density lipoprotein oxidation. *Mol. Nutr. Food Res.* **2007**, *51*, 956–961.
24. Liu, K.; Juan, X.; Liu, Y.; Zhao, X. A novel bud mutation that confers abnormal patterns of lycopene accumulation in sweet orange fruit (*Citrus sinensis* L Osbeck). *J. Exp. Bot.* **2007**, *58*, 4161–4171.
25. Alquézar, B.; Rodrigo, M.J.; Zacarías, L. Regulation of carotenoid biosynthesis during fruit maturation in the red-fleshed orange mutant Cara Cara. *Phytochemistry* **2008**, *69*, 1997–2007.
26. Rodrigo, M.J.; Cilla, A.; Barberá, R.; Zacarías, L. Carotenoid bioaccessibility in pulp and fresh juice from carotenoid-rich sweet oranges and mandarins. *Food Funct.* **2015**, *6*, 1950–1959.
27. Lu, P.J.; Wang, C.Y.; Yin, T.T.; Zhong, S.L.; Grierson, D.; Chen, K.S.; Xu, C.J. Cytological and molecular characterization of carotenoid accumulation in normal and high-lycopene mutant oranges. *Sci. Rep.* **2017**, *7*, 761.
28. Chen, J.; Zhang, H.; Pang, Y.; Cheng, Y.; Deng, X.; Xu, J. Comparative study of flavonoid production in lycopene-accumulated and blonde-flesh sweet oranges (*Citrus sinensis*) during fruit development. *Food Chem.* **2015**, *184*, 238–246.
29. Brasili, E.; Chaves, D.F.; Xavier, A.A.; Mercadante, A.Z.; Hassimotto, N.M.; Lajolo, F.M. Effect of Pasteurization on Flavonoids and Carotenoids in *Citrus sinensis* (L.) Osbeck cv. 'Cara Cara' and 'Bahia' Juices. *J. Agric. Food Chem.* **2017**, *65*, 1371–1377.
30. De Ancos, B.; Cilla, A.; Barberá, R.; Sánchez-Moreno, C.; Cano, M.P. Influence of orange cultivar and mandarin postharvest storage on polyphenols, ascorbic acid and antioxidant activity during gastrointestinal digestion. *Food Chem.* **2017**, *225*, 114–124.
31. Cilla, A.; Rodrigo, M.J.; Zacarías, L.; de Ancos, B.; Sánchez-Moreno, C.; Barberá, R.; Alegría, A. Protective effect of bioaccessible fractions of citrus fruit pulps against H_2O_2-induced oxidative stress in Caco-2 cells. *Food Res. Int.* **2018**, *103*, 335–344.
32. de Oliveira, R.; Orlando, C.; Cadavid, M.; Carmona, L.; Peña, L.; De, R.; Oliveira, P.; Scotti, L. Pasteurized Orange Juice Rich in Carotenoids Protects Caenorhabditis elegans against Oxidative Stress and β-Amyloid Toxicity through Direct and Indirect Mechanisms. *Oxid. Med. Cell. Longev.* **2019**, *2019*, 5046280.

33. Pan, Z.; Liu, Q.; Yun, Z.; Guan, R.; Zeng, W.; Xu, Q.; Deng, X. Comparative proteomics of a lycopene-accumulating mutant reveals the important role of oxidative stress on carotenogenesis in sweet orange (*Citrus sinensis* [L.] osbeck). *Proteomics* **2009**, *9*, 5455–5470.
34. Xu, Q.; Yu, K.; Zhu, A.; Ye, J.; Liu, Q.; Zhang, J.; Deng, X. Comparative transcripts profiling reveals new insight into molecular processes regulating lycopene accumulation in a sweet orange (*Citrus sinensis*) red-flesh mutant. *BMC Genet*. **2019**, 10, 540.
35. Kafkas, E.; Polatöz, S.; Koç, N.K. Quantification and comparison of sugars, carboxylic acids and vitamin c components of various citrus species by HPLC techniques. *J. Agric. Sci. Technol.* **2011**, *5, 175-180*.
36. Yu, K.; Xu, Q.; Da, X.; Guo, F.; Ding, Y.; Deng, X. Transcriptome changes during fruit development and ripening of sweet orange (*Citrus sinensis*). *BMC Genet.* **2012**, *13*, 10.
37. Liu, C.; He, M.; Wang, Z.; Xu, J. Integrative analysis of terpenoid profiles and hormones from fruits of red-flesh citrus mutants and their wild types. *Molecules* **2019**, *24*, 3456.
38. Zacarías-García, J.; Cronje, P.; Diretto, G.; Zacarias, L.; Rodrigo, M.J. A comprehensive analysis of carotenoids metabolism in two red-fleshed mutants of Navel and Valencia oranges (*Citrus sinensis*). *Front. Plant Sci.* **2022**, *accepted*.
39. Barry, G.H.; Caruso, M.; Gmiter, F.G. Commercial Scion Varieties. In The Genus *Citrus*; Talón, M., Caruso, M., Gmitter, F., Eds.; Woodhead Publishing: Cambridge, UK, 2020; pp. 83–104.
40. Pérez-Través, L.; Lopes, C.A. González, R.; Barrio, E.; Querol, A. Physiological and genomic characterisation of Saccharomyces cerevisiae hybrids with improved fermentation performance and mannoprotein release capacity. *Int. J. Food Microbiol*. **2015**, *205*, 30–40.
41. Alòs, E.; Rodrigo, M.J.; Zacarías, L. Differential transcriptional regulation of L-ascorbic acid content in peel and pulp of citrus fruits during development and maturation. *Planta* **2014**, *239*, 1113–1128.
42. Singleton, V.L.; Rossi, J.A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144−158.
43. Lafuente, M.T.; Alférez, F.; Romero, P. Postharvest ethylene conditioning as a tool to reduce quality loss of stored mature sweet oranges. *Postharvest Biol. Technol.* **2014**, *94*, 104–111.
44. Rey, F.; Zacarías, L.; Rodrigo, M.J. Carotenoids, vitamin c, and antioxidant capacity in the peel of mandarin fruit in relation to the susceptibility to chilling injury during postharvest cold storage. *Antioxidants* **2020**, *9*, 1296.
45. Benzie, I.F.F.; Strain, J.J. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of 'Antioxidant Power': The FRAP Assay. *Anal. Biochem.* **1996**, *239*, 70–76.
46. Legua, P.; Hernández, F.; Díaz-Mula, H.M.; Valero, D. Serrano, M. Quality, bioactive compounds, and antioxidant activity of new flat-type peach and nectarine cultivars: A Comparative Study. *J. Food Sci.* **2011**, *76*, 729–736.
47. Bain, J.M. Morphological anatomical and physiological changes in the developing fruit of the Valencia orange, *Citrus sinensis* L. Osbeck. *Aust. J. Bot.* **1968**, *6*, 1–23.
48. Lado, J.; Rodrigo, M.J.; Zacarías, L. Maturity indicators and citrus fruit quality. *Stewart Postharvest Rev.* **2014**, *10*, 1–6.

49. Ladaniya, M.S. *Citrus Fruit: Biology, Technology and Evaluation*; Elsevier Inc.: Atlanta, GA, USA, 2008.
50. Bermejo, A.; Cano, A. Analysis of nutritional constituents in twenty citrus cultivars from the mediterranean area at different stages of ripening. *Food Sci. Nutr.* **2012**, *03*, 639–650.
51. Guo, L.-X.; Shi, C.-Y.; Liu, X.; Ning, D.-Y.; Jing, L.-F.; Yang, H.; Liu, Y.Z. Citrate accumulation-related gene expression and/or enzyme activity analysis combined with metabolomics provide a novel insight for an orange mutant. *Sci. Rep.* **2016**, *6*, 29343.
52. Hussain, S.B.; Shi, C.-Y.; Guo, L.-X.; Kamran, H.M.; Sadka, A.; Liu, Y-Z. Recent advances in the regulation of citric acid metabolism in citrus fruit. *Crit. Rev. Plant Sci.* **2017**, *36*, 241–256.
53. Rey, F.; Rodrigo, M.J.; Diretto, G.; Zacarías, L. Effect of fruit shading and cold storage on tocopherol biosynthesis and its involvement in the susceptibility of Star Ruby grapefruit to chilling injury. *Food Chem.* **2021**, *3*, 100037.
54. Rey, F.; Rodrigo, M.J.; Zacarias, L. Regulation of tocopherol biosynthesis during fruit maturation of different Citrus species. *Front. Plant Sci.* **2021**, *12*, 743993.
55. Dong, X.; Hu, Y.; Li, Y.; Zhou, Z. The maturity degree, phenolic compounds and antioxidant activity of Eureka lemon [*Citrus limon* (L.) Burm. f.]: A negative correlation between total phenolic content, antioxidant capacity and soluble solid content. *Sci. Hortic.* **2019**, *243*, 281–289.
56. De Ancos, B.; Rodrigo, M.J.; Sánchez-Moreno, C.; Cano, M.P.; Zacarías, L. Effect of high-pressure processing applied as pretreatment on carotenoids, flavonoids and vitamin C in juice of the sweet oranges 'Navel' and the red-fleshed 'Cara Cara'. *Food Res Int.* **2020**, *132*, 109105.
57. Gironés-Vilaplana, A.; Moreno, D.A.; García-Viguera, C. Phytochemistry and biological activity of Spanish Citrus fruits. *Food Funct.* **2014**, *5*, 764–772.
58. Rodríguez-Amaya, D.B. Quantitative analysis in vitro assessment of bioavailability and antioxidant activity of food carotenoids—A review. *J. Food Compos. Anal.* **2010**, *23*, 726–740.
59. Yoo, K.M.; Lee, K.W.; Park, J.B.; Lee, H.J.; Hwang, I.K. Variation in major antioxidants and total antioxidant activity of Yuzu (Citrus Junos Sieb ex Tanaka) during maturation and between cultivars. *J. Agric. Food Chem*. **2004**, *52*, 5907–5913.
60. Huang, R.; Xia, R.; Hu, L.; Lu, H.; Wang, M. Antioxidant activity and oxygen-scavenging system in orange pulp during fruit ripening and maturation. *Sci. Hortic.* **2007**, *113*, 166–172.
61. Sánchez-Moreno, C.; Plaza, L.; De Ancos, B.; Cano, P. Quantitative bioactive compounds assessment and their relative contribution to the antioxidant capacity of commercial orange juices. *J. Sci. Food Agric.* **2003**, *83*, 430–439.
62. Long, X.; Zeng, X.; Yan, H.; Xu, M.; Zeng, Q.; Xu, C.; Xu, Q.; Liang, T.; Zhang, J. Flavonoids composition and antioxidant potential assessment of extracts from Gannanzao Navel Orange (*Citrus sinensis* Osbeck Cv. Gannanzao) peel. *Nat. Prod. Res.* **2021**, *35*, 702–706.
63. Gulo, K.N.; Suhartomi, S.; Saragih, A.D.; Raif, M.A.; Ikhtiari, R. Antioxidant Activity of flavonoid compounds in ethanol and ethyl acetate extract from Citrus sinensis. In Proceedings of the 2021 International Conference on Artificial Intelligence and Mechatronics Systems (AIMS), Jakarta, Indonesia, 28–30 April 2021; pp. 1–6.

64. Di Mascio, P.; Kaiser, S.; Sies, H. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. *Arch. Biochem. Biophys.* **1989**, *274*, 532–538.
65. Martínez, A.; Stinco, C.M.; Meléndez-Martínez, A.J. Free radical scavenging properties of phytofluene and phytoene isomers as compared to lycopene: A combined experimental and theoretical study. *J. Phys. Chem.* **2014**, *118*, 9819–9825.
66. Arnao, M.B.; Cano, A.; Acosta, M. The hydrophilic and lipophilic contribution to total antioxidant activity. *Food Chem.* **2001**, *73*, 239–244.
67. Liu, D.; Shi, J.; Ibarra, A.C.; Kakuda, Y.; Xue, S.J. The scavenging capacity and synergistic effects of lycopene, vitamin E, vitamin C, and b-carotene mixtures on the DPPH free radical. *LWT* **2008**, *41*, 1344–1349.
68. Chen, X.; Deng, Z.; Zheng, L.; Zhang, B.; Luo, T.; Li, H. Interaction between flavonoids and carotenoids on ameliorating oxidative stress and cellular uptake in different cells. *Foods* **2021**, *10*, 3096.
69. Ouchi, A.; Aizawa, K.; Iwasaki, Y.; Inakuma, T.; Terao, J.; Nagaoka, S.I.; Mukai, K. Kinetic study of the quenching reaction of singlet oxygen by carotenoids and food extracts in solution. Development of a singlet oxygen absorption capacity (SOAC) assay method. *J. Agric. Food Chem.* **2010**, *58*, 9967–9978.
70. Stahl, W.; Sies, H. Antioxidant activity of carotenoids. *Mol. Asp. Med.* **2003**, *24*, 345–351.

3.3. CAPÍTULO 3

Antioxidant capacity in fruit of Citrus cultivars with marked differences in pulp coloration: contribution of carotenoids and vitamin C

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Abstract

The purpose of this study was to evaluate the specific contribution of carotenoids and vitamin C to the lipophilic and hydrophilic antioxidant capacity (LAC and HAC), respectively, of the pulp of citrus fruits using the genetic diversity in pigmentation and in the carotenoid complement. To this end six citrus varieties were selected: two mandarins, Clemenules (*Citrus clementina*) and Nadorcott (*Citrus reticulata*); two grapefruits (*Citrus paradisi*), Marsh and Star Ruby; and two sweet oranges (*Citrus sinensis*), Valencia late and Valencia Ruby. Total carotenoid content and composition in the pulp of fruits were very different, in relation to their color singularities. Valencia Ruby and Nadorcott had the highest carotenoid content, accumulating the former large amounts of linear carotenes (phytoene, phytofluene and lycopene) and Nadorcott of β-cryptoxanthin. Orange fruits contained the highest amount of vitamin C while in Nadorcott mandarin was more that 50% lower. Analysis of antioxidant capacity, evaluated by ABTS and DPPH assays, in the pulp of the different fruit varieties indicated a high and positive correlation between vitamin C content and hydrophilic antioxidant capacity (HAC). Nevertheless, a weak correlation was observed between carotenoids content and lipophilic antioxidant capacity (LAC) in the pulp extracts assayed by ABTS. Overall, vitamin C in the pulp of citrus fruit had an important contribution to the HAC, whereas that of carotenoids to LAC was very variable; being the highest that of Valencia Ruby orange, with large concentrations of lycopene and phytoene, followed by Nadorcott mandarin, with high β-cryptoxanthin content.

Keywords: Antioxidant capacity, Carotenoids, Citrus fruit, Vitamin C

1. Introduction

Citrus fruit are one of the primary fruit crops in international trade in terms of value, and are highly demanded worldwide for fresh consumption, juice processing, freeze-concentrates and also as food additives for dishes and beverages (Talón et al., 2020). Color is one of the most remarkable features among the different Citrus species and cultivars and a key factor of external and internal quality and consumer's acceptance. The genus Citrus shows a high diversity in external and internal fruit coloration: from the yellow color of lemons (*Citrus limon*), pummelos (*Citrus maxima*), many grapefruits cultivars (*Citrus paradisi*) and citrons (*Citrus medica*), to the orange of mandarins (*Citrus reticulata*, *C. clementina* or C. *unshiu*) and sweet oranges (*Citrus sinensis*), and red or pink in some lycopene-accumulating grapefruits or new sweet orange cultivars (Liu et al., 2007; Alquézar et al., 2008; Lu et al., 2017).

Citrus fruit are good sources of useful phytochemicals, such as vitamins A, C and E, mineral elements, flavonoids, phenolic acids, coumarins, limonoids, anthocyanins (blood oranges), carotenoids and pectins, among others (Lv et al., 2015; Patil et al., 2017; Zou et al., 2016). Evidence from several in vitro and in vivo studies have related the consumption of citrus fruit with important health benefits and the reduction in the risk of chronic diseases (Silveira et al., 2015; De Oliveira et al., 2019; Ma et al., 2020; Llopis et al., 2019; Jing et al., 2020). The beneficial effects of these compounds in human health have been partially associated with their antioxidant activity (Barros et al., 2018; Rajendran et al., 2014). In general, antioxidants are natural or synthetic compounds that may prevent or delay the oxidative cell damage caused by physiological oxidants, scavenging or quenching reactive oxygen (ROS) or nitrogen species (RNS), products of respiration, including free radicals (Barros et al., 2018).

Carotenoids are among the most important phytochemicals in citrus fruit. They are lipophilic pigments responsible for the coloration of mature fruit in most Citrus species and cultivars, and therefore, their content and composition have a strong impact on their commercial acceptability (Gross et al., 1987; Tadeo et al., 2020). Citrus fruit are one of the most complex sources of carotenoids with a large diversity of them among the different species and cultivars in terms of types and amounts (Kato, 2012; Rodrigo et al., 2013a). Carotenoid content and profile are influenced by genotype and, in general, mandarin and orange cultivars

accumulate larger concentrations of carotenoids than grapefruits, pummelos and lemons (Kato et al., 2004; Alquézar et al., 2008; Rodrigo et al., 2013a).

In citrus fruit, the antioxidant activity mainly depends on a complex combination of hydrophilic (e.g., ascorbic acid and polyphenols) and lipophilic compounds (e.g., carotenoids and tocopherols) (reviewed by Zou et al., 2016). In plants, carotenoids play important roles protecting biological systems against photooxidative processes and oxidative damage, as they are efficient antioxidants scavenging singlet molecular oxygen ($^1O_2$) and peroxyl radicals (Stahl and Sies, 2003; Müller et al., 2011). The efficiency of carotenoids for physical quenching is related to the number of conjugated double bonds of the molecule, being lycopene the most efficient carotenoid with eleven (Di Mascio et al., 1989; Stahl and Sies, 2003). Furthermore, the presence of ionone rings, hydroxyl, epoxy or keto functional groups, modulates the ability of carotenoids to scavenge free radicals (Di Mascio et al., 1989; Stahl., 2003; Müller et al., 2011; Apak et al., 2013). A large number of studies have analyzed over the years the antioxidant activities of citrus fruit extracts, focusing on the antioxidant assessment by a single extraction of the bioactive compounds in specific solvents, irrespectively of the selective solubility of each family of compound in the extraction mixture (Cano et al., 2004). The general conclusions emerging from several works suggest positive correlations between total antioxidant activity and vitamin C content and phenolic compounds in pulp and juice of different Citrus species and cultivars (Franke et al., 2004; Rapisarda et al., 2008; Gironés-Vilaplana et al., 2014; Yoo and Moon, 2016; Sicari et al., 2016; De Ancos et al., 2017, 2020). Despite the well-recognized antioxidant capacity of some carotenoids, their relative contribution to the total antioxidant capacity of foodstuff is still controversial (Rodríguez-Amaya, 2010). Different studies indicated that carotenoids contribute to the antioxidant capacity of Citrus (Sánchez-Moreno et al., 2003; De Ancos et al., 2020). Sánchez-Moreno et al. (2003) reported a positive correlation between β-cryptoxanthin content and the antioxidant capacity of orange juices but a lack of correlation with other carotenoids. However, other works have not found a relationship between carotenoid content and the antioxidant capacity (De Ancos et al., 2002), or lower than that of phenolic compounds or vitamin C (Gardner et al., 2000; Sánchez-Moreno et al., 2003; Cano et al., 2004; Yoo & Moon, 2016). It seems that the extraction solvents and the antioxidant assay may be critical factors for the discrepancy among the different studies (Sánchez-Moreno et al. 2003; Rodríguez-Amaya et al., 2010; Müller et al., 2011). Moreover, the evaluation of the

antioxidant capacity of both the water-soluble and lipid-soluble fractions of citrus fruit extracts has been scarcely studied (Cano et al., 2004).

The aim of this study was to use the genetic diversity in coloration and carotenoid composition of citrus fruits to evaluate the antioxidant capacity of the lipophilic (LAC) and hydrophilic (HAC) fraction independently, through the scavenging DPPH and ABTS radical assays. To that end, we have used fruits of two genotypes of oranges, two grapefruits and two mandarins with contrasting differences in pulp coloration, and determined carotenoids content and composition, as well as vitamin C content (Vit C), and analyzed their contribution to the lipophilic and hydrophilic antioxidant capacity, respectively.

2. Materials and Methods

2.1. Plant material

Fruits of two genotypes of sweet orange (Citrus sinensis), Valencia late and Valencia Ruby, two grapefruits (Citrus paradisi) Marsh and Star Ruby and two mandarins Clemenules (Citrus clementina) and Nadorcott (Citrus reticulata), with contrasting differences in pulp coloration were selected (Figure 1).

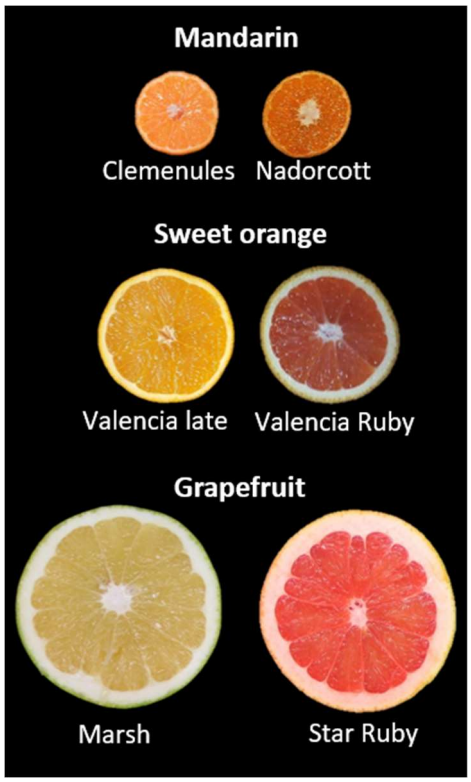

Figure 1. Internal appearance of the Navel (A), Kirkwood (B), Valencia (C) and Ruby (D) orange fruits used in this study.

Fruits of each genotype were harvested from adult trees developed under standard agronomical and growing conditions. Fruits were harvested at commercial maturity, and dates and growing locations were as follows: January and February for Clemenules and Nadorcott mandarins, respectively, from a commercial orchard located in Lliria (Valencia, Spain); both grapefruits were harvested in February at The Citrus Germplasm Bank from the Instituto Valenciano de Investigaciones Agrarias (IVIA, Moncada, Valencia, Spain), and the late-harvesting Valencia late and Valencia Ruby oranges were harvested in April from the Fundación ANECOOP (Museros, Valencia, Spain). At least 60 fruits for each genotype were harvested and immediately delivered to the laboratory. Fruits were selected for size uniformity and free of any external damage or defect. Fruits were sliced into halves and after determination of pulp color, pulp tissue was obtained by excising small cube pieces of approximately 1 cm2 containing juice vesicles free of segment membranes, immediately frozen in liquid nitrogen and stored at -80 °C until analysis. Juice was extracted from the remaining pulp with a household electric hand reamer (Citromatic MPZ22, Braun, Barcelona, Spain), filtered through a metal sieve with a pore size of 0.8 mm, immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

2.2. Color and internal maturity index determinations

Color of the pulp was measured using a CR-400 Minolta chromameter on three different positions. Hunter parameters a (negative to positive corresponds from green to red) and b (negative to positive, from blue to yellow) were determined, and color was expressed as the a/b Hunter ratio, a classical relationship for color measurement in citrus fruit (Stewart and Wheaton, 1972). Data of color index for each cultivar are the means ± SD of at least 10 fruits.

Total soluble solids (TSS) and total titratable acidity (TA) of the juices were determined using a digital refractometer PAL-BX/ACID1 (ATAGO, Japan). TSS is expressed as °BRIX and TA as mg of citric acid/100 mL of juice and maturity index (MI) was calculated as the TSS/TA ratio.

2.3. Carotenoid extraction and analysis by HPLC-DAD

Carotenoids were extracted and analyzed essentially as described by Rodrigo et al. (2015). Briefly, freeze-ground pulp (2 g) was weighed in screw-capped polypropylene tubes, 4 mL of methanol (MeOH) plus 5 mL Tris-HCl (50 mM, pH 7.5) (containing 1 M NaCl) were added

and sample was sonicated 5 min in XUBA3 ultrasonic water bath (Grant Instruments, England) at room temperature. Dichloromethane (DCM) (10 mL) was added to the mixture, stirred for 5 min at 4°C and centrifuged at 3000 g for 10 min at 4°C. The hypophase was recovered and the aqueous phase re-extracted with DCM until it was colorless. The pooled DCM extracts were dried on a rotatory evaporator at 40°C. Samples were saponified in methanolic KOH (6%, w/v) overnight. Saponified carotenoids were recovered from the upper phase after adding water and petroleum ether:diethyl ether (9:1) to the mixture. Extracts were dried and kept at −20 °C until further analysis. Each sample was extracted at least twice and results are mean ± SD.

The carotenoid composition of each sample was analyzed by HPLC with a Waters liquid chromatography system equipped with a 600E pump and a photodiode array detector (DAD) model 2998, and Empower3 software (Waters, Spain). A C30 carotenoid column (250 × 4.6 mm, 5 μm) coupled to a C30 guard column (20 × 4.0 mm, 5 μm) (YMC, Teknokroma, Spain) was used. Chromatographic conditions used are described in Lado et al. (2015) and Rodrigo et al. (2015). The carotenoids were identified by absorbance spectra and retention time, peaks integrated at their individual maximal wavelength, and their contents were calculated using the appropriate calibration curves, as described elsewhere (Lado et al., 2015; Rodrigo et al., 2015).

2.4. Ascorbic acid determination

Ascorbic acid was extracted and determined essentially as described in Alós et al. (2014). Briefly, pulp tissue (0.5 g) were homogenized for 1 min using a Polytron PT-1035 GT (Kinematica AG) homogenizer with 0.1 % metaphosphoric acid (4 ml). The homogenate was centrifuged for 10 min at 4000 g at 4 °C. The supernatant was filtered through a C18 cartridge (SepPak, Waters, Spain), previously activated with 4 ml of MeOH, 4 ml of MilliQ water and 4 ml of 2 % metaphosphoric acid. The extract was subsequently filtered through a 0.45 μm nylon filter (25 mm diameter, Análisis Vínicos, Spain). The filtrate was directly injected in the HPLC-DAD for ascorbic acid determination. Dehydroascorbic acid (DHA) content was calculated from the difference between total vitamin C and the ascorbic acid contents. To determine total vitamin C, we adapted the protocol described by Alós et al. (2014). Thus, a 200 μl aliquot of the above-mentioned filtrate was incubated for 15 min at room temperature with 100 μl 200

mM DTT in 400 mM Tris-base. Then, the reaction was stopped by acidification with 100 µl of 8.5 % ortho-phosphoric acid.

Sample analysis by HPLC was carried out using a HPLC system Waters ACQ Arc SysCore with DAD and Empower 3 software, using an Ultrabase C18 column (100 × 4.6 mm, 2.5 µm) (Análisis Vínicos, Spain) and a mobile phase of MeOH:water pH 2.5 (adjusted with metaphosphoric acid, 15:85, v/v), 0.2 ml min-1 flow and injection volume 10 µl. The temperature of the column was set at 35 °C.

2.5. Determination of antioxidant capacity: ABTS and DPPH assays

The 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate) (ABTS) assay was employed to determine antioxidant capacity. The method is based on the capacity of different components to scavenge the ABTS radical cation (ABTS•+) compared to a standard antioxidant (Trolox). Total antioxidant capacity (TAC) was quantified as described Legua et al. (2011) with slight modifications, which enables to determine antioxidant capacity due to both hydrophilic and lipophilic compounds in the same extraction. Briefly, for each sample, 1.5 g of pulp were homogenized with Polytron PT-1035 GT (Kinematica AG) in 12 ml of 50 mM phosphate buffer pH 7.8 and 12 ml of ethyl acetate, and then centrifuged at 4000 g for 15 min at 4°C. The upper fraction was used to determine the antioxidant activity due to lipophilic compounds (LAC) and the lower phase to hydrophilic compounds (HAC). In both cases, antioxidant capacity was determined in duplicate in each extract using the enzymatic system composed of ABTS, the horseradish peroxidase enzyme (Sigma, Madrid, Spain) and the oxidant substrate (hydrogen peroxide) in which ABTS•+ radicals are generated. For HAC, a reaction mixture containing 10 mM ABTS, H2O2 (30%) and 10 mM peroxidase in 120 mM glycine buffer solution (pH 4.5) in a total volume of 285 µl. For LAC, the same reaction mixture in pure ethanol was used, in a total volume of 250 µl. Then, 15 µl of the aqueous phase and 50 µl of the organic phase were added to the reaction medium for HAC and LAC determination, respectively, and the decrease in absorbance which is proportional to the ABTS quenched, was determined after 5 min. The absorbance change of the mixture was determined in a UV/Vis microplate spectrophotometer (Fluostar Omega, BMG Labtech) monitored at 730 nm. A calibration curve was performed with Trolox ((R)-(+)-6-hydroxy-2,5,7,8-tetramethyl-croman-2-carboxylic acid) in the range from 1 to 25 mmol (Sigma, Madrid, Spain), and results are

expressed as Trolox equivalent antioxidant capacity (TEAC) per fresh weight of fruit pulp (mg 100 g-1).

The hydrophilic antioxidant activity was also determined using DPPH free radical assay (2,2-diphenyl-1-picrylhydrazyl) as described by Girennavar et al. (2007), with slight modifications. Samples of 0.3 g of pulp were homogenized for 1 min using a Polytron PT-1035 GT (Kinematica AG) homogenizer in 6 ml of MeOH:water (80:20, v/v). The extract was centrifuged for 10 min at 4000 g at 4 °C and the supernatant collected into a new a tube. In a 96-well plate 10 μl of each sample was mixed with 290 μl of 100 mM DPPH solution (in 80% MeOH) and incubated at room temperature in darkness for 30 min. Thereafter, the absorbance change of the mixture was determined in a UV/Vis microplate spectrophotometer (Multiskan FC, Thermo Scientific) at 517 nm. Methanol (80%) was used as a control (10 μl MeOH 80% + 290 μl of DPPH) and each reaction was replicated in three wells. The assay was replicated twice and a curve of Trolox solution with concentrations ranging from 25 to 200 μg ml-1 was used as a standard. Absorbance was measured at 515 nm and compared to the absorbance obtained for the control (blank without samples). DPPH scavenging capacity was expressed as inhibition percentages by the formula:

% DPPH scavenging capacity = [(515nmAcontrol – 515nmAsample)/ 515nmAcontrol] x 100

2.6. Contribution of vitamin C and carotenoids to antioxidant capacity

Müller et al. (2011) determined the relative antioxidant capacity (RAC) of several bioactive compounds as equivalents of Trolox. Taking these values as a reference, we have determined the contribution of the carotenoids and vitamin C present in the lipophilic and hydrophilic extracts of the citrus samples to the antioxidant capacity. The RAC value of the carotenoids were: lycopene, 3.9; β-cryptoxanthin, 3.2; β-carotene, 3.1; anteraxanthin, 2; zeaxanthin, 1.9; violaxanthin, 1.6; and phytoene and phytofluene, 1. The RAC of Vitamin C is 1.0, which means that Trolox and vitamin C have the same antioxidant activity (Arnao et al., 2001; Cano et al., 2000). Thus, after determination of the HAC and LAC (as Trolox equivalents) and the concentration of the different compounds, their relative contribution to the antioxidant capacity was calculated.

2.7. Statistical analysis

Statistical significance was calculated by one-way analysis of variance (ANOVA) by XLSTAT software and the Tukey's test was used to determine any significant difference among cultivars at $p < 0.05$.

3. Results and Discussion

3.1. Pulp color and internal quality parameters

The quality parameters (color of the pulp, TSS, TA and maturity index) of the fruit of the six citrus genotypes selected for this study are shown in Table 1. Color of the pulp (determined as the a/b Hunter ratio) and differences between cultivars of the same species were the main criteria for the selection of these genotypes. Accordingly, for mandarin fruit, Nadorcott displayed a bright orange coloration (a/b, 0.50±0.01) more intense than the pale orange pigmentation of Clemenules (0.34±0.02). Nadorcott is a mandarin derived from the traditional Murcott mandarin in Morocco, characterized by a late harvest, excellent internal quality and the intense orange coloration of both peel and pulp (Nadori, 2004). By contrast, despite the excellent fruit quality and commercial relevance of Clemenules mandarin, its internal and external coloration is less intense than other mandarin cultivars (Barry et al., 2020). TSS was slightly higher in Nadorcott than in Clementine mandarin, indicating a late ripening, but the maturity index at the harvest time was similar in both genotypes (Table 1).

Table 1. Color index (a/b Hunter ratio) of the pulp, and total soluble solids (TSS, º Brix), total acidity (TA, mg citric acid 100 ml^{-1}) and maturity index (TSS/TA) of the juice of fruit of the six Citrus genotypes used in this study.

Cultivar	Pulp color	TSS	TA	IM
Clemenules	0.34±0.02d	9.83±0.06d	0.53±0.01d	18.59±0.92a
Nadorcott	0.50±0.01c	10.90±0.20c	0.70±0.20d	15.57±0.50a
Marsh	0.01±0.03f	11.60±0.01b	1.88±0.01b	6.19±0.60c
Star Ruby	1.04±.013a	12.27±0.06a	2.24±0.02a	5.48±0.40c
Valencia late	0.06±0.01e	11.50±0.10b	1.00±0.10c	11.50±0.90b
Valencia Ruby	0.60±0.03b	10.90±0.10c	1.10±0.20c	9.90±1.10b

M.I: maturity index.

Data are expressed as mean ± standard deviation (n=3). Different letters in the same column indicate significant differences ($p<0.05$) by Tukey's test.

These two grapefruit varieties are very common and cultivated worldwide, and differences in pulp coloration have been long described, despite the important effect of the climatic conditions in grapefruit pigmentation (Xu et al., 2006; Alquézar et al., 2013; Barry el at., 2020). Valencia Ruby is the first red-fleshed mutant derived from the Valencia late sweet orange (Zacarias, 2017). This phenotypic difference is well exemplified by the reddish (0.60±0.03) coloration of the pulp in comparison with the pale-orange tint (0.06±0.01) of the Valencia late orange fruit harvested at that same maturity stage and environmental conditions. Valencia late is a classical orange cultivar cultivated worldwide with moderate to low coloration and carotenoid content (Fanciullino et al., 2008; Kato, 2012). The characteristic pulp pigmentation of the Valencia Ruby mutant resembles that of the red-fleshed Cara Cara, a mutant of Navel orange more intensively studied, with an altered carotenoid complement (Xu et al., 2008; Alquézar et al., 2008). However, parameters of internal maturation were statistically similar in both Valencia and Valencia Ruby oranges, indicating that other ripening processes in the mutant are not affected (Table 1).

3.3. Carotenoid content and composition

Differences in pulp coloration in fruits of the six citrus genotypes selected were assessed in relation to their carotenoid content and composition. By HPLC-DAD ten major carotenoids were identified and quantified in our samples. Total carotenoid content in the pulp showed a high variability among the six genotypes (Table 2). Then, carotenoid content in the pulp of Nadorcott was 3.2-times higher than that of Clemenules. Interestingly, carotenoids were substantially higher in the red-fleshed grapefruit and orange than in the corresponding counterpart. In the white Marsh grapefruit, carotenoids were extremely low (< 0.1 mg 100 g-1 FW) in comparison with the red Star Ruby. Similarly, total carotenoids in the pulp of Valencia Ruby was near 20-times higher than in the pulp of Valencia late (Table 2).

A detailed analysis of the carotenoid complement in the pulp of the six varieties revealed significant differences that may account for the particular coloration of each genotype (Table 2). Thus, the pulp of Clemenules contained moderate amounts (between 0.25 and 0.31 mg 100 g-1 FW) of phytoene, phytofluene, β-cryptoxanthin and violaxanthin. It is remarkable that the pulp of Nadorcott mandarin contained 6- and 4-times more β-cryptoxanthin and violaxanthin, respectively, than that of Clemenules, whereas other carotenoids remained nearly similar.

Table 2: Carotenoid content and composition (mg 100 g^{-1} FW, fresh weight) in the pulp of six Citrus cultivars. Data are expressed as mean ± SD.

Carotenoid (mg 100 g^{-1} FW)	Mandarins		Grapefruits		Oranges	
	CM	ND	MS	ST	VL	VR
Phytoene	0.26±0.03	0.27±0.14	0.03±0.01	0.81±0.02	0.10±0.01	13.31±0.11
Phytofluene	0.31±0.27	0.28±0.13	0.01±0.01	0.32±0.01	0.02±0.01	1.84±0.36
ζ-Carotene	0.07±0.01	0.26±0.13	nd	0.03±0.01	0.03±0.01	0.06±0.01
Lycopene	nd	nd	nd	0.74±0.08	nd	0.86±0.02
β-Carotene	tr	0.24±0.12	nd	0.57±0.02	nd	0.04±0.01
β-Cryptoxanthin	0.31 ±0.03	1.83±0.50	nd	0.01±0.01	0.06±0.02	0.03±0.01
Zeaxanthin	0.04±0.01	0.09±0.02	nd	0.03±0.03	0.07±0.02	0.06±0.01
Anteraxanthin	0.10±0.01	0.32±0.02	0.01±0.01	0.02±0.02	0.14±0.01	0.11±0.03
Luteoxanthin	0.02±0.01	0.11±0.03	nd	nd	0.06±0.02	0.02±0.01
Violaxanthin	0.25±0.01	1.00±0.04	0.01±0.01	nd	0.38±0.02	0.23±0.01
Total carotenoids[α]	1.44±0.26d	4.69±1.06b	0.06±0.01f	2.60±0.15c	0.86±0.03e	16.51±0.28a

Tr, traces; nd, no detected.

CM, Clemenules mandarin; ND, Nadorcott mandarin; MS, Marsh grapefruit; SR, Star Ruby grapefruit; VL, Valencia late orange; VR, Ruby Valencia orange.

Data are expressed as mean ± SD. For total carotenoids, different letters indicate significant differences ($p<0.05$) by Student's t test.

[a]Total carotenoids are the sum of the main identified and quantified carotenoids.

A detailed analysis of the carotenoid complement in the pulp of the six varieties revealed significant differences that may account for the particular coloration of each genotype (Table 2). Thus, the pulp of Clemenules contained moderate amounts (between 0.25 and 0.31 mg 100 g-1 FW) of phytoene, phytofluene, β-cryptoxanthin and violaxanthin. It is remarkable that the pulp of Nadorcott mandarin contained 6- and 4-times more β-cryptoxanthin and violaxanthin, respectively, than that of Clemenules, whereas other carotenoids remained nearly similar. These major differences in these two xanthophylls (especially β-cryptoxanthin) may explain the deeper orange color observed in the pulp of Nadorcott compared with Clemenules (Table 1). β-cryptoxanthin is the main carotenoid in the pulp and juice of mandarins, providing their intense orange coloration and being one of the main dietary sources of this compound (Ma et al., 2020). The higher accumulation of deep orange carotenoids, such as β-cryptoxanthin, is a distinctive feature of mandarin fruits and it has been proposed as a criterion to distinguish from sweet orange fruits (Goodner et al., 2001; Kato et al., 2004; Fanciullino et al., 2006). It is remarkable the high carotenoid and β-cryptoxanthin content detected in the pulp of Nadorcott mandarin. This mandarin is currently appreciated in the fresh-fruit market by the intense

orange coloration of both peel and pulp, but a detailed analysis of its carotenoid content and composition has not been reported yet. In this study, we found that carotenoid content was in the range reported for other mandarin hybrids, such as its parental Murcott (Alquézar et al., 2008; Petry et al., 2019). Moreover, the content of β-cryptoxanthin in Nadorcott pulp account for about 40% of total carotenoids, while in Clemenules is 21% (Table 2), therefore, the high content and proportion of β-cryptoxanthin in Nadorcott may explain the intense orange coloration of its pulp.

Star Ruby was selected in this study as a pigmented grapefruit by the distinctive red coloration of the flesh due to the large accumulation of lycopene compared with other red-pigmented grapefruit cultivars (Xu et al. 2006; Alquézar et al., 2013). Analysis of carotenoids profile in the pulp of the Star Ruby revealed that the carotenes phytoene (31% of total carotenoids), lycopene (28.5%), β-carotene (22%) and phytofluene (12%) were the most abundant. By contrast, the pulp of the white Marsh was almost devoid of carotenoids, with only minor amounts of phytoene, phytofluene and xanthophylls (Table 2). These results are in good agreement with those reported in other studies for the same grapefruit varieties (Xu et al., 2006; Fanciullino et al., 2008, Alquézar et al. 2008, 2013) and corroborate the motion that the usual accumulation of lycopene in red grapefruits appears to be the result of a partial blockage in the cyclization of lycopene leading an accumulation of earlier linear carotenes (Alquézar et al., 2008).

Comparison of carotenoid profiling in the pulp of Valencia late orange and its red-fleshed Valencia Ruby mutant revealed a completely altered carotenoid content and composition (Table 2). First, total carotenoids were 20-times higher in Valencia Ruby than in Valencia late; second, Valencia late accumulated almost exclusively the β,β-xantophylls violaxanthin (44.2%), anteraxanthin (16.3) and β-cryptoxanthin (7%); and third, Valencia Ruby accumulated large amounts of linear carotenes phytoene (80.6%), phytofuene (6%) and lycopene (5.2%), whereas total xanthophylls were less than 3% of total carotenoids (Table 2). This unusual carotenoid profiling in the pulp of Valencia Ruby and the accumulation of lycopene resembles that reported for the two other red-fleshed oranges, Hong Anliu and Cara Cara, so far characterized (Liu et al., 2007; Alquézar et al., 2008; Rodrigo et al., 2015; Lu et a., 2017). It is interesting to remark that the concentration of lycopene reported in the pulp of mature fruit of these mutants is about 0.5 to 1.0 mg 100 g-1 FW and in the same range of that found in the

present study for Valencia Ruby (Xu et al., 2006; Alquézar et al., 2008; Rodrigo et al., 2015). However, total carotenoids in Valencia Ruby were substantial higher, mainly due to the accumulation of the early linear carotenes phytoene and phytofluene that reached concentrations extremely high (13.31 and 1.84 mg 100 g-1 FW, respectively) (Table 2). To our knowledge, this is the first report of the analysis of carotenoids in a red-fleshed mutant of Valencia orange and, it is worth to remark that such large amount of phytoene in its flesh is the highest so far reported for a citrus fruit (Alquézar et al., 2008; Rodrigo et al., 2003, 2019). Since accumulation of significant amounts of phytoene is not common in fruits of other species (Alquézar et al., 2008) and that important health-related benefits have been assigned to this carotene (Rao and Rao et al., 2007; Meléndez-Martínez et al., 2018, 2019), Valencia Ruby is an useful and promising orange cultivar in order to provide fresh fruit or derived products with added value and potential health benefits.

3.3. Vitamin C content

Vitamin C (Vit C) is one of the most important antioxidant compounds in plant cells and a major contributor to the health-related benefits attributed to citrus fruit (Alós et al., 2014, Zou et al., 2016). The concentration of Vit C in the pulp of the six cultivars selected for the current study were between 19.04 and 45.29 mg 100 g-1 FW (Figure 2). Valencia Ruby and Valencia late sweet oranges, and Marsh grapefruit showed the highest Vit C content followed by Star Ruby grapefruit and Clemenules mandarin. Nadorcott mandarin was the cultivar with the lowest vitamin C concentration (45% lower than Clemenules mandarin) (Figure 2). These results are in agreement and in the same range than that reported by other authors in different Citrus species and varieties (Cano et al., 2008; Martí et al., 2009; Alos et al., 2014; Kafkas et al., 2011). Conclusions from these studies indicated that Vit C concentration in the fruit appear to be related to the genotype and/or to the stage of development (Alós et al., 2014). Moreover, oranges contain the highest Vit C concentrations among the Citrus genus, followed by lemons, grapefruits and mandarins, with a higher variability among mandarins than in other species (Martí et al., 2009). Interestingly, comparison of Vit C content in several mandarin varieties indicated that Murcott (Cano et al., 2008) and Nadorcott mandarin (Nardello et al., 2018) contained about half of that of other Clementine mandarins, similarly to the differences obtained in the current work (Figure 2). It is important to note that other studies have detected between 39% and 15% reduction in Vit C concentration in the juice of the lycopene-

accumulating sweet orange Cara Cara compared to its parental Navel juice (De Ancos et al., 2020; Kafkas et al., 2011). However, the Vit C content in the red-fleshed Valencia Ruby is similar to Valencia and are the highest values among the genotypes analyzed in this study.

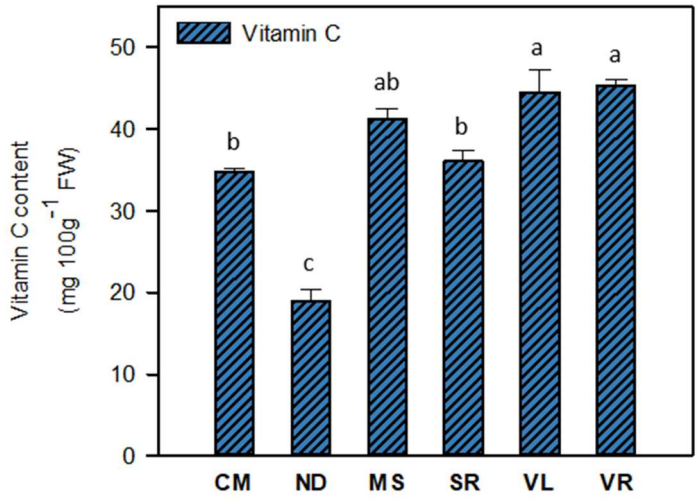

Figure 2. Vitamin C concentration (mg 100 g^{-1} fresh weight, FW) in pulp of mature fruits of six Citrus cultivars; CM, Clemenules mandarin; ND, Nadorcott mandarin; MS, Marsh grapefruit; SR, Star Ruby grapefruit; VL, Valencia late orange; VR, Ruby Valencia orange. Data are expressed as mean ± SD (n=3). Different letters indicate significant differences ($p<0.05$) by Tukey's test.

3.4. Antioxidant capacity in hydrophilic and lipophilic pulp extracts of citrus cultivars

In order to evaluate the contribution of carotenoids and Vit C content to the antioxidant capacity of citrus fruit, the antioxidant capacity of the pulp of the six citrus varieties was analyzed. The antioxidant capacity of the hydrophilic (HAC) fraction was evaluated by two methods, DPPH and ABTS scavenging assays, while the antioxidant capacity of lipophilic (LAC) fraction was determined by ABTS. The HAC of extracts showed a significant variability among cultivars (Figure 3). By the characteristics of each assay, values of the HAC obtained by ABTS were quantitatively higher than those of DPPH, but results of the different varieties were consistent between both assays. Comparison of HAC between cultivars of the same species revealed that Nadorcott and Star Ruby had lower capacity than Clemenules mandarin and Marsh grapefruit, respectively, and in sweet oranges only a minor reduction in HAC in Valencia late pulp with respect to Valencia Ruby was detected by the ABTS assay (Figure 3).

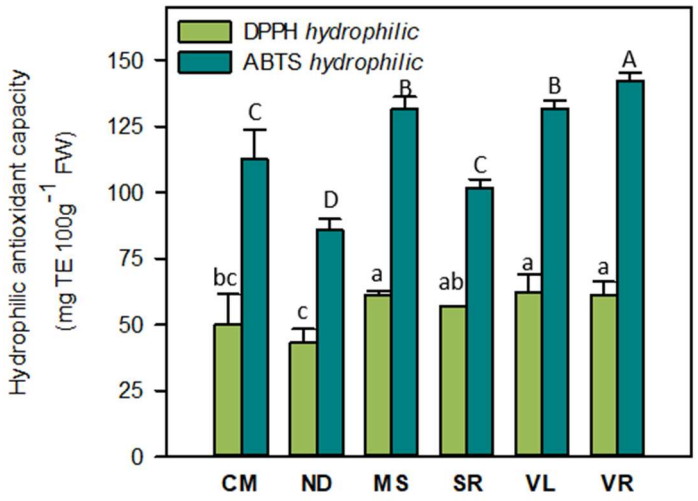

Figure 3. Antioxidant capacity of the hydrophilic fraction, assayed by DPPH and ABTS (mg of Trolox equivalents 100 g^{-1} FW) in the pulp of fruits of six Citrus cultivars. CM, Clemenules mandarin; ND, Nadorcott mandarin; MS, Marsh grapefruit; SR, Star Ruby grapefruit; VL, Valencia late orange; VR, Ruby Valencia orange. Small and capital letters indicate significant differences ($p<0.05$) for DPPH and ABTS, respectively, by Tukey's test.

Evaluation of antioxidant capacity in different food matrix are usually performed by several methods, such as ABTS, DPPH, FRAP (Ferric Reducing Antioxidant Power), and ORAC (Oxygen Radical Absorbance Capacity) (Müller et al., 2011; Apak et al., 2013, 2016). The ABTS assay has the advantage of being used over a wide range of pH, hence, it is more useful to study and compare foods at different pH. Moreover, the ABTS assay requires a shorter reaction time compared to the DPPH assay. In addition, ABTS is soluble in polar and non-polar solvents and can be therefore used to assess both water- and lipid-soluble antioxidants (Yoo and Moon, 2016). The scavenging ability of the different bioactive compounds varies widely by the mode of action of each radical (Gironés-Vilaplana et al., 2014), but, in general, the values of antioxidant capacity obtained by ABTS use to be higher than those by DPPH.

Analysis of the HAC in the pulp of the six citrus varieties analyzed revealed a parallelism with the profiling of Vit C content (Figure 2). The correlation analysis between HAC and Vit C for the six citrus varieties studied indicates a high correlation with r^2 values of 0.85 and 0.91 for the DPPH and ABTS assays, respectively. These results point out that in the pulp of mandarins, grapefruits and sweet oranges the concentration of Vit C is closely related to their hydrophilic antioxidant activity. Our results highly support previous observations indicating that Vit C is a major contributor to the total antioxidant capacity in fruits and juices of many

Citrus species and varieties (Sicari et al., 2016; Yoo and Moon, 2016; Elkhatim et al., 2018; Sánchez-Moreno et al., 2002, 2003; Martí et al., 2009).

By contrast, a virtual absence of correlation between the hydrophilic activity by both DPPH and ABTS assays, and carotenoid content in the six varieties was obtained (r^2= 0.01, r^2= 0.13, respectively). These results are not unexpected, since the extraction for the HAC determination was performed with polar solvents and only a minor fraction, if any, of the carotenoids present in the tissue would be extracted. Guddadarangavvanahally et al. (2008) have also discussed the influence of the extraction solvents in the analysis of the antioxidant capacity of grapefruit and oranges. Then, special attention should be taken in the interpretation of the potential contribution of carotenoids to the antioxidant capacity of foods or plant tissues extracts.

In order to explore more in detail, the involvement of the different carotenoid content and composition in the antioxidant activity of the pulp, we independently extracted and determined the scavenging capacity of the lipophilic fraction by ABTS assay. The lipophilic extracts of the pulp of both sweet orange cultivars showed the highest LAC (Figure 4). Comparison of LAC in grapefruit and mandarin varieties revealed that the red Star Ruby had higher capacity than Marsh, however, no significant differences were detected between Nadorcott and Clemenules (Figure 4). Together, the antioxidant capacity of the analyzed citrus pulp lipophilic extracts showed a weak positive correlation (r^2=0.40) with total carotenoids content. Moreover, we did not observe either significant correlation between individual carotenoids and LAC (data not shown). Carotenoids are fat-soluble compounds and have potential antioxidant properties because they quench singlet oxygen (Stahl and Sies, 2003). Nevertheless, the contribution of carotenoids to the antioxidant capacity of citrus fruit is still a matter of debate, since controversial results have been obtained (Arnao et al., 2001; Sánchez-Moreno et al., 2003; De Ancos et al., 2002, 2020). Despite the antioxidant activity in the lipophilic fraction has received little attention (Cano et al., 2000, 2004; Arnao et al., 2001; Rodríguez-Amaya et al., 2010), our results indicate that other recognized antioxidant compounds (such as tocopherols), in addition to carotenoids, may also significantly contribute to the LAC of the pulp of Citrus fruit (Rodríguez-Amaya et al., 2010; Arnao et al., 2001; Sánchez-Moreno et al., 2003).

Comparison of HAC and LAC assayed by ABTS indicated that the former was 5- to 10-times higher than LAC (Figure 3 and 4). This result is similar to that reported in other studies and reinforces the conclusion that hydrophilic compounds are the major contributors to the total antioxidant capacity of citrus fruit pulp (Arnao et al., 2001; Sánchez-Moreno et al., 2003; Cano et al., 2004; Wu et al., 2009; Yoo and Moon, 2016).

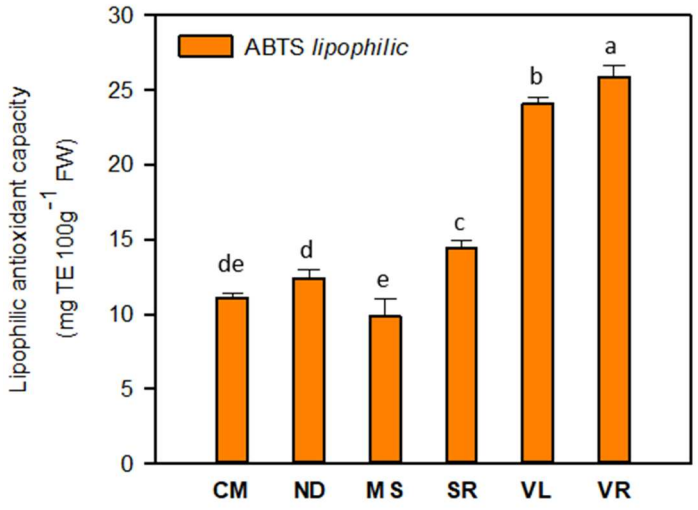

Figure 4. Antioxidant capacity of the lipophilic fraction determined by ABTS (mg of Trolox equivalents 100 g-1 FW) in the pulp of fruits of six Citrus cultivars. CM, Clemenules mandarin; ND, Nadorcott mandarin; MS, Marsh grapefruit; SR, Star Ruby grapefruit; VL, Valencia late orange; VR, Ruby Valencia orange Different letters indicate significantly differences ($p<0.05$) by Tukey's test.

3.5. Analysis of the potential contribution of vitamin C and carotenoids to total antioxidant capacity

Total antioxidant capacity in a food matrix is the combined activity of different antioxidants compounds such as carotenoids, vitamin C, polyphenols, tocopherols and others (Patil et al., 2017). One of the main goals of this study was to evaluate the specific contribution of carotenoids and Vit C to the lipophilic and hydrophilic antioxidant capacity, respectively, using the genetic diversity in pulp pigmentation and in the carotenoid complement found in Citrus species and cultivars. Carotenoid analysis in the pulp of the six varieties selected in this study revealed important differences in the total content but also an enrichment in specific carotenoids: lycopene in Star Ruby or Valencia Ruby, phytoene in Valencia Ruby, or β-cryptoxanthin in Nadorcott mandarin (Table 2). To estimate the contribution of carotenoids and Vit C of each variety to the LAC and HAC, respectively, we used the relative antioxidant capacity value of pure carotenoids and vitamin C described by Müller et al. (2011). Then, total

antioxidant capacity is calculated as the sum of the relative contribution of each specific carotenoid or xanthophyll (expressed as % of the total) present in the pulp of the citrus fruit. This approach showed that Vit C contributed to the HAC between 18% in Nadorcott mandarin and 35% in Valencia orange (Figure 5A). These values indicate that other components (such as phenolic compounds) are greater contributors than Vit C to the HAC of the pulp of citrus fruit. Similar results have been also obtained in fruits of different citrus cultivars (Yoo and Moon, 2016; Shin, 2012 and Sicari et al., 2016), but other studies estimated that Vit C is the predominant antioxidant in Citrus (Shin, 2012; Xu et al., 2008; Yoo et al., 2004, Sánchez-Moreno et al., 2003).

On the other hand, the relative contribution of carotenoids to LAC showed a wide variability among genotypes (Figure 5B). As expected, the cultivars with high total carotenoids content exhibited high contribution to LAC: Valencia Ruby orange and Nadorcott mandarin (77%) followed by Star Ruby (41%), Clemenules (20%) and Valencia late (5%), while that of Marsh grapefruit was almost negligible (Figure 5B). However, this sequence did not follow the carotenoid content of each variety (Table 2). This discrepancy indicates that other lipophilic compounds (as tocopherols) are likely contributing to the LAC and that synergistic effects among them may modify total antioxidant activity (Rodríguez-Amaya, 2010).

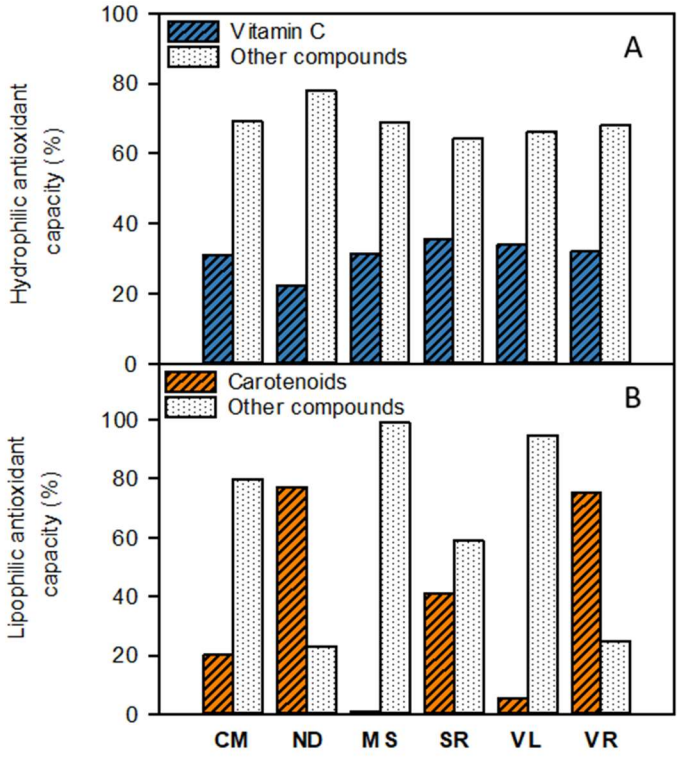

Figure 5. Percentage of the contribution of vitamin C and other compounds to the total hydrophilic antioxidant capacity (A); and of total carotenoids and other compounds to the total lipophilic antioxidant capacity (B) in the pulp of fruits of six Citrus cultivars. CM, Clemenules mandarin; ND, Nadorcott mandarin; MS, Marsh grapefruit; SR, Star Ruby grapefruit; VL, Valencia late orange; VR, Ruby Valencia orange

It is worth to mention that the varieties with higher contribution of carotenoids to LAC are Valencia Ruby sweet orange, which contains lycopene and also an extremely high concentration of phytoene, both carotenes have been postulated to have antioxidant properties (Bailey et al., 2015; Meléndez-Martínez et al., 2018); and the mandarin Nadorcott, with the largest content of β-cryptoxanthin, a xanthophyll with demonstrated protection against oxidative stress by using an *in vivo* assay (Llopis et al., 2019). Then, it is likely that the contribution of carotenoids to LAC is not only dependent of their total amount but also of the concentration of specific carotenoids, and thus, our results may explain the lack of consistency between carotenoid content and the antioxidant capacity observed in different studies in citrus fruit (reviewed by Zou et al., 2016).

In conclusion, in vitro antioxidant assays may be useful indicators to estimate the healthy properties of the edible part of fruits. However, the lack of standardization, the particular

features of the different methods and the ability of compounds to scavenge/quench different radicals, may generate discrepancies and over- and underestimate the specific contribution of the different hydro and lipophilic components of a food sample (Rodríguez-Amaya et al., 2010; Apak et al., 2013; 2016). In this work, we have used two antioxidant assays to determine HAC and LAC in the pulp of six citrus varieties with contrasting coloration and carotenoid content and composition. Results indicated a positive correlation between Vit C content and HAC, while total carotenoids did not parallel LAC. The contribution of Vit C was estimate to be 15-30% of the total HAC. Carotenoids had a very variable contribution to LAC, being the highest that of Valencia Ruby orange, with large concentrations of lycopene and phytoene, and Nadorcott mandarin, with high β-cryptoxanthin content.

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References

Alós E, Rodrigo MJ, and Zacarias L (2014) Differential transcriptional regulation of L-ascorbic acid content in peel and pulp of citrus fruits during development and maturation. Planta 239: 1113–1128.

Alquézar B, Rodrigo MJ and Zacarías L (2008) Carotenoid biosynthesis and their regulation in citrus fruits. Tree for Science Biotechnology 2: 23-35.

Alquézar B, Rodrigo MJ, Lado J and Zacarías L (2013) A comparative physiological and transcriptional study of carotenoid biosynthesis in white and red grapefruit (Citrus paradisi Macf). Tree Genetics and Genomes 9: 1257–1269.

Apak R, Gorinstein S, Böhm V, Schaich KM, Özyürek M and Güçlü K (2013) Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report). Pure and Applied Chemistry 85(5): 957–998.

Apak R, Oʻzyuʻrek M, Guʻcl̨uʻ K and Cap̨anogʻlu E (2016) Antioxidant Activity/Capacity Measurement 1 Classification Physicochemical Principles Mechanisms and Electron Transfer (ET)-Based Assays. Journal of Agricultural and Food Chemistry 64: 997−1027.

Arnao MB, Cano A and Acosta M (2001) The hydrophilic and lipophilic contribution to total antioxidant activity. Food Chemistry 73: 239-244.

Bailey JR (2015) Lycopene: Food sources potential role in human health and antioxidant effects New York: Nova Publishers.

Barros M, Rodrigo MJ and Zacarias L (2018) Dietary Carotenoid Roles in Redox Homeostasis and Human Health. Journal of Agricultural and Food Chemistry 66: 5733-5740.

Barry GH, Caruso M, Gmitter FG (2020) Commercial scion varieties. In: Talón M, Caruso M and Gmitter FG (eds) The Genus Citrus. Cambridge: Woodhead Publishing, pp. 495-508.

Cano A and Arnao MB (2004) Lipophilic and hydrophilic antioxidant activity and vitamin C content of commercial orange juices: correlation with organoleptic parameters. Ciencia y Tecnolología Alimentaria 4(3): 185-189.

Cano A, Acosta M and Arnao M B (2000) A method to measure antioxidant activity in organic media: application to lipophilic vitamins. Redox Report 5(6): 365-370.

Cano A, Medina A and Bermejo A (2008) Bioactive compounds in different citrus varieties. Discrimination among cultivars. Journal of Food Composition and Analysis 21: 377-381.

De Ancos B, Cilla A, Barberá R, Sánchez-Moreno C and Cano M P (2017) Influence of orange cultivar and mandarin postharvest storage on polyphenols ascorbic acid and antioxidant activity during gastrointestinal digestion. Food Chemistry 225: 114–124.

De Ancos B, Rodrigo MJ, Sánchez-Moreno C, Cano P and Zacarías L (2020) Effect of high-pressure processing applied as pretreatment on carotenoids flavonoids and vitamin C in juice of the sweet oranges 'Navel' and the red-fleshed 'Cara Cara'. Food Research International 132: 109105.

De Ancos B, Sgroppo S, Plaza L and Cano MP (2002) Possible nutritional and health related value promotion in orange juice preserved by high-pressure treatment. Journal of the Science of Food and Agriculture 82: 790–796.

De Oliveira R, Orlando Muñoz C, Carmona L, Peña L and de Paula Oliveira R (2019) Pasteurized Orange Juice Rich in Carotenoids Protects Caenorhabditis elegans against Oxidative Stress and β-Amyloid Toxicity through Direct and Indirect Mechanisms. Oxidative Medicine and Cellular Longevity 5046280.

Di Mascio P, Kaiser S and Sies H (1989) Lycopene as the most efficient biological carotenoid singlet oxygen quencher. Archives of Biochemistry and Biophysics 274: 532–538.

Elkhatim K, Elagib R, Amro B and Hassan A (2018) Content of phenolic compounds and vitamin C and antioxidant activity in wasted parts of Sudanese citrus fruits. Food Science and Nutrition 6: 1214–1219.

Fanciullino A, Asanova JOC, Orillon RAM, Llitrault PAO (2006). Carotenoid diversity in cultivated citrus is highly influenced by genetic factors. Journal of Agricultural and Food Chemistry 54: 4397–406.

Fanciullino AL, Cercos M, Dhuique-Mayer C, Froelicher Y, Talón M, Ollitrault P and Morillon R (2008) Changes in carotenoid content and biosynthetic gene expression in juice sacs of four orange varieties (Citrus sinensis) differing in flesh fruit color. Journal of Agricultural and Food Chemistry 56: 3628–3638.

Franke SIR, Ckless K, Silveira JD, Rubensam G, Brendel M and Erdtmann B (2004) Study of antioxidant and mutagenic activity of different orange juices. Food Chemistry 88(1): 45–55.

Gardner PT, White TAC, McPhail DB and Duthie GG (2000) The relative contributions of vitamin C, carotenoids and phenolics to the antioxidant potential of fruit juices. Food Chemistry 68: 471–474.

Girennavar B, Jayaprakasha GK, Jadegoud Y, Nagana Gouda GAS and Patil B (2007) Radical scavenging and cytochrome P450 3A4 inhibitory activity of bergaptol and geranylcoumarin from grapefruit. Bioorganic & Medicinal Chemistry 15: 3684-3691.

Gironés-Vilaplana A, Moreno DA and García-Viguera C (2014) Phytochemistry and biological activity of Spanish Citrus fruits. Food and Function 5: 764-772.

Goodner KL, Rouseff RL and Hofsommer HJ (2001) Orange mandarin and hybrid classification using multivariate statistics based on carotenoid profiles. Journal of Agricultural and Food Chemistry 49: 1146–1150.

Gross J (1987) Pigments in fruits. International Journal of Food Science and Technology. Academic Press.

Guddadarangavvanahally KJ, Girennavar B and Patil S (2008). Antioxidant capacity of pummelo and navel oranges: Extraction efficiency of solvents in sequence. Swiss Society of Food Science and Technology 41: 376–384.

Jing WJ, Deng N, Wang H, Li T, Chen L, Zheng B and Liu RH (2020) Effects of orange extracts on longevity, healthspan, and stress resistance in Caenorhabditis elegans. Molecules 25(2): 351.

Kafkas E, Polatöz SN and Koç K (2011) Quantification and comparison of sugars carboxylic acids and vitamin c components of various citrus species by HPLC techniques. Journal of Agricultural Science and Technology 5(2): 175-180.

Kato M (2012) Mechanism of carotenoids accumulation in Citrus fruit. Japanese Society of Horticultural Science 81(3): 219–233.

Kato M, Ikoma Y, Matsumoto H, Sugiura M, Hyodo H and Yano M (2004) Accumulation of carotenoids and expression of carotenoid biosynthetic genes during maturation in citrus fruit. Plant Physiology 134: 824–837.

Lado J, Cronje P, Alquézar B, Page A, Mazif M, Gómez-Cadenas A, Steag AD, Rdrigo MJ and Zacarias L (2015) Fruit shading enhances peel color, carotenes accumulation and chromoplast differentiation in red grapefruit. Physiologia Plantarum 154: 469–484.

Legua P, Hernández F, Díaz-Mula HM, Valero D and Serrano M (2011) Quality, bioactive compounds, and antioxidant activity of new flat-type peach and nectarine cultivars: A Comparative Study. Journal of Food Science 76(5): 729-736.

Liu K, Juan X, Liu Y and Zhao X (2007) A novel bud mutation that confers abnormal patterns of lycopene accumulation in sweet orange fruit (Citrus sinensis L Osbeck). Journal of Experimental Botany 58(15): 4161–4171.

Llopis S, Rodrigo M, González N, Genovés S, Zacarías L, Ramón D and Martorell P (2019) β-cryptoxanthin reduces body fat and increases oxidative stress response in Caenorhabditis elegans model. Nutrients 11(2): 232.

Lu P, Wang C, Yin T, Zhong S, Grierson D, Chen K and Xu C (2017) Cytological and molecular characterization of carotenoid accumulation in normal and high-lycopene mutant oranges. Scientific reports 7: 761.

Lv X, Zhao S, Ning Z, Zeng H, Shu Y, Tao O, Xiao C, Lu C and Liu Y (2015) Citrus fruits as a treasure trove of active natural metabolites that potentially provide benefits for human health. Chemistry Central Journal 9: 68.

Ma G, Zhang L, Sugiura M and Kato M (2020) Citrus and health. In: Talón M, Caruso M and Fred Gmitter FG (eds) The Genus Citrus. Cambridge: Woodhead Publishing, pp, 495-508.

Martí M, Mena P, Cánovas JA, Micol V and Saura D (2009) Vitamin C and the role of citrus juices as functional food. Natural Product Communications 4(5) 678-700.

Meléndez-Martínez AJ, Stinco CM and Mapelli-Brahm P (2019) Skin carotenoids in public health and nutricosmetics: the emerging roles and applications of the UV radiation-absorbing colourless carotenoids phytoene and phytofluene. Nutrients 11: 1093.

Meléndez-Martínez, AJ, Mapelli-Brahm P and Stinco CM (2018) The colourless carotenoids phytoene and phytofluene: from dietary sources to their usefulness for the functional foods and nutricosmetics industries. Journal of Food Composition and Analysis 68: 91–103.

Müller L, Fröhlich K and Böhm V (2011) Comparative antioxidant activities of carotenoids measured by ferric reducing antioxidant power (FRAP) ABTS bleaching assay (αTEAC) DPPH assay and peroxyl radical scavenging assay. Food Chemistry 129: 139-148.

Nadori E (2004) Nadorcott Mandarin: a promising new variety. Proceedings of the International Society of Citriculture 1: 353-359.

Nardello IC, Cantillano RFF, Seifert M and Mello-Farias M (2018) postharvest quality during refrigerated storage of 'Nadorcott' mandarin. American Journal of Experimental Agriculture 9: 1-10.

Patil BS, Jayaprakasha GK and Murthy KNC (2017) Beyond vitamin C: the diverse, complex health-promoting properties of citrus fruits. Citrus Research and Technology 38(1): 107:121.

Petry FC, de Nadai FB, Cristofani-Yaly M, Latado RR, Mercadante AZ (2019). Carotenoid biosynthesis and quality characteristics of new hybrids between tangor (Citrus reticulata x C. sinensis) cv. 'Murcott' and sweet orange (C. sinensis) cv. 'Pêra'. Food Research International 122: 461–70.

Rajendran P, Nandakumar N, Rengarajan T, Palaniswami R. Gnanadhas E and NLakshminarasaiah U (2014) Antioxidants and human diseases. Clinica Chimica Acta 436: 332–347.

Rao A and Rao LG (2007) Carotenoids and human health. Pharmacological Research 55: 207–216.

Rapisarda P, Lo Bianco M, Pannuzzo P and Timpanaro N (2008) Effect of cold storage on vitamin C phenolics and antioxidant activity of five orange genotypes (Citrus sinensis (L) Osbeck). Postharvest Biology and Technology 49: 348–354.

Rodrigo MJ, Alquézar B, Alós E, Lado J and Zacarias L (2013a) Biochemical bases and molecular regulation of pigmentation in the peel of Citrus fruit. Scientia Horticulturae 163: 46–62.

Rodrigo MJ, Cilla A, Barberá R and Zacarías L (2015) Carotenoid bioaccessibility in pulp and fresh juice from carotenoid-rich sweet oranges and mandarins. Food and function 6: 1950-1959.

Rodrigo MJ, Lado J, Alòs E, Alquézar B, Dery O, Hirschberg J and Zacarias L (2019) A mutant allele of ζ-carotene isomerase (Z-ISO) is associated with the yellow pigmentation of the "Pinalate" sweet orange mutant and reveals new insights into its role in fruit carotenogenesis. BMC Plant Biology 19: 465.

Rodrigo MJ, Marcos JF, Alférez F, Mallent MD and Zacarías L (2003) Characterization of Pinalate a novel Citrus sinensis mutant with a fruit-specific alteration that results in yellow pigmentation and decreased ABA content. Journal of Experimental Botany 54: 727–738.

Rodríguez-Amaya DB (2010) Quantitative analysis in vitro assessment of bioavailability and antioxidant activity of food carotenoids—A review. Journal of Food Composition and Analysis 23: 726–740.

Sánchez-Moreno C (2002) Review: Methods used to evaluate the free radicals scavenging activity in foods and biological systems. Food Science and Technology International 8: 121–137.

Sánchez-Moreno C, Plaza L, De Ancos B and Cano P (2003) Quantitative bioactive compounds assessment and their relative contribution to the antioxidant capacity of commercial orange juices. Journal of the Science of Food and Agriculture 83: 430–439.

Shin Y (2012) Correlation between antioxidant concentrations and activities of Yuja (Citrus junos Sieb ex Tanaka) and other citrus fruit. Food Science and Biotechnology 21(5): 1477-1482.

Sicari V, Pellicano TM, Giuffrè AM, Zappia C and Capocasale M (2016) Bioactive compounds and antioxidant activity of citrus juices produce from varieties cultivated in Calabria. Food Measure 10: 773-780.

Silveira JQ, Grace KZS, Cesar D and Cesar TB (2015) Red-fleshed sweet orange juice improves the risk factors for metabolic syndrome. International Journal of Food Sciences and Nutrition 66(7): 830-836.

Stahl W and Sies H (2003) Antioxidant activity of carotenoids. Molecular Aspects of Medicine 24: 345–351.

Stewart I and Wheaton TA (1972). Carotenoids in citrus: their accumulation induced by ethylene. Journal of Agricultural and Food Chemistry 20: 448–449.

Tadeo FR, Terol J, Rodrigo MJ, Licciardelo C and Sadka A (2020) Fruit growth and development. In: Talón M, Caruso M and Fred Gmitter FG (eds) The Genus Citrus. Cambridge: Woodhead Publishing, pp, 495-508.

Talón M, Caruso M and Gmitter F (2020) The Genus Citrus. Cambridge: Woodhead Publishing.

Wu G, Peng MJ, and Peng S (2009) Free radical scavenging effects of extracts from various parts of Citrus. Food Science 23: 008.

Xu C, Fraser P, Wang WJ and Bramley PM (2006) Differences in the carotenoid content ordinary citrus and lycopene-accumulating mutants. Journal of Agricultural and Food Chemistry 54: 5474-548.

Xu G, Liu D, Chen J, Ye X, Maa Y and Shi J (2008) Juice components and antioxidant capacity of citrus varieties cultivated in China. Food Chemistry 106: 545–551.

Yoo KM and Moon B (2016) Comparative carotenoid compositions during maturation and their antioxidative capacities of three citrus varieties. Food Chemistry 196: 544-549.

Yoo KM, Lee KW, Park JB, Lee HJ and Hwang IK (2004) Variation in major antioxidants and total antioxidant activity of Yuzu (Citrus Junos Sieb ex Tanaka) during maturation and between cultivars. Journal of Agricultural and Food Chemistry 52: 5907–5913.

Zacarias J (2017). Caracterización fisiológica y molecular del metabolismo de carotenoides en dos variedades de naranja (Citrus sinensis) de pulpa roja. Máster Universitario en Calidad y Seguridad Alimentaria, 8ª Edición. Departamento de Medicina Preventiva i Salut Pública, Ciències de l'Alimentació, Toxicologia i Medicina Legal, Universidad de Valencia, España.

Zou Z, Xi W, Hu Y, Nie C and Zhiquin Z (2016) Antioxidant activity of Citrus fruits. Food Chemistry 196: 885-896.

3.4. CAPÍTULO 4

Physiological Responses, Nutritional Quality and Aroma Volatiles of the Red-fleshed Kirkwood Navel and Ruby Valencia Oranges during Postharvest Cold Storage.

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Postharvest Biology and Technology

(En revisión)

Abstract

This study evaluates the postharvest performance and responses of the red-fleshed Kirkwood Navel and Ruby Valencia oranges to cold storage (28 d at 2 °C) and shelf-life (5 d at 20 °C) in comparison with the standard varieties Washington Navel and Valencia late, respectively. Fruit of Kirkwood and Ruby exhibited lower chilling injury than the reference varieties while maintained similar weight loss and peel firmness. Moreover, the large concentration and the unusual composition of carotenoids in the pulp of Kirkwood and Ruby was preserved during the postharvest storage. Similarly, the content of vitamin C, phenolics and flavonoids, and the higher lipophilic antioxidant capacity and the capacity to quench singlet oxygen of the red-fleshed oranges remained unaltered after the whole storage period. The content of other compounds as tocopherols and sugars increased at the end of the storage period in fruit of the red-fleshed varieties. Monoterpenes, norisoprenoids, ethanol, aldehydes and ethyl esters were the compounds that exhibited the major changes during the postharvest storage, especially in the red-fleshed varieties. The profile of volatile compounds of Kirkwood and Ruby fruit was characterized by higher levels of norisoprenoids, monoterpenes, long-chain aldehydes, and variable content of aliphatic esters compared to the reference varieties. Altogether, our results indicated that Kirkwood Navel and Ruby Valencia oranges show good postharvest performance and attitude for long-term cold storage, ensuring their quality and retaining their enhanced properties and add value to consumers.

Keywords: red-fleshed oranges; postharvest storage; fruit quality; carotenoids; antioxidants; volatiles

1. Introduction

Citrus is the first fruit-tree crop in the world, with a total production of more than 158 Million Tm in the season 2021-2022, disseminated in more than 100 countries over the world. Destination of this large production is very uneven, since in Brazil, the 2nd world producer of citrus fruit, dedicates 70 % of the total production to the processing industry. Contrastingly, in other countries like Spain, the 6th world producer, more than 80 % is destined to the fresh-fruit markets. Moreover, Spain is the world leader in exportation of fresh citrus fruit, and around 40 % of the total production of oranges and 61 % of mandarins are destined to the exportation (FAOSTAT, 2022). Then, the external and internal quality of the fruit, absence of defects and an attractive appearance are essential features to ensure marketability and consumers acceptance.

In a scenario of increasing competitiveness and globalized market, the citrus industry is searching for new varieties that may extend the marketing period, differentiating and diversifying the offer and importantly, providing fruit with enhanced nutritional value and additional health-related properties. One of the promising alternatives is the red-fleshed sweet oranges varieties accumulating lycopene in the pulp, as an alternative to the blood-oranges with anthocyanin. Cara Cara and Hong-Anliu are two red-fleshed spontaneous mutants from the original Washington Navel and Anliu oranges, respectively, that have been intensively studied. Mature fruit of both genotypes accumulate lycopene in the pulp, but the pattern of carotenoids accumulation is differently between them, suggesting specific metabolic mechanism leading to lycopene accumulation (Liu et al., 2007; Alquézar et al., 2008; Lu et al., 2017). Recently, we have reported the characterization of two novel red-fleshed orange varieties, named Kirkwood Navel (K) and Ruby Valencia (V), with an altered carotenoid content and composition. Fruit of both mutants displayed some common features: 1) higher content of total carotenoids, 2) accumulation of lycopene in the pulp from early stages of fruit development, 2) accumulation of large amounts of the colourless carotenes phytoene and phytofluene and 3) reduced levels of downstream xanthophylls and ABA (Zacarías-García et al., 2022a). Moreover, internal quality parameters and the content and composition of several bioactive compounds (other than carotenoids) seem to be similar in the red oranges K and R as compared to those of the standards varieties. The pulp of these oranges displayed higher

lipophilic antioxidant capacity and specifically, higher singlet oxygen antioxidant capacity (SOAC) than the corresponding standard oranges, which might enhance the functional properties of these new varieties (Zacarías-García et al., 2022b). Then, K and R varieties, which belong to the Navel and Valencia group, respectively, offer promising opportunities to the citrus industry, since Navel are mainly destined to the fresh-fruit market while those of Valencia are usually employed for juice processing. Nevertheless, the effect of postharvest cold storage and shelf life on the maintenance of quality and the health-promoting properties of these red-fleshed oranges has not yet been evaluated, and it would be of special interest to assess their potential for the entire Citrus market chain.

Low temperature is the most effective system of postharvest storage of citrus fruit, since reduces the respiration rate and water loss, pathogens infection, and maintains the quality of the fruit (Zacarías et al., 2020). Moreover, quarantine treatment at temperature between 1-2 °C is a phytosanitary requirement for disinfestation of the Mediterranean fruit fly for the exportation of citrus fruit to several countries, as USA, Japan or China (Zacarías et al., 2020). However, fruit of many citrus species and varieties are sensitive to develop chilling injury when stored below of a critical threshold temperature (Lado et al., 2019a). In orange fruit, chilling injury symptoms initiate as a superficial scalding and bronzing that after prolonged low temperature exposure developed as dark pits and depressed areas affecting a large extension of the fruit peel. The incidence and intensity of these symptoms are very variable, depending on the variety, rootstock, environmental conditions, and the temperature and extension of the storage period (Lado et al., 2019a).

Besides the impact on physiological disorders, storage temperature highly influences fruit quality, and therefore, the content and composition of relevant bioactive compounds during postharvest cold storage (Lado et al., 2018; Strano et al., 2022). In particular, carotenoids content in the pulp are differentially affected by the storage temperature in citrus fruit. In Navel oranges, the content of β-criptoxanthin, antheraxanthin and violaxanthin experienced a higher increase at 12 °C than a 2 °C (Carmona et al., 2012). In the pulp of Satsuma mandarin, cold storage stimulated carotenoids content and the expression of carotenoids biosynthesis genes up to 14 d and declined thereafter (Matsumoto et al., 2009). Similarly, fruit of the red-fleshed orange Cara Cara stored at 2 °C increased the carotenoid content in the pulp during the first week of storage and declined after prolonged periods (Tao et al., 2012).

Citrus fruit are an excellent source of bioactive compounds among which vitamin C and flavonoids, in addition to carotenoids, are the most relevant and recognized phytochemicals for their potential health benefits (Lv et al., 2015; Saini et al., 2022). However, the content of these compounds may be differentially affected by cold storage, depending on the variety, maturity, and storage conditions (Baltazari et al., 2020]. For instance, the content of flavonoids increased during cold storage in blood oranges (Habibi et al., 2021), declined in the red Star Ruby grapefruit (Chaudhary et a., 2016) but remained virtually unchanged in mandarins (Lafuente et al., 2011). The influence of storage temperature on vitamin C content of citrus fruit has also been extensively examined (Mditshwaa et al., 2017). It seems that, in general, vitamin C content is more stable in oranges than in mandarins subjected to low temperatures. Vitamin C levels in Satsuma mandarin were higher in fruit stored at 4 °C than at 20 °C, but decreased after prolonged storage (Mditshwaa et al., 2017). Nonetheless, in Valencia oranges and several blood oranges varieties, vitamin C remained nearly unchanged after more than 2 moth at 6 °C (Rapisarda et al., 2008).

Volatile organic compounds (VOCs) are crucial components determining the flavor and aroma of citrus fruit and then, have a strong impact on their organoleptic quality and consumer acceptance (Pérez-Cacho and Rouseff, 2008; González-Mas et al., 2019). Different studies have evaluated the influence of cold storage on volatile compounds in the pulp/juice of citrus fruit, revealing that low temperatures influence diversely the VOCs profile, depending on the citrus variety and duration of the storage conditions. It has been described an increase of alcohols and esters, and a reduction of aldehydes, monoterpenes and sesquiterpenes in fruit of mandarin stored a 6 °C (Goldenberg et al., 2016). Furthermore, Obenland et al. (2013) reported a marked increase of esters and reduction of terpenes in mandarins stored 14 d at 20 °C after 28 d at 5 °C. Since monoterpenes are the most abundant volatiles in citrus fruit, it was proposed a correlation between the reduction of monoterpenes levels and the development of off-flavors during cold storage (Tietel et al., 2011). Moreover, it has been suggested that the losses in flavor quality in fruit of different mandarin varieties may be prevented by maintaining the fruit at cold temperature (5–10 °C) following packing and until the time of consumption (Obenland et al., 2013). Other study in Navel oranges determined an increment in the emission of some esters, such as ethyl butanoate, methyl and ethyl hexanoate and ethyl octanoate, suggesting that these compounds may be indicators of

freeze-damage (Obenland et al., 2003) and the main responsible of the flavour induced by packing (Obenland et al., 2008). A comparative metabolomic analysis of terpenoids in the red-fleshed Hong Anliu and the standard Anliu orange revealed significant differences in the volatiles profile in freshly-harvested fruit, with increasing levels of total volatiles and monoterpenes, but lower content of sesquiterpenes in the red orange. Nevertheless, the potential relationship between the volatiles composition and the alteration in the carotenoids content has not been established (Liu et al., 2019).

This objective of this study was to evaluate the postharvest performance of the novel red-fleshed oranges Kirkwood Navel and Ruby Valencia, in comparison with the standard orange varieties Navel and Valencia, respectively, during cold storage. To that end, we investigated the physiological responses of the fruit, and the changes in the content of relevant bioactive compounds, antioxidant capacity and aroma volatiles in fruit stored 28 d at 2 °C and subsequent shelf-life for 5 d at 20 °C.

2. Materials and methods

2.1. Plant material and storage conditions

Fruit used in this study were harvested from trees of the two blond sweet oranges (*Citrus sinensis* L. Osbeck), Washington Navel (cv. Foios, referred as Navel or N) and Valencia late (cv. Midknight, referred as Valencia or V) and the two red-fleshed genotypes, Kirkwood Navel (K) and Ruby Valencia (R), grafted onto Citrange Carrizo rootstock (Citrus sinensis x Poncirus trifoliata) and planted in experimental orchards of the Foundation ANECOOP (Museros, Valencia, Spain; 39°34'10.8"N 0°21'28.8"W). Trees of both standard varieties were located in adjacent rows to the red-fleshed varieties and then, all varieties were grown under identical environmental conditions and agronomical practices. About 150-200 fruit of each genotype were picked up around the external canopy of the trees at their corresponding commercial maturity: Navel and Kirkwood were harvested during the second week of January and those of Valencia and Ruby in mid-April. Fruit were delivered to the laboratory, inspected for colour uniformity and free of any defect or damage, and randomly separated into two lots and stored at 2 °C and 85 % RH (relative humidity) for up to 28 d (cold storage, 28 d 2 °C). After that period, fruit were transferred to 20 °C and 90 % RH for additional 5 d to simulate shelf-life (SL, +5 d 20 °C) conditions. The first lot of fruit was used for the analysis of fruit firmness and to

collect samples of flavedo (external coloured layer of the peel) and pulp (juice vesicles) tissue, while the second lot, compromising 3 replicates of 10 fruit each one, was used for evaluation of weight loss (WL) and chilling injury (CI). Samples of flavedo and pulp of fruit were taken at harvest, upon cold storage and cold storage + SL, and immediately frozen in liquid nitrogen. Samples were stored at -80 °C until analysis. Juice was extracted from the remaining pulp of the fruit with a domestic electric squeezer (Citromatic MPZ22, Braun, Barcelona, Spain), filtered through a metal sieve (0.8 mm pore size) and stored at −20 °C until analysis.

2.2. Evaluation of chilling injury, fruit weight loss, firmness and color

Fruit from each variety were weekly inspected for CI symptoms during cold storage and SL. Fruit were scored according to the extension and severity of the peel damage on the following scale: 0 (no CI), 1 (low CI, < 25 % peel surface), 2 (high CI, 25-50 % peel surface) and 3 (severe CI > 50 % peel surface). Results were expressed as CI index, which was calculated by adding the product of the number of fruit in each category multiplied by the score for each category and dividing it by the number of total fruit evaluated (Rey et al., 2020). Accumulative percentage of fruit weight loss (WL) was determined by weighing individual fruit before storage and at weekly intervals during cold storage and after SL. The CI and WL were evaluated in 3 replicate samples of 10 fruit each. Fruit firmness was measured with a texture analyzer Model TA-XT2 (Stable Micro Systems, UK) by compression of 10 % of the equatorial area. Approximately 10 to 15 fruit of each variety were used for each determination and results are reported as N. Fruit external and internal color was determined by a Minolta CR-400 colorimeter (Minolta, Osaka, Japan) and expressed as the a/b Hunter ratio.

2.3. Internal maturity

Maturity index (MI) was calculated as the ratio between total soluble solids (TSS)/ titratable acidity (TA) of the juice. TSS and TA of the juices were determined using a digital refractometer PAL-BX/ACID1 (ATAGO, Japan) as described by Zacarías-García et al. (2020b).

2.4. Carotenoids analysis by HPLC-DAD

Carotenoids were extracted and analyzed in the pulps of the four varieties at harvest, cold storage and SL following the protocol described by Zacarías-García et al. (2022a). The carotenoids content and composition was analyzed using a Waters liquid chromatography system (HPLC) equipped with a 600E pump and a photodiode array detector (DAD) model

2998, and Empower3 software (Waters, Spain). A C30 carotenoid column (250 × 4.6 mm, 5 μm) coupled to a C30 guard column (20 × 4.0 mm, 5 μm) (YMC, Teknokroma, Spain) was used. Chromatographic conditions used are described in Zacarías-García et al. (2022a). The carotenoids were identified by absorbance spectra and retention time, peaks integrated at their individual maximal wavelength, and their contents were calculated using the appropriate calibration curves, as described elsewhere Zacarías-García et al. (2022a). Results are expressed as mg kg-1 of fresh weight. Samples were extracted twice, and the results are the mean of two independent replicates (mean ± SD).

2.5. Sugars and organic acids analysis by HPLC-RI-DAD

Sugars and organic acids were determined in extracts of the pulp of the four varieties as previously described Zacarías-García et al. (2022b). Concentration of glucose, fructose, citric acid, malic acid, quinic acid and succinic acid was determined by HPLC (Thermo Fisher Scientific, Waltham, MA) and equipped with a refraction index (RI) and diode array detector (DAD). A HyperREZTM XP Carbohydrate H+ 8μm column (Thermo Fisher Scientific) was used protected by a HyperREZTM XP Carbohydrate (Thermo Fisher Scientific) guard column. For sucrose determination, a Pb column was used (Hi-Plex Pb, 300 x 7.7 mm, Agilent Technologies (Santa Clara, CA, USA) Chromatographic conditions used are described in Zacarías-García et al. (2022b). Results are expressed as g kg-1 of fresh weight. Samples were extracted twice, and the results are the mean of two independent replicates (mean ± SD).

2.6. Vitamin C and tocopherols analysis by HPLC

Ascorbic acid and tocopherols in the pulp of fruit of the four varieties were extracted and determined as described by (Zacarías-García et al., 2022b). Vitamin C content was determined using a HPLC system (Acquity® Arc™, Waters, Barcelona, Spain) with DAD (Acquity® Arc™, Waters, Barcelona, Spain) and Ultrabase C18 column (100 × 4.6 mm, 2.5 μm) (Análisis Vínicos, Spain). Vitamin C is expressed as mg kg-1 of fresh weight. Tocopherols content was determined using a HPLC coupled with a fluorescence detector (Acquity® Arc™, Waters, Barcelona, Spain). Separation of tocopherols was carried out with a YMC C30 column (150 × 4.6 mm, 3 μm) (Teknokroma, Barcelona, Spain). Identification and quantification of the different tocopherols was achieved by comparison with the retention times and peak areas of authentic standards of δ-, γ- and α-tocopherol (Sigma-Aldrich, Barcelona, Spain). Tocopherol

content is expressed ad mg kg-1 of fresh weight. For both type of compounds, samples were extracted twice, and the results are the mean of two independent replicates (mean ± SD).

2.7. Determination of total phenolic

Total phenolics were extracted and determined in extracts of the pulp of the four orange varieties by the Folin-Ciocalteu assay as described Zacarías-García et al. (2022b). The absorbance of each extract was measured at 724 nm in a UV/Vis microplate spectrophotometer (Multiskan FC, Thermo Scientific) and compared with a standard curve of gallic acid (Sigma-Aldrich, Barcelona, Spain). Results are expressed as g of Gallic Acid Equivalents (GAE) kg-1 of fresh weight. Samples were extracted twice, and the results are the mean of two independent replicates (mean ± SD).

2.8. Flavonoids analysis by HPLC-DAD

Extraction and determination of the flavonoids composition was carried out following the procedure described in Zacarías-García et al. (2022b). Flavonoids content was determined by HPLC-DAD. Separation of flavonoids was carried out with a XBridgeTM BEH C18 (4.6 x 150 mm, 2.5 μm Column XP). Identification and quantification of the different flavonoids was achieved by comparison with the retention times and peak areas of authentic standards of hesperidin, narirutin, naringin, eriocitrin, dydimin and rutin (Sigma-Aldrich, Barcelona, Spain). Results are expressed as mg kg-1 of fresh weight. Samples were extracted twice, and the results are the mean of two independent replicates (mean ± SD).

2.9. Determination of the total antioxidant capacity and singlet oxygen absorption capacity (SOAC)

The 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate) (ABTS) assay was employed to total determine hydrophilic (ABTS-H) and lipophilic antioxidant (ABTS-L) capacity in comparison to a standard antioxidant Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma, Madrid, Spain) as described Zacarías-García et al. (2022b). ABTS-H and ABTS-L of pulp extracts of the four orange varieties were measured by a UV/Vis microplate spectrophotometer (SPECTROstar® Omega, BMG Labtech, Offenburg, Germany) monitored at 730 nm. A calibration curve was performed with Trolox (Sigma, Madrid, Spain) and results are expressed as g of Trolox equivalent antioxidant capacity (TEAC) kg-1 of fresh weight. Samples were extracted twice, and the results are the mean of two independent replicates (mean ± SD).

The determination of singlet oxygen absorption capacity (SOAC) was performed following previous procedures (Rey et al., 2020) and adapted to pulp of orange fruit (Zacarías-García et al., 2022b). To measure the extracts 1O2 quenching capacity, absorbance changes of DPBF were monitored at 413 nm during a 90 min reaction at 35 °C using a UV-Vis spectrophotometer microplate reader. α-Tocopherol (Sigma–Aldrich, Barcelona, Spain) was used as a standard compound and ethanol:chloroform:water (50:50:1, v:v:v) as a blank. Results were expressed as relative SOAC values based on g L-1 unit. The relative SOAC value for each sample was calculated with the following formula (1):

(t1/2 sample − t1/2 blank) / (t1/2 α-toc − t1/2 blank) × ([α-toc, g L−1] / [sample, g L−1]) (1)

Each sample was analyzed in two independent assays and samples were run in triplicate in each plate.

2.10. Analysis of volatile compounds by HS-SPME/GC-MS

Extraction, identification and quantification of volatile compounds were performed by headspace solid-phase micro-extraction (HS-SPME)/Gas chromatography-mass spectrometry (GC-MS) according to method previously reported by González-Mas et al. (2011) with some modifications. Briefly, the sample extraction was carried out using 0.5 g of frozen pulp into a 10 mL crimp cap headspace vial. Samples were mixed with 0.5 mL CaCl2 5M and 0.25 mL EDTA 0.5M pH 7.5 and incubated at 25 °C during 5 min in a water bath. Volatiles from the headspace were extracted using an SPME fiber (50/30 μm DVB/CAR/PDMS; Supelco, Bellefonte, PA, USA). The SPME fiber was put into the headspace vial, and it was exposed for 20 min at 40 °C (to avoid thermal degradation of carotenoids) under agitation, after 10 min incubation under the same conditions of temperature and agitation. Desorption was performed for 1 min at 250 °C in splitless mode. VOCs trapped on the fiber were analyzed by GC-MS using a COMBI-PAL autosampler (CTC Analytics, Zwingen, Switzerland), a 6890N GC Agilent Technologies (Santa Clara, CA, USA) and a 5975B Inert XL MSD Agilent, equipped with an DB-5MS fused silica capillary column (5 %-phenyl- 95 %-dimethylpolysiloxane as stationary phase, 60 m length, 0.25 mm i.d., and 1 mm thickness film) (Agilent J&W Scientific). Oven ramp was 40 °C for 2 min, then the temperature was increased 5 °C/min until 250 °C, then maintained at 250 °C for 5 min. Helium was used as carrier gas at 1.2 mL/min constant flow. Mass/z detection was obtained by an Agilent mass spectrometer operating in the EI

mode (ionization energy, 70 eV; source temperature 230 °C). Data acquisition was performed in scanning mode (mass range m/z 35– 250; six scans per second). Chromatograms and mass spectra were recorded using the Enhanced ChemStation E.02.02 software for GC-MS (Agilent).

Identification of compounds was performed by comparison of the mass spectra from the reference mass spectra in NIST 2005 Mass Spectral library, the Kovats Retention Index calculated on DB-5 column (Flavornet database, www.flavornet.org; Pherobase database, www.pherobase.com.) (Supplementary Table 3) and mass spectra custom library generated using available standards supplied by Sigma-Aldrich (Barcelona, Spain) and Extrasynthese (Genay, France) (González-Mas et al., 2011). For compound quantification a specific ion (m/z) was chosen for each compound based on ion specificity and the highest signal-to-noise ratio (Supplementary Table 3), and the corresponding peak area was integrated. A reference sample was prepared by mixing equal amounts of each of the samples analyzed and injected as any other sample on a daily basis. This reference was used for normalization to correct for detector variation and fiber aging along the experiment. Finally, the normalized results for each compound were expressed as the relative ratio to the average level present in Navel at harvest unless otherwise indicated.

3. Results and Discussion

In order to evaluate the performance and aptitude of the red-fleshed orange Kirkwood Navel and Ruby Valencia to postharvest cold storage, we determined their weight loss, fruit firmness and chilling injury during storage for 28 d at 2 °C following a shelf-life of 5 d at 20 °C in comparison with the standard genotypes Navel and Valencia, respectively. In addition, we evaluated the changes in internal fruit quality parameters, bioactive compounds, antioxidant capacity, and volatile compounds in the four orange genotypes at harvest, during cold storage and shelf life.

3.1. Physiological and internal quality parameters

During cold storage, the rate of weight loss was similar in N (0.4-1.7 %) and K (0.3-1.4 %) oranges but after simulation of SL, it increased markedly in both varieties, and it was slightly higher in N (5.6 %) than in K (4.5 %) oranges. In V and R oranges, only minor differences in weight loss were detected after 21 and 28 d of storage at 2 °C. After subsequent transference to 20 °C, the increase in weight loss was similar in both genotypes (3.4 %) but lower than that occurring in Navel oranges (Fig. 1A). Consistently with these results, peel firmness decreased during cold storage and remained nearly constant after SL in the four varieties. Only fruit of N after the SL period had lower fruit firmness than K fruit. In general, firmness values were slightly higher in Valencia fruit, consistent with the early maturation stages of these varieties relative to the Navel oranges (Fig. 1B).

Weight loss during postharvest storage is mainly due to transpiration by the diffusion of water from the peel surface to the environment and is one of the main factors that may produce softening and shriveling, leading to the loss of fruit quality (Ben-Yehoshua, 1987). The rate of water loss during cold storage observed in our study was rather low and in agreement with that reported for citrus fruit (Lado et al., 2018, Habibi et al., 2020a). The marked increment in water loss observed in fruit of the two Navel varieties after transfer to 20 °C may be related to their more advanced stage of maturity relative to Valencia oranges, since it has been observed that the water potential of the peel and the transpiration rate evolves progressively during fruit maturation (Zacarías and Alférez, 2014). Overall, these results show that the red-fleshed K and R oranges had a rate of water loss and fruit firmness during cold storage similar to that of the corresponding standard orange varieties.

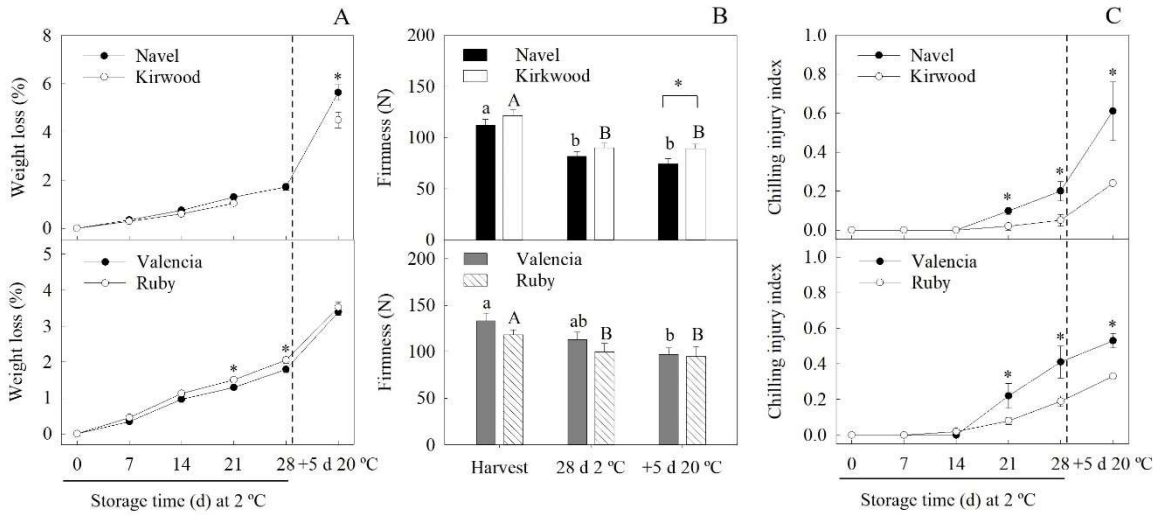

Fig. 1. Weight loss (A), firmness (B) and chilling injury index (C) at harvest, 28 d at 2 °C (cold storage) and 5 d at 20 °C (shelf-life) of Navel, Kirkwood, Valencia and Ruby orange fruit. Vertical lines separate between cold storage and shelf-life simulation period. Different lower letters indicate significant differences between each postharvest stage for the standard Navel and Valencia varieties by one-way ANOVA ($p < 0.05$). Different uppercase letters indicate significant differences between each postharvest stage for the red-fleshed Kirkwood and Ruby varieties by one-way ANOVA ($p < 0.05$). Asterisks indicate significant differences between Navel and Kirkwood; or Valencia and Ruby for each postharvest stage by t-test ($p < 0.05$).

Development of chilling-induced damage is a major physiological disorder affecting mostly the external fruit quality and therefore their marketability (Lado et al., 2019a). After 14 d at 2 °C, fruit of the four orange varieties developed incipient CI symptoms, although, they were more intense in N and V (Fig. 1C). These symptoms were manifested as superficial bronzing on the peel surface and small-depressed pits which turned brown-dark and increased in extension with the time of cold exposure. The CI index after 28 d at 2 °C was around 0.2 and 0.4 in N and V oranges, respectively. However, the red-fleshed fruit showed only minor CI index, which was <0.1 in K and 0.2 in R (Fig. 1C). Nevertheless, fruit of all varieties exhibited more chilling damage during SL, but at the end of the storage period, the fruit of the red-fleshed have less CI index than the standard oranges (Fig. 1C). Similarly, the percentage of affected fruit after cold storage was higher in the blond N (13 %) and V (29 %) than in the red-fleshed oranges (2 % and 17 % in K and R, respectively). The comparison of the incidence of CI in orange fruit among the different studies is not straightforward due to the natural variability, the different methodologies used to evaluate external damage and the storage time and conditions. Manzi et al. (2022) obtained similar results in fruit of Washington Navel and Valencia with a 5 % and 15 %, respectively, of chilling incidence after 28 d at 1 °C. Our results

indicate that K and R oranges had better response to low temperatures storage in both CI severity and incidence than the corresponding standard varieties. Previous studies have suggested a positive relationship between carotenoids content and the tolerance of mandarin fruit to CI due to their antioxidant properties (Rey et al., 2021). Carotenoid content in the peel of both K and R oranges is substantially higher and with a different complement, than that of the standard oranges (Zacarías et al., 2022a). Then, it is reasonable that the tolerance of the red-fleshed genotypes to CI during cold storage might be related to their altered carotenoid content and composition.

Analysis of total soluble solids (TSS) and total acidity (TA) of the juice in the four varieties revealed that the red-fleshed oranges maintained a high maturity index during cold storage+SL (Table 1). TSS and TA remained constant during the cold and SL period in V and R fruit, and therefore, the maturity index did not vary during the entire storage period. On the other hand, N oranges experienced a reduction in TA (28 % over the initial), a slight increase in TSS (4.4 %) and therefore, the maturity index was 27 % superior after storage (Table 1). By contrast, in K oranges, TSS increased slightly but TA and MI remained stable. Other studies also reported slow increases in TSS during cold storage but minor changes in TA (Lado et al., 2018). Therefore, it seems that the changes in TA and TSS are genotype-specific in Navel varieties, and the red-fleshed K displayed a slower maturation rate than the bold N orange during postharvest storage.

Color of citrus fruit is a key quality parameter for fresh market and processing industry (Lado et al., 2014). The a/b Hunter ratio of the peel and pulp was monitored in fruit of the four genotypes at harvest, upon cold storage and SL (Table 1). Color index of the peel at harvest was similar in the red-fleshed varieties and their blond counterparts. However and as expected, the red pigmentation of the pulp originated a significantly higher color index in K and R fruit as competed with the standard oranges (Table 1). Interestingly, regardless the genotype, the peel and pulp color index of fruit of the four varieties was not affected by the postharvest storage (Table 1).

Table 1. Effect of cold storage for 28 d at 2 °C and after simulation of shelf life for 5 d at 20 °C on total soluble solids (TSS, %), titratable acid (TA, % citric acid), maturity index (MI, TSS/TA), and peel and pulp color (Hunter a/b) in Navel, Kirkwood, Valencia and Ruby oranges.

Parameter	Navel			Kirkwood		
	Harvest	28 d 2 °C	+5 d 20 °C	Harvest	28 d 2 °C	+5 d 20 °C
TSS	12.60 ± 0.01b	12.63 ± 0.05b	13.16 ± 0.11a*	12.20 ± 0.10b	12.20 ± 0.10b	12.66 ± 0.0
TA	0.84 ± 0.02a	0.78 ± 0.04a	0.69 ± 0.01b	0.84 ± 0.04a	0.86 ± 0.01a	0.82 ± 0.0
MI	14.95 ± 0.44b	16.19 ± 0.94b	19.08 ± 0.42a*	14.58 ± 1.16a	14.18 ± 0.29a	15.43 ± 0.7
Peel color	0.81 ± 0.03a	0.88 ± 0.03a	0.84 ± 0.03a	0.89 ± 0.02a	0.83 ± 0.02a	0.86 ± 0.0
Pulp color	0.10 ± 0.01a*	0.10 ± 0.01a*	0.10 ± 0.01a*	0.56 ± 0.03a	0.56 ± 0.01a	0.62 ± 0.0
Parameter	Valencia			Ruby		
	Harvest	28 d 2 °C	+5 d 20 °C	Harvest	28 d 2 °C	+5 d 20 °C
TSS	11.50 ± 0.01a*	11.23 ± 0.05a	11.51 ± 0.15a	10.95 ± 0.20a	11.03 ± 0.05a	11.33 ± 0.1
TA	0.99 ± 0.07a	1.20 ± 0.10a	0.99 ± 0.10a	1.00 ± 0.04b	1.20 ± 0.06a	0.95 ± 0.0
MI	11.60 ± 0.87a	9.35 ± 0.87b	11.62 ± 0.25a	10.95 ± 0.54a	9.18 ± 0.51b	11.92 ± 0.5
Peel color	0.66 ± 0.04a	0.67 ± 0.03a	0.69 ± 0.01a	0.66 ± 0.02a	0.60 ± 0.01a	0.64 ± 0.0
Pulp color	0.05 ± 0.01b	0.09 ± 0.01a*	0.10 ± 0.01a*	0.57 ± 0.01a	0.56 ± 0.01a	0.55 ± 0.0

Different lowercase letters indicate significant differences between each postharvest stage for the same variety by one-way ANOVA ($p < 0.05$). Asterisks indicate significant differences for each parameter between Navel and Kirkwood; or Valencia and Ruby for each postharvest stage by t-test ($p < 0.05$).

3.2. Carotenoids content and composition

The most remarkable feature of the K and R oranges is the red coloration of their pulp. We have previously described that this phenotype is associated with a greater accumulation of total carotenoids, particularly, large amounts of the red carotenoid lycopene and also other colorless carotenes (phytoene and phytofluene) as compared with the standard orange (Zacarías-García et al., 2022a). In this work, we evaluated the effect of cold storage + SL on carotenoid content and composition in the pulp of fruit of the four varieties. Total carotenoids of K and R fruit at harvest were 9- and 18-times higher than in the corresponding N and V, respectively (Table 2 and 3). In agreement with previous reports, the major differences were mainly related to the larger content of phytoene and phytofluene, the presence of lycopene and the low levels of xanthophylls, especially violaxanthin, in the red-fleshed oranges (Table 2 and 3) (Zacarías-García et al., 2021, 2022a). Exposure of standard oranges at 2 °C produced minor changes in carotenoid content and composition, but after fruit rewarming, the content of total carotenoids increased 50 % in N fruit (Table 2). Nevertheless, in V fruit, the individual and total content of carotenoids remained relatively stable, supporting the notion that fruit of Valencia oranges are less carotenogenic than those of Navel as previously suggested (Table 3) (Rodrigo et al. 2013). These results are consistent with those found in fruit of other standard

citrus varieties in which carotenoids content are maintained or reduced at low temperatures but increased at temperatures of storage around 10-15 °C or higher (Matsumoto et al., 2009; Carmona et al., 2012).

Table 2. Effect of cold storage for 28 d at 2 °C and simulation of shelf life for 5 d at 20 °C on carotenoid content and composition (mg kg^{-1}) in the pulp of Navel and Kirkwood oranges.

Carotenoids	Navel			Kirkwood		
	Harvest	28 d 2 °C	+5 d 20 °C	Harvest	28 d 2 °C	+5 d 20 °C
Phytoene	1.33 ± 0.04b*	1.47 ± 0.22b*	2.02 ± 0.01a*	84.85 ± 5.03a	72.89 ± 3.30b	90.28 ± 5.31a
Phytofluene	0.16 ± 0.04c*	0.44 ± 0.11a*	0.25 ± 0.01b*	13.96 ± 0.03b	13.18 ± 0.28b	16.38 ± 0.52a
ζ-carotene	0.11 ± 0.04c*	0.20 ± 0.01b	0.34 ± 0.03a	0.57 ± 0.10a	0.16 ± 0.19b	0.48 ± 0.10a
Neurosporene	nd	nd	nd	0.53 ± 0.11a	0.34 ± 0.04b	0.25 ± 0.08b
Lycopene	nd	nd	nd	8.14 ± 0.35b	8.04 ± 0.01b	11.81 ± 0.07a
δ-carotene	nd	nd	nd	0.10 ± 0.06a	0.11 ± 0.04a	0.07 ± 0.01a
Lutein	0.55 ± 0.04b	0.61 ± 0.10b*	0.87 ± 0.02a*	0.45 ± 0.11a	0.31 ± 0.04a	0.32 ± 0.07a
β-carotene	0.02 ± 0.01*	traces	traces	0.62 ± 0.21b	traces	1.08 ± 0.10a
β-	0.85 ± 0.15b	0.97 ± 0.15ab*	1.60 ± 0.38a*	0.94 ± 0.08a	0.54 ± 0.02b	0.99 ± 0.10a
Zeaxanthin	0.43 ± 0.03b*	0.50 ± 0.08b	0.90 ± 0.12a	0.78 ± 0.15a	0.59 ± 0.04b	0.85 ± 0.11a
Anteraxanthin	1.43 ± 0.11b	2.06 ± 0.27ab*	3.05 ± 0.49a*	1.94 ± 0.46a	1.11 ± 0.02b	1.16 ± 0.53a
Violaxanthin	6.48 ± 0.34b*	7.13 ± 0.25ab*	11.19 ± 2.62a*	3.66 ± 0.16a	2.11 ± 0.28b	3.15 ± 0.25a
Luteoxanthin	0.69 ± 0.08a*	0.53 ± 0.01a*	0.58 ± 0.01a*	0.37 ± 0.07a	0.27 ± 0.03a	0.29 ± 0.01a
Mutatoxanthin	0.16 ± 0.01	nd	nd	0.20 ± 0.09a	0.31 ± 0.24a	0.20 ± 0.02a
Total	12.2 ± 0.81b*	14.08 ± 1.30b*	21.31 ± 3.92a*	117.14 ± 3.26a	100.50 ± 3.20b	127.4 ± 7.31a
RE	0.07 ± 0.01b*	0.08 ± 0.01b	0.14 ± 0.02a*	0.16 ± 0.02b	0.11 ± 0.02b	0.26 ± 0.02a

Different lowercase letters indicate significant differences between each postharvest stage for the same variety by one-way ANOVA ($p < 0.05$). Asterisks indicate significant differences for each carotenoid between the standard Navel and the red-fleshed Kirkwood for each postharvest stage by t-test ($p < 0.05$). Traces indicate amounts lower than 0.05 mg kg^{-1}. The results are the mean of two biological replicates ± SD. Nd: no detected. RE: retinol equivalents.

Cold storage produced similar pattern of changes in both red-fleshed varieties, which were different to their standard varieties. In general, more relevant effects on the carotenoid profile of K and R fruit were: 1) a reduction of total carotenoids content (15 and 10 % in K and R, respectively), 2) phytoene was the most affected carotenoid by low temperatures, 3) lycopene was significantly reduced in R. On the other hand, SL had also relevant effects in the pulp of K and R fruit: 1) total carotenoids content increased by 27 % and 10 % in K and R, respectively, 2) phytoene suffered a remarkable increase, 3) lycopene concentration was enhanced about 30 % respect to cold-stored fruit (Table 2 and 3). It is also relevant to mention that the pro-vitamin A activity expressed as retinol equivalents (RE) was significantly higher in both red-fleshed varieties because of their higher β-carotene content (Table 2 and 3). The differential effects of

cold storage on the carotenoid profiling in the red-fleshed and the ordinary varieties highlight the motion of an altered carotenoid metabolism in the K and R mutant, which is also manifested during postharvest storage. Our results, however, are different to that found in the red-fleshed variety Cara Cara, in which phytoene, phytofluene, β-cryptoxathin and other xanthophylls increased during storage at 4 °C, in accordance with the induction of the expression of carotenoid biosynthetic genes (Tao et al., 2012). Thus, storage at low temperatures (4 °C and 2 °C) produced different carotenoid effects between Cara Cara, and K and R fruit These differences could be to metabolic singularities, or differences related to the maturation stages, or to the agronomical or environmental growing-conditions.

Table 3. Effect of cold storage for 28 d at 2 °C and simulation of shelf life for 5 d at 20 °C on carotenoid content and composition (mg kg^{-1}) in the pulp of Valencia and Ruby oranges.

Carotenoids	Valencia			Ruby		
	Harvest	28 d 2 °C	+5 d 20 °C	Harvest	28 d 2 °C	+5 d 20 °C
Phytoene	1.05 ± 0.04b*	1.65 ± 0.27a*	1.48 ± 0.13a*	133.1 ± 1.08a	117.4 ± 5.07c	127.00 ± 1.12b
Phytofluene	0.18 ± 0.01b*	0.31 ± 0.03a*	0.45 ± 0.20a*	18.42 ± 3.60a	17.91 ± 0.39b	19.99 ± 0.47a
ζ-carotene	0.29 ± 0.02b*	0.45 ± 0.04a*	0.23 ± 0.11b*	0.62 ± 0.13a	0.65 ± 0.01a	0.61 ± 0.15a
Neurosporene	nd	nd	nd	0.33 ± 0.02a	0.39 ± 0.09a	0.18 ± 0.06b
Lycopene	nd	nd	nd	8.58 ± 0.14b	7.34 ± 0.18c	10.32 ± 0.38a
δ-carotene	nd	nd	nd	0.13 ± 0.01a	0.13 ± 0.05a	0.08 ± 0.02b
Lutein	0.61 ± 0.08a*	0.58 ± 0.01a*	0.61 ± 0.04a*	0.32 ± 0.05a	0.40 ± 0.01a	0.47 ± 0.13a
β-carotene	traces	traces	traces	0.41 ± 0.03b	0.38 ± 0.03b	0.65 ± 0.07a
β-	0.58 ± 0.16a	0.62 ± 0.07a	0.72 ± 0.01a*	0.30 ± 0.10b	0.45 ± 0.08a	0.54 ± 0.02a
Zeaxanthin	0.66 ± 0.15a	0.67 ± 0.11a	0.73 ± 0.01a	0.61 ± 0.02b	0.75 ± 0.07a	0.87 ± 0.09a
Anteraxanthin	1.20 ± 0.12a	1.45 ± 0.18a	1.10 ± 0.02b	0.98 ± 0.26a	1.08 ± 0.12a	0.75 ± 0.37a
Violaxanthin	3.70 ± 0.11a*	2.66 ± 0.70a	3.71 ± 0.17a*	2.30 ± 0.03a	2.31 ± 0.41a	1.46 ± 0.15b
Luteoxanthin	0.58 ± 0.17a*	0.57 ± 0.01a	0.75 ± 0.10a*	0.23 ± 0.03b	traces	0.40 ± 0.06a
Mutatoxanthin	0.18 ± 0.13a	0.10 ± 0.01b	0.24 ± 0.01a	0.10 ± 0.01a	0.25 ± 0.14a	0.23 ± 0.10a
Total	9.12 ± 0.92a*	9.06 ± 1.00a*	10.17 ± 0.28a*	166.50 ± 2.81a	149.80 ± 5.65b	163.60 ± 1.84a
RE	0.05 ± 0.01c*	0.06 ± 0.01b*	0.09 ± 0.01a*	0.09 ± 0.01c	0.12 ± 0.01b	0.15 ± 0.01a

Different lowercase letters indicate significant differences between each postharvest stage for the same variety by one-way ANOVA ($p < 0.05$). Asterisks indicate significant differences for each carotenoid between the standard Valencia and the red-fleshed Ruby for each postharvest stage by t-test ($p < 0.05$). Traces indicate amounts lower than 0.05 mg kg^{-1}. The results are the mean of two biological replicates ± SD. Nd: no detected. RE: retinol equivalents.

Interestingly, the recovery of the content of phytoene and lycopene during the SL period may be related to the raise of ethylene production usually occurring after rewarming of the fruit (Zacarías et al., 2020), that would induce the expression of carotenoid biosynthesis genes responsive to ethylene in citrus fruit (Rodrigo et al., 2007). In summary, our results indicate

that under the postharvest conditions assayed (28 d 2 °C + 5 d 20 °C) the pulp of K and R fruit maintained their high content and singular carotenoid composition and, therefore, retained their carotenoid associated nutritional and health values.

Since carotenoids composition in the flavedo (fruit external coloured layer) of sweet orange determines fruit external pigmentation (Rodrigo et al., 2013) and this have been related with protective effects against environmental stress such as low temperature (Rey et al., 2020), the carotenoid profile in the flavedo of the four varieties was also evaluated (Supplementary Table 1 and 2). Interestingly, the total content of carotenoids in the peel remained stable during cold storage in the four varieties but after SL it remarkably increased between 51-69 % (Supplementary Table 1 and 2). It is well known that citrus fruit rewarming after prolonged cold storage triggers fruit endogenous ethylene production (Lado et al., 2014) and, on the other hand, carotenoid biosynthesis is enhanced by ethylene upregulating the expression of key genes of the pathway, such as phytoene synthase or β-carotene hydroxylase responsible for phytoene and β,β-xanthophylls synthesis, respectively (Rodrigo et al., 2007). Therefore, it is reasonable to propose that the substantial increment in phytoene and xanthophylls during the SL period is a direct effect of ethylene stimulating carotenoid biosynthesis. This promoting effect on carotenoids content in the flavedo has been also reported for other Navel oranges (Carmona et al., 2012) or Star Ruby grapefruit (Lado et al., 2014) in which carotenoids maintained stable at low temperatures but their contents increased when stored above 12 °C. The most remarkably feature between the red and the blond oranges was that the total carotenoids content was between 10-27 % and 30-45 % higher in K and R, respectively, than in the corresponding standard varieties (Supplementary Table 1 and 2). This exceptionally high content of carotenoids in the peel of K and R was mainly due to the large levels of phytoene. Nevertheless, N and V varieties presented significant higher contents of violaxanthin. These results are in accordance with those described for the peel of the same orange varieties during fruit maturation (Zacarías et al., 2022a). Therefore, as it was mentioned above, the better tolerance to CI observed in the red-fleshed genotypes during postharvest storage may be related to their higher carotenoids content in the flavedo, which may act as effective quencher of particular ROS species (Martínez et al., 2014; Rey et al., 2020).

3.3. Sugars, organic acids and other bioactive compounds

Sucrose, fructose and glucose were the main sugars accumulating in the four orange varieties. At harvest, significant differences in the sugar content were detected between the red and the standard varieties, with higher sucrose but lower glucose and fructose in the red-fleshed varieties than in the standard oranges (Table 4 and 5). Other studies have reported variable results in the sugar content in fruit of other red-fleshed orange, suggesting that, in addition to the genetic diversity, other factors, such as maturity, growing conditions, among others, may also be involved in this discrepancy (Pan et al., 2014). The content of sugars during cold storage significantly increased for all varieties but in a variable degree. Sucrose is the sugar that experienced main changes, increasing its content up to 70 %. However, the levels of sucrose showed virtually no changes during SL (Table 4 and 5). By the end of the postharvest storage, the content of the three sugars substantively increased in the four varieties, with only significant differences in sucrose between V and R (Table 5). In fruit of other orange varieties sugars content increased after few weeks of cold storage and progressively declined after prolonged storage periods (Gao et al., 2019; Habibi et al., 2020a; Zhang et al., 2022). These responses appear to be rather variable, depending on the variety, maturity, and may be related to the respiratory activity and the senescence rate of each genotype (Tang et al., 2016). Then, it is concluded that under the postharvest storage conditions evaluated in this study the red-fleshed oranges experienced similar changes and maintained the sugar content as the standard genotypes.

Citric acid was the major organic acid found in the four genotypes, followed by quinic, malic and succinic acid (Table 4 and 5). The two red-fleshed genotypes showed specific differences in the acids content with respect to their respective standard oranges. Thus, N fruit had less citric acid and more quinic acid than K at harvest, but at SL, the levels of these acids were similar in both varieties, and those of the malic and succinic acid were slightly higher in K (Table 4). Interestingly, changes in organic acids during the postharvest storage were notably different between V and R fruit. In V fruit, citric acid increased while the other acids decreased at the end of SL. In contrast, the levels of all acids increased from harvest to SL in R fruit. Thus, the main organics acids (citric and quinic acid) were higher in R fruit at the end of SL (Table 5).

Table 4. Effect of cold storage for 28 d at 2 °C and after simulation of shelf life for 5 d at 20 °C on the content of sugars (g L-1), organic acids (g L-1), ascorbic acid (mg kg-1), tocopherols (mg kg-1) and total phenolic (TP, g gallic acid equivalents kg-1) in the pulp of Navel and Kirkwood oranges.

Compound	Navel			Kirkwood		
	Harvest	28 d 2 °C	+5 d 20 °C	Harvest	28 d 2 °C	+5 d 20 °C
Sugars						
Sucrose	35.13±0.91b*	60.06±4.89a	59.41±3.25a	40.75±0.66b	71.62±4.57a	66.93±2.18a
Glucose	24.65±0.53b*	29.36±1.82a	37.50±1.96a	19.27±0.31b	31.06±0.54b	33.78±2.88a
Fructose	26.90±0.61ab*	23.40±1.24b	31.72±1.53a	21.67±0.88b	25.74±0.63a	28.61±2.27a
Organic						
Citric acid	10.10±0.22a*	9.50±0.31a*	10.06±0.33a	11.20±0.06a	12.15±0.37a	10.15±0.78a
Quinic acid	8.45±0.30b*	8.13±0.50b	11.06±0.48a	6.99±0.13b	9.35±0.35a	10.18±1.01a
Malic acid	2.33±0.27a	1.68±0.03b*	1.60±0.07b*	2.44±0.04a	2.27±0.02ab	2.06±0.12b
Succinic	1.50±0.41a	2.09±0.06a*	1.97±0.06a*	1.35±0.14b	2.54±0.03a	3.00±0.35a
Ascorbic	527.55±7.51b	507.77±5.20b*	575.11±13.44a*	492.72±8.00a	468.44±1.45b	508.37±2.74a
Tocopherols	2.89±0.12c	3.50±0.03b	4.22±0.39a*	3.23±0.02c	4.31±0.01b	5.67±0.36a
TP	4.09±0.23a	3.77±0.36a	4.33±0.12a	4.18±0.24a	4.35±0.28a	4.02±0.13a

Different lowercase letters indicate significant differences between each postharvest stage for the same variety by one-way ANOVA ($p < 0.05$). Asterisks indicate significant differences for each compound between the standard Navel and the red-fleshed Kirkwood for each postharvest stage by t-test ($p < 0.05$). The results are the mean of two biological replicates ± SD.

Citric acid was the major organic acid found in the four genotypes, followed by quinic, malic and succinic acid (Table 4 and 5). The two red-fleshed genotypes showed specific differences in the acids content with respect to their respective standard oranges. Thus, N fruit had less citric acid and more quinic acid than K at harvest, but at SL, the levels of these acids were similar in both varieties, and those of the malic and succinic acid were slightly higher in K (Table 4). Interestingly, changes in organic acids during the postharvest storage were notably different between V and R fruit. In V fruit, citric acid increased while the other acids decreased at the end of SL. In contrast, the levels of all acids increased from harvest to SL in R fruit. Thus, the main organics acids (citric and quinic acid) were higher in R fruit at the end of SL (Table 5). These results are in accordance with previous studies showing that the organic acids content evolved differentially during fruit maturation between V and R fruit, with higher citric acid but lower malic and quinic acid in R (Zacarías-García et al., 2022b). Changes in organic acids during cold storage in citrus fruit are very variable and, in most instances, declined progressively along the storage time (Sun et al., 2012; Habibi et al., 2020a) and may be determined by the genotype and other exogenous factors as well (Lado et al., 2018). In other red-fleshed oranges, as Red Anliu, it was detected very low levels of citric acid associated with

several down-regulated genes of the citrate cycle compared to its parental, suggesting an altered tricarboxylic acids metabolism in this mutant (Xu et al., 2019). However, this alteration in acids observed in Red Anliu does not occur in V and R, which may be explained by the different genetic background and origin of the mutations.

Table 5. Effect of cold storage for 28 d at 2 °C and after simulation of shelf life for 5 d at 20 °C on the content of sugars (g L-1), organic acids (g L-1), ascorbic acid (mg kg-1), tocopherols (mg kg-1) and total phenolic (TP, g gallic acid equivalents kg-1) in the pulp of Valencia and Ruby oranges.

Compound	Valencia			Ruby		
	Harvest	28 d 2 °C	+5 d 20 °C	Harvest	28 d 2 °C	+5 d 20 °C
Sugars						
Sucrose	31.05 ± 0.39b*	52.15 ± 2.23a	51.14 ± 1.09a*	36.58 ± 2.20b	50.34 ± 6.46a	44.04 ± 0.93a
Glucose	17.61 ± 0.23b*	26.10 ± 0.45a	26.29 ± 0.15a	15.74 ± 0.90b	26.60 ± 1.24a	28.16 ± 0.52a
Fructose	19.23 ± 0.21b	21.84 ± 0.45a	22.91 ± 0.06a	17.66 ± 0.88b	21.97 ± 0.95a	23.68 ± 0.58a
Organic acids						
Citric acid	14.42 ± 0.84a*	12.36 ± 0.28b*	12.33 ± 0.02b*	12.61 ± 0.08b	13.96 ± 0.08a	13.77 ± 0.71a
Quinic acid	6.02 ± 0.07b	9.26 ± 0.43a	9.49 ± 0.04a*	5.97 ± 0.01b	9.25 ± 0.48a	10.35 ± 0.18a
Malic acid	2.64 ± 0.16b	3.23 ± 0.07a*	3.09 ± 0.02a*	2.42 ± 0.02a	2.37 ± 0.12a	2.03 ± 0.03b
Succinic	1.57 ± 0.22b	2.28 ± 0.01a	2.37 ± 0.01a	1.65 ± 0.02c	2.11 ± 0.08b	2.35 ± 0.01a
Ascorbic	456.82 ± 6.60a	439.13 ± 0.41b	451.28 ± 0.33a*	482.66 ± 7.62a	457.60 ± 1.44a	508.99 ± 13.43a
Tocopherols	4.28 ± 0.02a	3.79 ± 0.01b*	4.37 ± 0.13a*	4.06 ± 0.10c	5.61 ± 0.13b	6.26 ± 0.05a
TP	3.87 ± 0.03b*	4.51 ± 0.16a	4.26 ± 0.22a	4.37 ± 0.19a	4.32 ± 0.18a	4.11 ± 0.06a

Different lowercase letters indicate significant differences between each postharvest stage for the same variety by one-way ANOVA ($p < 0.05$). Asterisks indicate significant differences for each compound between the standard Valencia and the red-fleshed Ruby for each postharvest stage by t-test ($p < 0.05$). The results are the mean of two biological replicates ± SD.

Sugars and organic acids are the main compounds responsible of the taste of citrus fruit. Then, changes in their concentration largely affect the taste characteristics and organoleptic quality (Lado et al., 2018). Considering the results reported above for sugars and organic acids, we can suggest that the postharvest conditions assayed essentially maintain the levels of these compounds in the red-fleshed oranges similarly than in the standard oranges and then, their fruit quality characteristics.

The effects of cold storage + SL on the content of two relevant vitamins (C or ascorbic acid, and E or tocopherols) were analyzed. The concentration of vitamin C was slightly higher in N than in K (Table 4) while there were not differences between the red-fleshed and the standard Valencia oranges (Table 5). Ascorbic acid remained changeless in the four varieties after SL in comparison with the fruit at harvest (Table 4 and 5), indicating that vitamin C appears to be

stable or minimally affected by low temperatures storage (Rapisarda et el., 2008; Mditshwaa et al., 2017; Zhang et al., 2022). However, other studies indicated that vitamin C in citrus fruit decreased at temperatures above 10 °C. Hence, the variety and the time of cold storage seem to be critical factors determining the stability of vitamin C during postharvest storage (Mditshwaa et al., 2017). Our results clearly indicated that ascorbic acid in the pulp of intact fruit was not affected after one month of cold storage and rewarming and then, fruit can reach the markets with similar levels of this important vitamin than that of freshly harvested fruit.

Tocopherols are natural compounds exhibiting vitamin E activity in animal cells (Muñoz and Munne-Bosch, 2019), being α-tocopherol the most abundant form of vitamin E in citrus fruit (Rey et al., 2021). To our knowledge, this is the first study that evaluates the effect of postharvest storage on tocopherol content in red-fleshed oranges. Tocopherols content at harvest were similar between the red and the blond oranges in agreement previous results (Zacarías-García et al. 2021b). Except for V fruit, the content of tocopherols increased during cold storage + SL, reaching concentrations about 46 % to 60 % higher compared to fruit at harvest (Table 4 and 5). However, at the end of the postharvest storage, tocopherols levels were significantly larger in K and R fruit than in the blond oranges (Table 4 and 5), suggesting that tocopherols in the red oranges had a better response to postharvest storage than in the standard varieties. Studies performed in other citrus species showed that storage at 2 °C increased total tocopherols content in the peel of Star Ruby grapefruit but not in mandarin fruit (Rey et al., 2021a; 2021b), suggesting being a variety-dependent effect.

The levels of total phenolic found in N, K and R fruit did not exhibit significant changes during postharvest storage, whereas cold storage led to an increase of 15 % in V fruit (Table 4 and 5). Habibi et al. (2020a) found that total phenolics were maintained or moderately increased in blood oranges stored for 30 d at 2 °C. The effect of low temperature on phenolic content have been addressed in different works, indicating that, in most cases, phenolic metabolism is enhanced under cold stress, but the responses are highly dependent on the storage conditions and the variety (Lattanzio et al., 1994). In this line, Rapisarda et al. (2008) found that phenolic content increased in the blood orange Tarocco and Moro after 65 d of storage at 6 °C while it was significantly reduced in the blond Valencia orange.

Flavonoids are one to the most important secondary metabolites in Citrus, playing essential roles in their antioxidant and nutritional properties (Deng et al., 2022). Thus, the individual contents of main flavonoids were analyzed in the pulp of the four orange genotypes. Six major individual flavonoids, five flavanones (eriocitrin, narirutin, naringin, hesperidin and dydimin) and one flavonol (rutin), were quantified and differentially affected by cold storage (Table 6 and 7). Hesperidin was the predominant flavonoid in all the samples, accounting between 80-90 % of the total flavonoid content, followed by narirutin (7-12 %) and rutin (3 %). The flavonoid profiles observed in these varieties are in accordance with that reported for other orange fruit (Peterson et al., 2006; Cheng et al., 2015; De Ancos et al., 2017). The concentration of total flavonoids in N and V oranges at harvest were similar of the red-fleshed oranges K and R, respectively, and only minor differences were detected in individual flavonoids (Table 6 and 7). Total flavonoids in N, V and R decreased at 2 °C followed by a further increase when the fruit were transferred to 20 °C, recovering the same levels or even higher than at harvest. In contrast, cold storage increased the levels of flavonoids in K, to decrease at the end of the storage to values similar that at harvest (Table 6). Hesperidin was the flavonoid most contributing to these changes all along the postharvest storage, while other minor flavonoids had lower effects on total flavonoid (Table 6 and Table 7). In agreement with our results, other studies observed no variation in the content of hesperidin in Newhall Navel at 4 °C (Lafuente et al., 2011; Zhang et al., 2022), despite other results showed different effects of cold storage in the pulp or the juice of the same varieties (Del Caro et al. 2004).

Table 6. Effect of cold storage for 28 d at 2 °C and after simulation of shelf life for 5 d at 20 °C on individual flavonoids (mg kg^{-1}) in the pulp of Navel and Kirkwood oranges.

Flavonoids	Navel			Kirkwood		
	Harvest	28 d 2 °C	+5 d 20 °C	Harvest	28 d 2 °C	+5 d 20 °C
Rutin	22.31±3.40a	20.24±1.62a	22.01±3.04a	20.22±0.10b	23.55±1.70a	23.03±0.70a
Eriocitrin	9.91±0.40a	9.00±0.77a	9.99±0.10a*	8.95±0.71a	10.21±0.81a	8.44±0.43a
Narirutin	82.72±3.91a*	66.73±5.55b*	74.96±2.77ab	66.42±0.71c	82.64±1.53a	74.18±2.70b
Naringin	nd	3.01±0.12b	4.10±0.30a	2.97±0.15a	2.94±0.11a	3.17±0.57a
Hesperidin	549.72±54.40a	437.27±45.27a*	524.08±23.81a	603.15±12.92ab	648.70±0.22a	546.54±33.70b
Dydimin	10.99±2.43a	5.11±0.90b*	7.12±1.92ab	8.70±0.60a	14.17±3.91a	9.52±3.37a
Total	675.43±58.90a	541.25±33.84b*	642.05±28.53a	710.2±13.72b	782.00±42.34a	664.55±41.32b

Different lowercase letters indicate significant differences between each postharvest stage for the same variety by one-way ANOVA ($p < 0.05$). Asterisks indicate significant differences for each flavonoid between the standard Navel and the red-fleshed Kirkwood for each postharvest stage by t-test ($p < 0.05$). The results are the mean of two biological replicates ± SD. Nd: no detected.

In different tissues of the two red-fleshed oranges Hong Anliu and Cara Cara, the content of flavonoids were different to that of the blond Anlui and Seike oranges, and authors suggest that accumulation of lycopene may have a direct or indirect effect on secondary metabolism, modifying the concentration of different metabolites (Chen et al,. 2015). Our results indicate that the alteration in the carotenoids accumulation in K and R mutants does not lead to alterations in the flavonoids metabolism since the content and composition of these compounds are comparable to that of the standard oranges.

Table 7. Effect of cold storage for 28 d at 2 °C and after simulation of shelf life for 5 d at 20 °C on individual flavonoids (mg kg^{-1}) in the pulp of Valencia and Ruby oranges.

Flavonoids	Valencia			Ruby		
	Harvest	28 d 2 °C	+5 d 20 °C	Harvest	28 d 2 °C	+5 d 20 °C
Rutin	28.62 ± 1.51a*	24.82 ± 2.44a	26.74 ± 1.83a	23.44 ± 1.31b	24.34 ± 0.30b	29.51 ± 1.31a
Eriocitrin	11.47 ± 0.73a*	10.00 ± 1.11a	9.81 ± 0.95a	8.30 ± 0.60a	8.50 ± 0.20a	9.72 ± 0.60a
Narirutin	49.88 ± 15.00a	38.55 ± 3.46a*	42.17 ± 3.12a*	62.96 ± 4.41a	45.72 ± 1.42b	67.75 ± 7.40a
Naringin	7.41 ± 1.14a*	1.23 ± 0.10b*	1.53 ± 0.13b*	4.42 ± 0.92a	5.54 ± 0.21a	6.93 ± 1.53a
Hesperidin	702.54 ± 28.22b	666.51 ± 69.11b	865.94 ± 34.61a	749.24 ± 98.21a	675.24 ± 14.23a	802.27 ± 87.96a
Dydimin	7.00 ± 1.40a	3.51 ± 0.70b*	3.80 ± 0.41b*	8.76 ± 3.23ab	6.94 ± 0.82b	9.74 ± 1.74a
Total	806.77 ± 75.11b	744.50 ± 61.73b	949.82 ± 40.92a	856.91 ± 108.32ab	766.25 ± 12.75b	925.74 ± 96.74a

Different lowercase letters indicate significant differences between each postharvest stage for the same variety by one-way ANOVA ($p < 0.05$). Asterisks indicate significant differences for each flavonoid between the standard Valencia and the red-fleshed Ruby for each postharvest stage by t-test ($p < 0.05$). The results are the mean of two biological replicates ± SD. Nd: no detected.

3.4. Total antioxidant capacity and antioxidant capacity against singlet oxygen

The hydrophilic antioxidant activity, determined by ABTS assay, of the pulp of the four orange genotypes did not change during the entire storage period and no differences were detected between the standard oranges and their corresponding red mutants (Fig. 2A,B). These results are consistent with those found for the same varieties during development and maturation (Zacarías et al., 2022b) and are not unexpected since the content of main hydrophilic compounds that contribute to this antioxidant capacity (ascorbic acid, phenolics, or/and flavonoids) were relatively similar in the pulp of all these fruit. However, the ABTS lipophilic antioxidant capacity was about 15-25 % higher in the extracts of K and R oranges, compared to N and V, respectively (Fig. 2C,D). Moreover, the singlet oxygen absorption capacity (SOAC) was 2- and 2.5-times higher in the red oranges than in the corresponding standard oranges (Fig. 2E,F). During cold storage + SL, the lipophilic antioxidant capacity and

SOAC remained unaltered (Fig. 2). The enhanced lipophilic antioxidant values observed in both red oranges are likely associated with their unusual high content and composition of carotenoids. Although lycopene is the carotenoid with the highest antioxidant activity against singlet oxygen (Stahl and Sies; 2003), the colorless phytoene and phytofluene have been proposed to be important contributors to these antioxidant capacities (Martinez et al., 2014). Then, it is likely that the presence of lycopene and the plentiful concentration of phytoene and phytofluene contribute to the enhanced lipophilic ad SOAC antioxidant capacity of the red oranges. It should be pointed out that neither the cold storage nor the subsequent shelf-life altered these antioxidant capacities and therefore the red-fleshed orange fruit could reach consumers with this added-value intact.

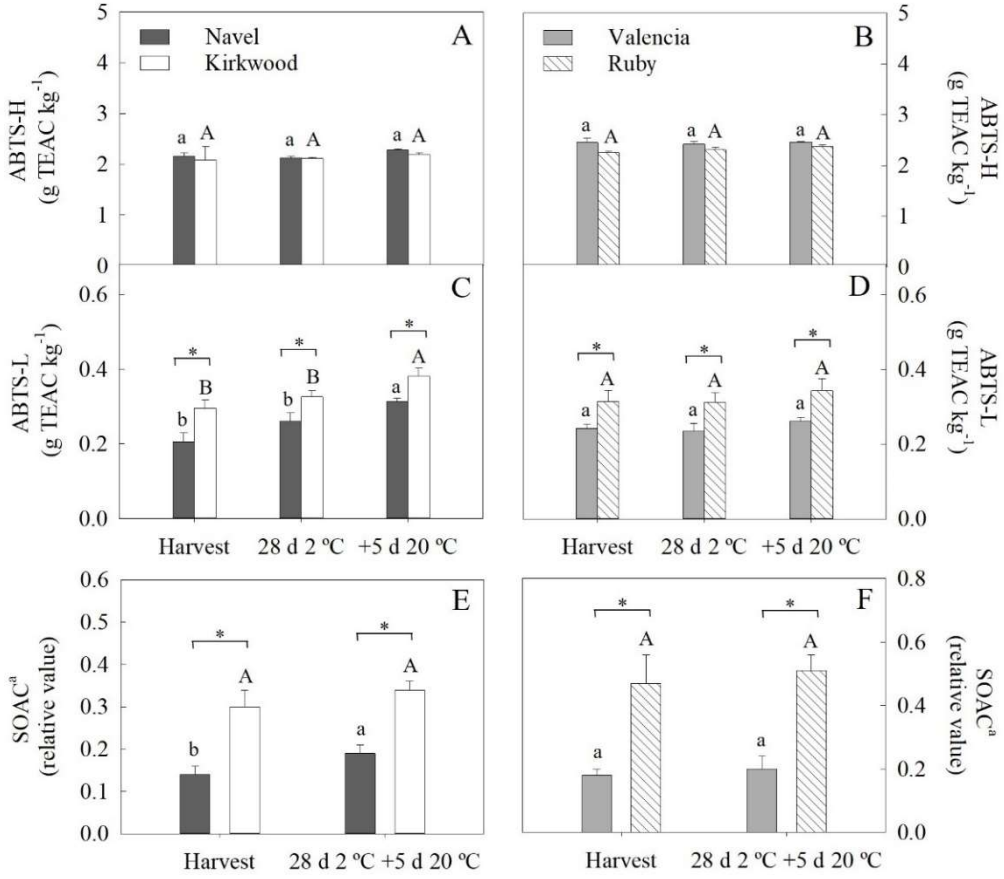

Fig. 2. Hydrophilic (ABTS-H) (A,B) and lipophilic (ABTS-L) (C,D) antioxidant capacity by ABTS, and singlet oxygen absorption capacity (SOAC) (E,F) of the pulp of Navel, Kirkwood, Valencia and Ruby orange fruit at harvest, 28 d at 2 °C (cold storage) and 5 d at 20 °C (shelf-life). Different lower letters indicate significant differences between each postharvest stage for the standard Navel and Valencia varieties by one-way ANOVA ($p < 0.05$). Different uppercase letters indicate significant differences between each postharvest stage for the red-fleshed Kirkwood and Ruby varieties by one-way ANOVA ($p < 0.05$). Asterisks indicate significant differences between Navel and Kirkwood; or Valencia and Ruby for each postharvest stage by t-test ($p < 0.05$). [a]Relative to g L^{-1} units.

3.5. Volatile compounds

Volatile organic compounds (VOCs) play essential function in flavor, taste and palatability of citrus fruit and their changes during postharvest storage may be determinants for the fresh-fruit market, juice and consumers perception (Pérez-Cacho and Rouseff, 2008b; Tietel et al., 2011). In the present study 92 volatile compounds were detected in the pulp of the four orange varieties. Eighty-four were unequivocally identified and the other eight were tentatively assigned to the sesquiterpene group based on their MS spectra and retention time (Supplementary Table 3). A total of 40 terpenes (11 monoterpene hydrocarbons, 8 monoterpene alcohols, 2 ketones, 2 aldehydes, 2 esters and 2 epoxy, and 13 sesquiterpenes), 5 aliphatic alcohols, 16 aliphatic aldehydes, 2 aliphatic ketones, 15 aliphatic esters and 4 furans were identified. In addition, 10 compounds were classified as norisoprenoids depending on the nature of the precursors (carotenoids) (Fig. 3). Eighty-seven compounds were detected in N and K (Fig. 3A; Supplementary Table 4), and 85 in V and R (Fig. 3B; Supplementary Table 5). The relative content of all VOCs in the pulp of the red-fleshed and the standard varieties during the postharvest storage is shown in Fig. 3 and Supplementary Tables 4 and 5. These results indicate that the main differences between the red and the standard oranges are associated with quantitative rather than qualitative changes (Fig. 3). A principal component analysis (PCA) was performed independently for Navel (N and K) (Fig. 4A,B) and Valencia (V and R) (Fig. 4C,D) varieties, including the 92 volatile compounds identified in fruit at harvest, cold storage and SL. The two first principal components, which explain the 63.1 % of the variability for the Navel oranges, and the 65.4 % for the Valencia oranges, separated the volatile profile of samples according to the postharvest condition. The main changes occurred during postharvest storage and the differences in the relative content of volatiles between the red-fleshed and the standard oranges are discussed below.

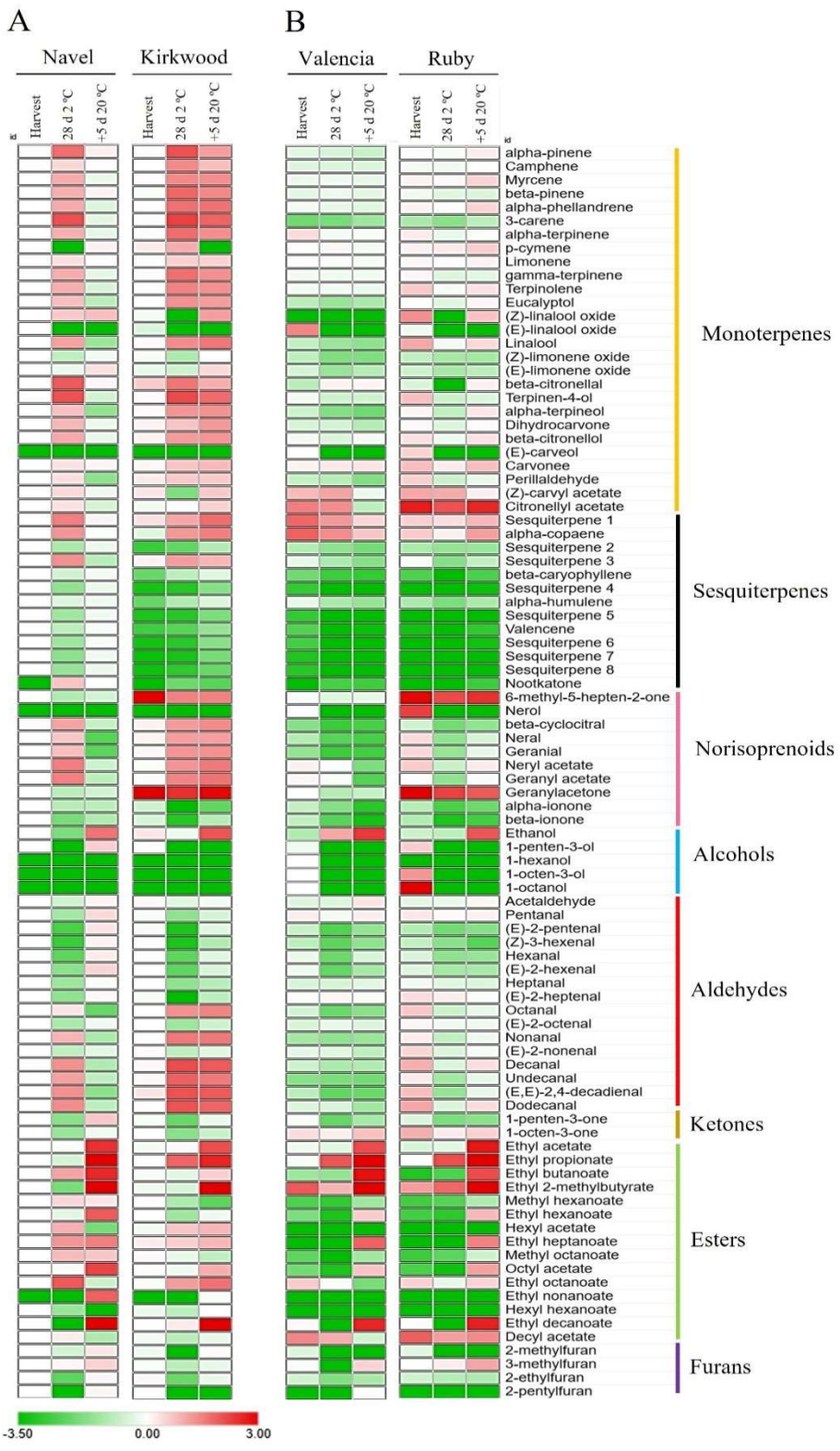

Fig. 3. Heat map of the relative abundance of the volatile compounds detected in Navel and Kirkwood (A) and Valencia and Ruby (B) at harvest, after 28 d at 2 °C (cold storage) and 5 d at 20 °C (shelf-life). Cells colors are based on the relative abundance; red represents high abundance and green represents low abundance. Data normalized with the mean of Navel at harvest. Nootkatone was normalized with the mean of Navel at shelf-life. (E)-carveol, nerol 1-hexanol, 1-octen-3-ol and 1-octanol were normalized with the mean values for Valencia at harvest.

The major classes of volatile compounds found in the four orange genotypes were terpenes hydrocarbons, and terpenic and non-terpenic alcohols, aldehydes, esters and furans, in agreement with previous studies in standard sweet oranges (Pérez-Cacho and Rouseff, 2008a; Wei et al., 2018). Our results revealed that the pattern of accumulation of the different volatiles compounds also depends on their biosynthetic origin and metabolic pathway (Fig. 4). For instance, monoterpenes, which are synthesized by monoterpenes synthases in plastids (Dudareva et al., 2013) are grouped relatively close, independently whether they present or not functional groups (alcohol, aldehyde, etc). Monoterpenes are the most abundant components in the orange aroma but their relative abundance varies among citrus species (González-Mas et al., 2019). They contribute to their natural citrus-like odor and masking off-flavors (Pérez-Cacho and Rouseff, 2008a; Tietel et al., 2011). However, its influence on orange flavour is controversial since monoterpenes as limonene and myrcene have high odor thresholds (Pérez-Cacho and Rouseff, 2008a). Particularly, most of monoterpenes are found in higher levels in N and K oranges and follow a similar trend during low temperature storage (Fig. 3A) while they remained relatively stable in V and R (Fig. 3B). However, upon transfer the fruit from 2 °C to 20 °C important upsurge of those monoterpenes were produced in the red-fleshed varieties, especially in K, being the content of most of monoterpenes significantly higher in the red oranges than in the standard varieties (Fig. 3A and 3B). In agreement with our results, increases in specific monoterpenes have also been detected in fruit of other citrus species in response to cold storage (Obenland, et al., 2008; Tietel et al., 2011; Habibi et al., 2020b). The different response of red-fleshed fruit to SL might indicate an alteration in monoterpene metabolism, as it has been reported in fruit of the red orange Anliu (Liu et al., 2019).

The levels of sesquiterpenes were remarkably lower in fruit of both red-fleshed varieties at harvest. Ten out of 13 sesquiterpenes decreased significantly during cold storage in N and K, and subsequently increased at SL. The relative content of most of these sesquiterpenes remained significantly lower in the pulp of K as compared to N by the end of the postharvest storage (Fig. 3A). Instead, the levels of sesquiterpenes were notably low in both V and R varieties and unlike the Navel group oranges, V and R fruit contained similar levels of sesquiterpenes during cold storage and SL (Fig. 3B).

Norisoprenoids constitute a family of terpenoids (C9-C13) that can be derived from degradation of carotenoids and most of them have an extremely low odor threshold (Mahattanatawee et al 2005). Particularly, in this category are included 6-methyl-5-hepten-2-one (MHE), neral, neryl acetate, geranial, geranyl acetate and geranylacetone which can be generated from the specific cleavage of linear carotenes (Lewinsohn et al., 2005a). After SL, most norisoprenoids decreased in N and V but slightly increased in the red-fleshed varieties (Fig. 3A,B). It is noteworthy that K and R accumulated higher levels of norisoprenoids potentially derived from carotenes during the entire postharvest storage.

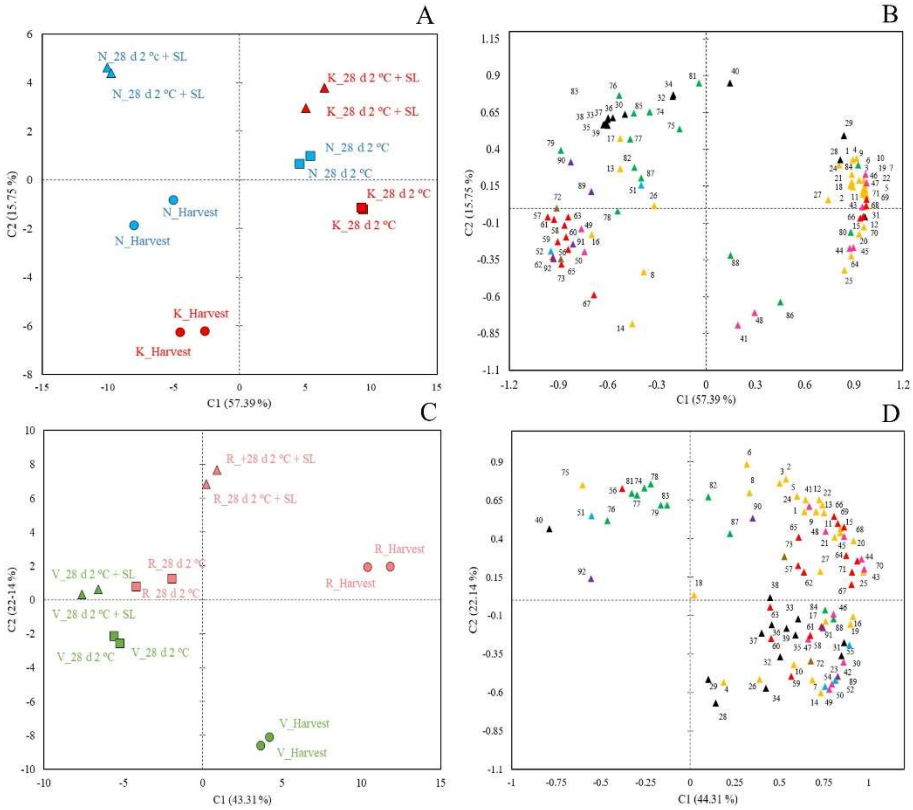

Fig. 4. Principal Component Analysis (PCA) score plot (A) of Navel (blue) and Kirkwood (red) and loading plot (B) of the volatile compounds detected during postharvest storage. PCA score plot (C) of Valencia (green) and Ruby (pink) and loading plot (D) of the volatile compounds detected at harvest, after 28 d at 2 °C (cold storage) and after cold storage + 5 d at 20 °C (SL, shelf-life). Different group of volatiles are colored as follow: yellow, monoterpenes (1-27); black, sesquiterpenes (28-40); pink, norisoprenoids (41-50); blue, alcohols (51-55); red, aldehydes (56-71); brown, ketones (72,73); green, esters (74-88); purple, furans (89-92).

Among them, MHE (4-7x), neral (4-11x), geranial (4-11x) and geranylacetone (6-11x) were those that displayed the major differences between the red and the standard varieties, being more abundant in K than in R (Fig. 3; Supplementary Tables 4 and 5). As the pulp of both red-fleshed varieties presented very higher amounts of phytoene and phytofluene and moderate levels of lycopene (Table 2 and 3), then, suggesting a direct precursor-product relationship between the levels of these carotenoids and their derived norisoprenoids in K and R fruit. Therefore, the unusual content of carotenoids in the red-fleshed oranges does not only affect the color of the fruit but also the aroma components. Other studies have also reported a direct impact of carotenoids content and composition on the volatiles profile in other fruit including orange, nectarine, tomato, watermelon and grapes (Mahattanatawee al., 2005; Lewinsohn et al., 2005b; Brandi et al., 2011).

Ethanol, a precursor of natural aroma compounds, may be responsible of development off-flavor in citrus fruit when produced in high amounts (Pérez-Cacho and Rouseff, 2008a). Ethanol is indicative of fermentation processes, which is highly influenced by the length of storage and by the storage temperature (Zacarías et al., 2020). Thus, ethanol accumulation in the fruit pulp during postharvest storage and SL was a relevant parameter for the aptitude of the red-fleshed varieties for postharvest storage. The content in the pulp of the four varieties experienced a remarkably increase after being transferred to 20 °C, although with no differences between varieties (Fig. 3).

It is recognized that aldehydes play a major role in orange flavor, contributing to citrus-like, floral and grassy notes (Pérez-Cacho et al., 2008a; Tietel et al., 2011). The fatty acid aldehydes are the compounds that exhibited the most variable changes during the postharvest storage between the different varieties. The cold storage had a similar effect in the aldehydes concentrations in both Navel oranges. However, the short-chain (C5-C7) and the long-chain (C8-C12) aldehydes showed an opposite pattern of accumulation between K and N fruit during SL. Interestingly, the levels of C5-C7 aldehydes in N fruit were about 2 times higher than in K fruit (Fig. 3A; Supplementary Table 4). In addition the content of C8-C12 aldehydes such as octanal, nonanal, decanal, (E,E)-2,4-decadienal, undecanal and dodecanal were between 6 and 11- times higher in K, and 2 and 3- times higher in R compared to N and V, respectively, by the end of the postharvest storage (Fig. 3; Supplementary Table 4 and 5). Our results therefore suggest a differential response in fatty acid aldehydes metabolism during

postharvest storage for the red-fleshed and the standard genotypes. Other studies in mandarins and oranges have indicated that the content of aldehydes decreased during the storage time (Tietel et al., 2011).

Volatile esters are responsible for the fruity top-notes and are some of the most important odor-active compounds in fresh citrus fruit and juices (Pérez-Cacho et al., 2008a,b). Important differences in the content of esters were also detected between the red-fleshed and the standard varieties. The SL increased markedly the amounts of almost all volatile esters in the four varieties. It should be noted the increase of ethyl 2-methylbutyrate (x24), ethyl decanoate (x15), ethyl propionate (x11), ethyl butanoate (x5.5) and ethyl acetate (x5.3) in N fruit. These increments were found at much lower extent in K, with the content of 11 esters being lower than in the standard N variety (Fig. 3A; Supplementary Table 4). In contrast, the relative amounts of esters showed only minor differences between V and R fruit (Fig. 3B). Our results indicate that among the volatile esters, the ethyl ester group experienced the major changes during the postharvest storage. These changes are likely due to the increment of its alcohol precursor ethanol (González-Mas et al., 2011). The increment in the esters content appear to be a common feature during postharvest storage of citrus fruit (Tietel et al., 2011) and it has been related to the incidence of chilling injury (Lado et al., 2019b; Habibi et al., 2021) or as predictors of freeze-damaged (Obenland et a., 2003).

In summary, during postharvest storage, the volatiles profile of the red-fleshed varieties K and R presented certain features in common and distinctive from the standard N and V oranges, such as higher levels of monoterpenes, norisoprenoids derived from linear carotenes breakdown and C8-C12 aliphatic aldehydes. Nevertheless, significantly larger content of C5-C7 aldehydes and aliphatic esters were found in N than in K. Altogether, these differences in VOCs generated during the postharvest storage in the red-fleshed fruit might impact their aroma and provide to the K and R oranges a distinctive flavor respect to that of the standard oranges.

5. Conclusions

This is the first report evaluating the postharvest performance of the red-fleshed orange varieties Kirkwood Navel and Ruby Valencia during cold storage (28 d at 2 °C) followed by an extended shelf-life at 20 °C for 5 d. Fruit of these varieties showed comparable values of weight loss and firmness, and lower CI index than ordinary oranges, indicating that the red-fleshed oranges had good physiological responses to the postharvest cold storage. Moreover, cold storage and shelf-life maintained the levels carotenoids, vitamin C, phenolics and flavonoids in the red-fleshed oranges and even increased the content of tocopherols, sugars and organic acids. The hydrophilic antioxidant activity was similar in the four genotypes and did not change during postharvest storage, but the lipophilic antioxidant capacity and the capacity quenching singlet oxygen (SOAC) was superior in the red-fleshed oranges and the differences were maintained during the cold storage. The aroma profile of all varieties was substantially modified during storage but different responses were observed between the Navel and Valencia oranges. Fruit of both red-fleshed varieties displayed higher levels of norisoprenoids, monoterpenes, long-chain aldehydes and variable content of aliphatic esters. Then, the unusual content of carotenoids does not only influence the color but also the aroma of the mutant fruit. Taken together, these results indicate that Kirkwood Navel and Ruby Valencia oranges display similar responses to cold storage and shelf-life than the ordinary oranges, with a good attitude for the postharvest management and long-term storage ensuring their quality and the potential added value for the consumers.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Carotenoid content and composition (mg kg^{-1} FW) in the flavedo of Navel and Kirkwood at harvest, 28 d at 2 °C (cold storage) and 5 d 20 °C (shelf-life). Violaxanthin is the sum of all E-violaxanthin and 9-Z-violaxanthin. Nd, no detected. Data are the mean of two biological replicates ± SD; Table S2: Carotenoid content and composition (mg kg^{-1} FW) in the flavedo of Valencia and Ruby at harvest, 28 d at 2 °C (cold storage) and 5 d 20 °C (shelf-life). Violaxanthin is the sum of all E-violaxanthin and 9-Z-violaxanthin. Nd, no detected. Data are the mean of two biological replicates ± SD; Table S3: Chromatographic characteristics of volatile organic compounds detected by HS-SPME/GC-MS in all orange samples.; Table S4: Relative levels (fold change) of VOCs detected in the pulp of Navel and Kirkwood fruit at harvest, 28 d at 2 °C (cold storage) and 5 d 20 °C (shelf-life); Table S5: Relative levels (fold change) of VOCs detected in the pulp of Valencia and Ruby fruit at harvest, 28 d at 2 °C (cold storage) and 5 d 20 °C (shelf-life).

Data availability

All data are available in the manuscript or supplementary materials.

CRediT authorship contribution statement

Jaime Zacarías-García: performed the experiments, methodology, data analysis, writing – original draft, review and editing. **Jose Luis Rambla**: supervised volatile analysis and data curation; writing – review; **Antonio Granell:** supervised volatile analysis, writing – review. **Mª Jesus Rodrigo** and **Lorenzo Zacarías:** conceptualization and coordination, project management, writing – review and editing. All authors have read the manuscript and agree to publish the current version

Declaration of Competing Interest

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

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References

Alquézar, B., Rodrigo, M.J., Zacarías, L., 2008. Regulation of carotenoid biosynthesis during fruit maturation in the red-fleshed orange mutant Cara Cara. Phytochem. 69, 1997–2007. https://doi.org/10.1016/j.phytochem.2008.04.020

Baltazari, A., Mtui, H.D., Mwatawala, M.W., Chove, L.M., Msogoya, T., Samwel, J., Subramanian, J., 2020. Effects of storage conditions, storage duration and post-harvest treatments on nutritional and sensory quality of orange (*Citrus sinensis* (L) Osbeck) Fruit. Int. J. Fruit Sci. 20(4), 737-749. https://doi.org/10.1080/15538362.2019.1673278

Ben-Yehoshua, S. 1987**.** Transpiration, water stress and gas exchange, in: Weichmann, J. (Eds.), Postharvest Physiology of Vegetables. Marcel Dekker, New York, pp. 113–170.

Brandi, F., Bar, E., Mourgues, F., Horváth, G., Turcsi, E., Giuliano, G., Liverani, A., Tartarini, S., Lewinsohn, E., Rosati, C., 2011. Study of 'Redhaven' peach and its white-fleshed mutant suggests a key role of CCD4 carotenoid dioxygenase in carotenoid and norisoprenoid volatile metabolism. BMC Plant Biol. https://doi.org/10.1186/1471-2229-11-24

Carmona, L., Zacarías, L., Rodrigo, M.J., 2012. Stimulation of coloration and carotenoid biosynthesis during postharvest storage of 'Navelina' orange fruit at 12 °C. Postharvest Biol. Technol. 74, 108–117. https://doi.org/10.1016/j.postharvbio.2012.06.021

Chaudhary, P.R., Yu, X., Jayaprakasha, G.K., Patil, B.S., 2016**.** Influence of storage temperature and low-temperature conditioning on the levels of health-promoting compounds in Rio Red grapefruit. Food Sci Nutr. 5, 545–553. https://doi.org/10.1002/fsn3.429

Chen, J., Zhang, H., Pang, Y., Cheng, Y., Deng, X., Xu, J., 2015. Comparative study of flavonoid production in lycopene-accumulated and blonde-flesh sweet oranges (*Citrus sinensis*) during fruit development. Food Chem., 184, 238–246. https://doi.org/10.1016/j.foodchem.2015.03.087

De Ancos, B., Cilla, A., Barberá, R., Sánchez-Moreno, C., Cano, M.P., 2017. Influence of orange cultivar and mandarin postharvest storage on polyphenols, ascorbic acid and antioxidant activity during gastrointestinal digestion. Food Chem. 225, 114–124. https://doi.org/10.1016/j.foodchem.2016.12.098

Del Caro, A., Piga, A., Vacca, V., Agabbio, M., 2004. Changes of flavonoids, vitamin C and antioxidant capacity in minimally processed citrus segments and juices during storage. Food Chem. 84, 99–105. https://doi.org/10.1016/S0308-8146(03)00180-8

Deng, Y., Tu, Y., Lao, S., Wu, M., Yin, H., Wang, L., Liao, W., 2022. The role and mechanism of citrus flavonoids in cardiovascular diseases prevention and treatment. Crit. Rev. Food Sci. Nutr. 62, 7591–7614. https://doi.org/10.1080/10408398.2021.1915745

Dudareva, N., Klempien, A., Muhlemann, J.K., Kaplan, I., 2013. Biosynthesis, function and metabolic engineering of plant volatile organic compounds. New Phytol. 198, 16-32. https://doi.org/10.1111/nph.12145

FAOSTAT statistical database. 2022. Food and Agriculture Organization of the United Nations (FAO).

Gao, Y., Liu, Y., Kan, C., Chen, M., Chen, J., 2019**.** Changes of peel color and fruit quality in navel orange fruit under different storage methods. Sci. Hortic. 256, 108522. https://doi.org/10.1016/j.scienta.2019.05.049

Goldenberg, L., Yanivb, Y., Choic, H.I., Doron-Faigenboimb, A., Carmib, N., Porat, R., 2016. Elucidating the biochemical factors governing off-flavor perception in mandarins. Postharvest Biol. Technol. 120, 167-179. https://doi.org/10.1002/jsfa.8495

González-Mas, M.C., Rambla, J.L., Alamar, M.C., Gutiérrez, A., Granell, A., 2011. Comparative analysis of the volatile fraction of fruit juice from different citrus species. PLoS One 6, e22016. https://doi.org/10.1371/journal.pone.0022016

González-Mas, M.C., Rambla, J.L., López-Gresa, M.P., Blázquez, M.A., Granell, A., 2019. Volatile compounds in citrus essential oils: A comprehensive review. Front. Plant Sci. 10, 12. https://doi.org/10.3389/fpls.2019.00012

Habibi, F., Guillén, F., Serrano, M., Valero, D., 2021. Physicochemical changes, peel colour, and juice attributes of blood orange cultivars stored at different temperatures. Horticulturae 7, 320. https://doi.org/10.3390/horticulturae7090320

Habibi, F., Ramezanian, A., Guillén, F., Castillo, S., Serrano, M., Valero, D., 2020a**.** Changes in bioactive compounds, antioxidant activity, and nutritional quality of blood orange cultivars at different storage temperatures. Antioxidants 9, 1016. https://doi.org/10.3390/antiox9101016

Habibi, F., Ramezanian, A., Guillén, F., Serrano, M., Valero D., 2020b. Effect of various postharvest treatment on aroma volatile compounds of blood orange fruit exposed to chilling temperature after long-term storage. Food and Bioprocess Technol. 13, 2054-2064. https://doi.org/10.1007/s11947-020-02547-1

Lado, J., Cronje, P.J.R., Rodrigo, M.J., Zacarías, L., 2019a. *Citrus*, in: de Freitas, S.T., Pareek, S. (Eds.), Postharvest Physiological Disorders of Fruit and Vegetables. CRC Press: Boca Raton, FL, USA, pp. 321–342. https://doi.org/10.1201/b22001

Lado, J., Gambetta, G., Zacarías, L., 2018. Key determinants of citrus fruit quality: Metabolites and main changes during maturation. Sci. Hortic. 233, 238–248. https://doi.org/10.1016/j.scienta.2018.01.055

Lado, J., Gurrea, A., Zacarías, L., Rodrigo, M.J., 2019b. Influence of the storage temperature on volatile emission, carotenoid content and chilling injury development in Star Ruby red grapefruit. Food Chem. 295, 72-81. https://doi.org/10.1016/j.foodchem.2019.05.108

Lado, J., Rodrigo, M.J., Zacarías, L., 2014. Analysis of ethylene biosynthesis and perception during postharvest cold storage of Marsh and Star Ruby grapefruit. Food Sci. Technol. Inter. 21, 537–546. https://doi.org/10.1177/1082013214553

Lafuente, M.T, Ballester, A.R., Callejero, J., González-Candelas, L., 2011. Effect of high-temperature-conditioning treatments on quality, flavonoid composition and vitamin C of cold stored 'Fortune' mandarins. Food Chem. 128, 1080-1086. https://doi.org/1080-1086. 10.1016/j.foodchem.2011.03.129

Lattanzio, V., Cardinali, A., Palmieri, S., 1994. The role of phenolics in the postharvest physiology of fruit and vegetables: Browning reaction and fungal diseases. Ital. J. Food Sci. 1, 3-22.

Lewinsohn, E., Sitrit, S., Bar, E., Azulay Y., Meir, A., Zamir, D., Tadmor, Y., 2005b. Carotenoid pigmentation affects the volatile composition of tomato and watermelon fruit, as revealed by comparative genetic analyses. J. Agric. Food Chem. 53, 3142-3148. https://doi.org/10.1021/jf047927t

Lewinsohn, E., Sitrit, Y., Bar, E., Azulay, Y., Ibdah, M., Meir, A. Yosef, E., Zamir, E., Tadmor, Y. 2005a. Not just colors-carotenoid degradation as a link between pigmentation and

aroma in tomato and watermelon fruit. Trends Food Sci. Technol. 16, 407–415. https://doi.org/10.1016/j.tifs.2005.04.004

Liu, C., He, M., Wang, Z., Xu, J., 2019. Integrative analysis of terpenoid profiles and hormones from fruit of red-flesh citrus mutants and their wild types. Molecules 24, 3456. https://doi.org/10.3390/molecules24193456

Liu, Q., Xu, J., Liu, Y., Zhao, X., Deng, X., Guo, L., Gu, J., 2007. A novel bud mutation that confers abnormal patterns of lycopene accumulation in sweet orange fruit (*Citrus sinensis* L. osbeck). J. Exp. Bot., 58, 4161–4171. https://doi.org/10.1093/jxb/erm273

Lu, P.J., Wang, C.Y., Yin, T.T., Zhong, S.L., Grierson, D., Chen, K.S., 2017. Cytological and molecular characterization of carotenoid accumulation in normal and high-lycopene mutant oranges. Sci. Rep. 7, 1–15. https://doi.org/10.1038/s41598-017-00898-y

Lv, X., Zhao, S., Ning, Z., Zeng, H., Shu, Y., Tao, O., Xiao, C., Lu, C., Liu, Y., 2015**.** Citrus fruit as a treasure trove of active natural metabolites that potentially provide benefits for human health. Chem. Cent. J. 9, 68. https://doi.org/10.1186/s13065-015-0145-9

Mahattanatawee, K., Rouseff, R., Valim, M.F., Naim, M., 2005. Identification and aroma impact of norisoprenoids in orange juice. J. Agric. Food Chem. 53, 393-397. https://doi.org/10.1021/jf049012k

Manzi, M., Hernández-Mazzini, F., Pintos, P., Lado, J., 2022. Abscisic acid preharvest application alleviates chilling injury in oranges during cold storage. J. Hortic. Sci. Biotechnol. 97, 747-756. https://doi.org/10.1080/14620316.2022.2061383

Martínez, A., Stinco, C.M., Meléndez-Martínez, A.J., 2014. Free radical scavenging properties of phytofluene and phytoene isomers as compared to lycopene: A combined experimental and theoretical study. J. Phys. Chem. 118, 9819–9825. https://doi.org/10.1021/jp503227j

Matsumoto, H., Ikoma, Y., Kato, M., Nakajima, N., Hasegawa, Y., 2009. Effect of postharvest temperature and ethylene on carotenoid accumulation in the flavedo and juice sacs of Satsuma mandarin (*Citrus unshiu* Marc.) fruit. J. Agric. Food Chem. 57, 4724–4732. https://doi.org/10.1021/jf9005998

Mditshwaa, A., Magwazab, L.S., Tesfaya, S.Z., Oparac, U.L., 2017. Postharvest factors affecting vitamin C content of citrus fruit: A review. Sci. Hortic. 128, 95-104. https://doi.org/10.1016/j.scienta.2017.02.024

Muñoz, P., Munne-Bosch, S., (2019). Vitamin E in plants: biosynthesis, transport, and function. Trends Plant Sci. 24, 1040–1051. https://doi.org/10.1016/j.tplants.2019.08.006

Obenland, D., Collin, S., Sievert, J., Arpaia, M.L., 2013. Mandarin flavor and aroma volatile composition are strongly influenced by holding temperature. Postharvest Biol. Technol. 82, 6–14. https://doi.org/10.1016/j.postharvbio.2013.02.013

Obenland, D., Collin, S., Sievert, J., Fjeld, K., Doctor, J., Arpaia, M.L., 2008. Commercial packing and storage of navel oranges alters aroma volatiles and reduces flavour quality. Postharvest Biol. Technol. 47, 159–167. https://doi.org/10.1016/j.postharvbio.2007.06.015

Obenland, D.M., Aung, L.H., Bridges, D.L., Mackey, B.E., 2003. Volatile emissions of Navel oranges as predictors of freeze damage. J. Agric. Food Chem. 51, 3367–3371. https://doi.org/10.1021/jf021109o

Pérez-Cacho, P.R., Rouseff, R.L., 2008a**.** Fresh squeezed orange juice odor: a review. Crit. Rev. Food Sci. Nutr. 48, 681-695. https://doi.org/10.1080/10408390701638902

Pérez-Cacho, P.R., Rouseff, R.L., 2008b. Processing and storage effects on orange juice aroma: a review. J. Agric. Food Chem. 56, 9785-9796. https://doi.org/10.1021/jf801244j

Peterson, J.J., Dwyer, J.T., Beecher, G.R., Bhagwat, S.A., Gebhardt, S.E., Haytowitz, D.B., Holden, J.M., 2006. Flavanones in oranges, tangerines (mandarins), tangors, and tangelos: a compilation and review of the data from the analytical literature. J. Food Anal. 19, 66–73. https://doi.org/10.1016/j.jfca.2005.12.006

Rapisarda, P., Bianco, M.L., Pannuzzo, P., Timpanaro, N., 2008. Effect of cold storage on vitamin C, phenolics and antioxidant activity of five orange genotypes (*Citrus sinensis* (L.) Osbeck). Postharvest Biol. Technol. 49, 348–354. https://doi.org/10.1016/j.postharvbio.2008.02.002

Rey, F., Zacarías, L., Rodrigo, M.J., 2020**.** Carotenoids, vitamin C, and antioxidant capacity in the peel of mandarin fruit in relation to the susceptibility to chilling injury during postharvest cold storage. Antioxidants 9, 1296. https://doi.org/10.3390/antiox9121296

Rodrigo, M.J, Zacarías, L., 2007. Effect of postharvest ethylene treatment on carotenoid accumulation and the expression of carotenoid biosynthetic genes in the flavedo of orange (*Citrus sinensis* L. Osbeck) fruit. Postharvest Biol. Technol. 41, 14-22. https://doi.org/10.1021/jf9005998

Rodrigo, M.J., Alquézar, B., Alòs, E., Lado, J., Zacarías, L., 2013. Biochemical bases and molecular regulation of pigmentation in the peel of Citrus fruit. Sci. Hortic. 163, 46–62. https://doi.org/10.1016/j.scienta.2013.08.014

Saini, R.K., Ranjit, A., Sharma, K., Prasad, P., Shang, X., Gowda, K.G.M., Keum, Y.S., 2022**.** Bioactive compounds of Citrus fruit: A review of composition and health benefits of carotenoids, flavonoids, limonoids, and terpenes. Antioxidants 11, 239. https://doi.org/10.3390/antiox11020239

Stahl, W., Sies, H., 2003**.** Antioxidant activity of carotenoids. Mol. Asp. Med. 24, 345-351. https://doi.org/10.1016/s0098-2997(03)00030-x

Strano, M.C., Altieri, G., Allegra, M., Di Renzo, G.C., Paterna, G., Matera, A., Genovese, F., 2022. Postharvest technologies of fresh citrus fruit: advances and recent developments for the loss reduction during handling and storage. Horticulturae 8, 612. https://doi.org/10.3390/horticulturae8070612

Sun, X.H., Xiong, J.J., Zhu, A.D., Zhang, L., Ma, Q.L., Xu, J., Cheng, Y.J., Deng, X.X., 2012. Sugars and organic acids changes in pericarp and endocarp tissues of pumelo fruit during postharvest storage. Sci. Hortic. 142, 112–117. https://doi.org/10.1016/j.scienta.2012.05.009

Tang, N., Deng, W., Hu, N., Chen, N., Li, Z., 2016. Metabolite and transcriptomic analysis reveals metabolic and regulatory features associated with Powell orange pulp deterioration during room temperature and cold storage. Postharvest Biol. Technol. 112, 75–86. https://doi.org/10.1016/j.postharvbio.2015.10.008

Tao, N., Wang, C., Xu, J., Chen, Y., 2012. Carotenoid accumulation in postharvest Cara Cara navel orange (*Citrus sinensis* Osbeck) fruit stored at different temperatures was transcriptionally regulated in a tissue-dependent manner. Plant Cell Rep. 31, 1667–1676. https://doi.org/10.1007/s00299-012-1279-z

Tietel, Z., Plotto, A., Fallik, E., Lewinsohn, E., Porat, R., 2011. Taste and aroma of fresh and stored mandarins. J. Sci. Food Agric. 91, 14–23. https://doi.org/10.1002/jsfa.4146

Wei, X. Song, M., Chen, C., Tong, H., Liang, G., Gmitter, F.G., 2018**.** Juice volatile composition differences between Valencia orange and its mutant Rohde Red Valencia are associated with carotenoid profile differences. Food Chem. 245, 223-32. https://doi.org/10.1016/j.foodchem.2017.10.066

Xu, Q., Yu, K., Zhu, A., Ye, J., Liu, Q., Zhang, J., Deng, X., 2019. Comparative transcripts profiling reveals new insight into molecular processes regulating lycopene accumulation in a sweet orange (*Citrus sinensis*) red-flesh mutant. BMC Genet. 10, 540. https://doi.org/10.1186/1471-2164-10-540

Zacarías, L., Alférez, F., 2014. Influence of fruit maturity in the susceptibility of Navelina oranges to develop postharvest non-chilling peel pitting. Food Sci. Technol. Int. 20, 183-91. https://doi.org/10.1177/1082013213476077

Zacarías, L., Cronje, P.J., Palou, L., 2020. Postharvest technology of citrus fruit, in: Talon, M., Caruso, M., Gmitter, F.G., Jr. (Eds.), The Genus *Citrus*. Woodhead Publishing: Sawston, UK, pp. 421–446.

Zacarías-García, J., Cronje, P., Diretto, G., Zacarías, L., Rodrigo, M.J., 2022a. A comprehensive analysis of carotenoids metabolism in two red-fleshed mutants of Navel and Valencia oranges (*Citrus sinensis*). Front. Plant Sci. 13, 1034204. https://doi.org/10.3389/fpls.2022.1034204

Zacarías-García, J., Pérez-Través, L., Gil, J.V., Rodrigo, M.J., Zacarías, L., 2022b. Bioactive compounds, nutritional quality and antioxidant capacity of the red-fleshed Kirkwood Navel and Ruby Valencia oranges. Antioxidants 11, 1905. https://doi.org/10.3390/antiox11101905

Zhang, J., Liang, Y., He, L., Kaliaperumal, K., Tan, H., Jiang, Y., Zhong, B., Zhang, J., 2022. Effects of storage time and temperature on the chemical composition and organoleptic quality of Gannan navel orange (*Citrus sinensis* Osbeck cv. Newhall). J. Food Meas. Charact. 16, 935–944. https://doi.org/10.1007/s11694-021-0

Supplementary Material

Table Supplementary 1. Carotenoid content and composition (mg kg-1) in the flavedo of Navel and Kirkwood at harvest, 28 d at 2 °C (cold storage) and 5 d at 20 °C (shelf-life). Violaxanthin is the sum of all E-violaxanthin and 9-Z-violaxanthin. Nd, no detected. Data are the mean of two biological replicates ± SD.

Carotenoids	Navel			Kirkwood		
	Harvest	4w 2 °C	+5d 20 °C	Harvest	4w 2 °C	+5d 20 °C
Phytoene	66.93±7.59	61.54±12.38	90.62±3.93	163.26±9.05	124.85±30.82	237.77±38.97
Phytofluene	9.68±3.27	10.77±1.11	22.53±2.23	10.86±0.37	8.93±2.23	17.46±2.17
ζ-carotene	0.33±0.01	2.26±0.45	2.84±0.74	0.41±0.43	0.44±0.14	0.94±0.31
Lutein	3.78±3.29	3.67±1.24	2.46±0.31	0.44±0.12	0.51±0.16	0.91±0.43
β-cryptoxanthin	5.85±0.6	6.46±0.62	11.36±1.51	3.85±0.09	4.23±0.23	6.25±2.25
Zeaxanthin	ND	ND	1.24±0.25	0.74±0.02	0.57±0.09	0.95±0.49
β-citraurin	ND	ND	8.69±3.13	7.99±4.79	4.94±3.36	0.91±0.21
Anteraxanthin	1.14±1.61	3.2±0.67	5.96±1.14	6.33±0.45	5.13±0.19	5.14±0.94
Violaxanthin	104.35±9.60	105.37±17.64	159.63±27.01	75.3±3.58	67.7±5.07	95.86±21.63
Luteoxanthin	2.95±0.05	1.81±0.13	11.1±3.89	1.58±0.31	1.11±0.24	2.59±0.64
Mutatoxanthin	5.94±0.65	5.2±1.39	12.44±1.98	4.06±0.29	3.43±0.17	6.4±1.71
Total	200.96±1.74	200.28±33.41	328.88±36.04	274.79±0.63	221.77±42.64	375.12±8.23

Table Supplementary 2. Carotenoid content and composition (mg kg^{-1}) in the flavedo of Valencia and Ruby at harvest, 28 d at 2 °C (cold storage) and 5 d at 20 °C (shelf-life). Violaxanthin is the sum of all E-violaxanthin and 9-Z-violaxanthin. Nd, no detected. Data are the mean of two biological replicates ± SD.

Carotenoids	Valencia			Ruby		
	Harvest	4w 2 °C	+5d 20 °C	Harvest	4w 2 °C	+5d 20 °C
Phytoene	18.57±1.92	6.44±3.2	23.75±0.94	130.22±16.65	125.24±0.5	267.59±0.29
Phytofluene	2.41±0.12	2.42±0.61	6.33±0.41	5.09±1.36	6.53±2.11	17.02±1.31
Lutein	1.82±0.17	2.98±0.29	3.71±0.52	1.09±0.24	1.54±0.1	3.36±0.94
β-cryptoxanthin	2.77±0.22	4.53±0.68	5.23±0.45	1.56±0.48	2.61±0.19	3.62±0.01
Zeaxanthin	0.72±0.06	0.95±0.26	1.35±0.25	0.64±0.3	1.22±0.11	1.57±0.11
β-citraurin	1.66±0.30	2.95±1.42	4.31±0.27	ND	ND	ND
Anteraxanthin	3.21±0.13	4.76±0.58	6.68±1.18	5.1±1.78	6.97±0.84	7.82±0.36
Violaxanthin	119.75±1.33	135.8±19.94	186.05±23	79.38±17.74	85.16±2.02	143.98±7.43
Luteoxanthin	4.01±1.18	4.25±2.5	5.76±0.62	1.75±0.4	1.75±0.31	4.18±0.1
Mutatoxanthin	4.42±0.05	6.06±1.02	7.41±1.04	2.47±0.9	3.43±1.2	5.36±0.07
Total	159.30±1.45	171.11±27.61	250.56±26.23	227.27±39.81	235.83±6.74	454.46±8.57

Table Supplementary 3. Chromatographic characteristics of volatile organic compounds detected by HS-SPME/GC-MS in all orange samples.

Code	Volatile Organic Compound	RI$^{\alpha}$	RI$^{\beta}$	Family code	Specific ion (m/z)	RT (min)
1	α-pinene	946	939	Mt hd	93	21.57
2	Camphene	967	953	Mt hd	93	22.33
3	Myrcene	989	992	Mt hd	91	23.16
4	β-pinene	994	981	Mt hd	93	23.35
5	α-phellandrene	1018	1007	Mt hd	136	24.17
6	3-carene	1021	1009	Mt hd	93	24.31
7	α-terpinene	1028	1012	Mt hd	121	24.53
8	p-cymene	1029	1027	Mt hd	119	24.8
9	Limonene	1042	1033	Mt hd	108	25
10	γ-terpinene	1068	1074	Mt hd	93	25.92
11	Terpinolene	1097	1088*	Mt hd	121	26.93
12	Eucalyptol[b]	1051	1033*	Mnt	154	25.26
13	(Z)-linalool oxide[c]	1081	1070	Mnt	59	26.37
14	(E)-linalool oxide[c]	1170	1172	Mnt	59	26.9
15	Linalool[d]	1100	1100	Mnt	93	27.04
16	(Z)-limonene oxide[b]	1138	1137	Mnt	67	28.6
17	(E)-limonene oxide[b]	1140	1139	Mnt	94	28.78
18	β-citronellal[f]	1155	1159	Mnt	69	28.78
19	Terpinen-4-ol[d]	1200	1179	Mnt	93	30.26
20	α-terpineol[d]	1212	1195	Mnt	59	30.61
21	Dihydrocarvone[e]	1217	1202	Mnt	95	30.74
22	β-citronellol[d]	1226	1233	Mnt	81	31.01
23	(E)-carveol[d]	1234	1197	Mnt	137	31.27
24	Carvone[e]	1264	1217	Mnt	82	32.16
25	Perillaldehyde[f]	1300	1319	Mnt	68	33.27
26	(Z)-carvyl acetate[g]	1338	1411	Mnt	84	34.37
27	Citronellyl acetate[g]	1345	1357	Mnt	95	34.57
28	Sesquiterpene 1	undetermined		Sqt	93	35.28
29	α-copaene	1406	1377	Sqt	119	36.3
30	Sesquiterpene 2	undetermined		Sqt	93	36.48
31	Sesquiterpene 3	undetermined		Sqt	93	36.62
32	β-caryophyllene	1460	1467	Sqt	133	37.75
33	Sesquiterpene 4	undetermined		Sqt	93	38.31
34	α-humulene	1496	1467	Sqt	80	38.7
35	Sesquiterpene 5	undetermined		Sqt	189	39.37
36	Valencene	1530	1490	Sqt	67	39.56
37	Sesquiterpene 6	undetermined		Sqt	189	39.72
38	Sesquiterpene 7	undetermined		Sqt	119	39.84
39	Sesquiterpene 8	undetermined		Sqt	161	40.38
40	Nootkatone	1858	1814	Sqt	121	47.87
41	6-methyl-5-hepten-2-one[a,e]	982	985*	Nor	108	22.9
42	Nerol[a,d]	1129	1233	Nor	93	31.18
43	β-cyclocitral[a,f]	1241	1250	Nor	137	31.49
44	Neral[a,f]	1240	1247	Nor	109	31.66
45	Geranial[a,f]	1273	1277	Nor	69	32.46
46	Neryl acetate[g]	1355	1362	Nor	69	34.84
47	Geranyl acetate[g]	1374	1382	Nor	69	35.37
48	Geranylacetone[a,e]	1451	1448	Nor	43	37.49
49	α-ionone[a,e]	1438	1422	Nor	192	37.23
50	β-ionone[a,e]	1499	1493	Nor	177	38.78
51	Ethanol	667	668	Alc	45	5.02
52	1-penten-3-ol	756	767	Alc	57	11.24
53	1-hexanol	862	851	Alc	56	15.87

Table Supplementary 3. Continuation.

Code	Volatile Organic Compound	RI$^{\alpha}$	RI$^{\beta}$	Family code	Specific ion (m/z)	RT (min)
54	1-octen-3-ol	977	982	Alc	57	22.72
55	1-octanol	1067	1072	Alc	56	25.92
56	Acetaldehyde	400	427	Ald	43	4.31
57	Pentanal	688	732	Ald	58	11.82
58	(E)-2-pentenal	748	754	Ald	83	14.1
59	(Z)-3-hexenal	791	800	Ald	69	15.77
60	Hexanal	794	801	Ald	56	15.87
61	(E)-2-hexenal	852	854	Ald	83	18.01
62	Heptanal	901	903	Ald	70	19.86
63	(E)-2-heptenal	959	957	Ald	68	22.01
64	Octanal	1003	1006	Ald	57	23.64
65	(E)-2-octenal	1061	1060	Ald	70	25.66
66	Nonanal	1105	1104	Ald	57	27.17
67	(E)-2-nonenal	1164	1162	Ald	70	29.07
68	Decanal	1207	1209	Ald	57	30.46
69	Undecanal	1309	1291	Ald	57	33.27
70	(E,E)-2,4-decadienal	1328	1284	Ald	81	34.04
71	Dodecanal	1411	1407*	Ald	57	36.42
72	1-penten-3-one	675	680	Ket	55	11.33
73	1-octen-3-one	977	976	Ket	70	22.68
74	Ethyl acetate	608	628	Est	61	8.74
75	Ethyl propionate	397	713	Est	57	12.22
76	Ethyl butanoate	790	804	Est	88	15.74
77	Ethyl 2-methylbutyrate	842	776	Est	102	17.71
78	Methyl hexanoate	991	1000	Est	74	20.55
79	Ethyl hexanoate	992	1002	Est	88	23.26
80	Hexyl acetate	1119	1014	Est	56	23.73
81	Ethyl heptanoate	1091	1100	Est	88	26.7
82	Methyl octanoate	1119	1041	Est	74	27.62
83	Octyl acetate	1203	1149	Est	70	29.07
84	Ethyl octanoate	1189	1198	Est	88	29.92
85	Ethyl nonanoate	1289	1297	Est	88	33.05
86	Hexyl hexanoate	1299	1379	Est	117	35.58
87	Ethyl decanoate	1388	1398*	Est	88	35.8
88	Decyl acetate	1401	1408*	Est	70	36.16
89	2-methylfuran	602	602^{+}	Fur	82	8.49
90	3-methylfuran	610	610^{+}	Fur	82	8.81
91	2-ethylfuran	690	690^{+}	Fur	81	11.94
92	2-pentylfuran	992	993	Fur	138	23.25

Family code: Mt hd, monoterpene hydrocarbon; Mnt, monoterpenoids; Sqt, sesquiterpene; Nor, noisoprenoid; Alc; alcohol; Ald; aldehyde; Ket, ketone; Est, ester; Fur; furane; Ac, acid; Ar, aromatic hydrocarbon. RI: retention index. [a]Monoterperne derived compound. [b]Its cyclic ether group corresponds to an epoxy group. [c]It contains an alcohol group and a tetrahydrofuran group. [d]It contains an alcohol group. [e]It contains a ketone group. [f]It contains an aldehyde group. [g]It contains an ester group. [α]Calculated retention indices based on a series of n-alkanes. [β]Published retention indices on DB-5 column according to the Flavornet database (www.flavornet.org). *Published retention indices on DB-5 column according to the Pherobase database (www.pherobase.com). [+]Retenxion indices according to Sdiri et al. (2015).

Table Supplementary 4. Relative levels (fold change) of VOCs detected in the pulp of Navel and Kirkwood fruits at harvest, 28 d at 2 °C (cold storage) and 5 d at 20 °C (shelf-life).

			Navel			Kirkwood		
Code	VOCs	Family	Harvest	4w 2 °C	+5d 20 °C	Harvest	4w 2 °C	+5d 20 °C
1	α-pinene[1]	Mt hd	1.00±0.01b	3.20±0.80a	1.13±0.13b	1.01±0.18c	4.12±0.42a	2.21±0.03b
2	Camphene[1]	Mt hd	1.00±0.02a	1.38±0.52a	1.01±0.05a*	0.99±0.16c	2.57±0.12a	1.69±0.02b
3	Myrcene[1]	Mt hd	1.00±0.20b	1.95±0.11a	0.83±0.01b*	1.02±0.11b	2.61±0.02a	2.49±0.08a
4	β-pinene[1]	Mt hd	1.00±0.02a	1.92±0.64a	1.10±0.09a*	0.95±0.24c	3.40±0.17a	2.63±0.01b
5	α-phellandrene[1]	Mt hd	1.00±0.27b	1.97±0.25a	0.72±0.04b*	1.03±0.28b	3.17±0.20a	3.09±0.01a
6	3-carene[1]	Mt hd	1.00±0.23b	4.04±0.77a	0.77±0.05b*	1.04±0.15c	4.65±0.15a	3.53±0.15b
7	α-terpinene[1]	Mt hd	1.00±0.23a	1.91±0.49a	0.79±0.01a*	1.03±0.47b	3.26±0.04a	2.50±0.08a
8	p-cymene[1]	Mt hd	1.00±0.34a	nd	1.12±0.01a	1.17±0.49a	1.95±0.84a	nd
9	Limonene[1]	Mt hd	1.00±0.04b	1.30±0.01a	1.01±0.01b*	1.02±0.07b	1.47±0.01a	1.44±0.07a
10	γ-terpinene[1]	Mt hd	1.00±0.36a	1.93±0.56a	0.79±0.02a*	0.98±0.37b	3.19±0.13a	2.48±0.09a
11	Terpinolene[1]	Mt hd	1.00±0.23b	1.92±0.03a	0.69±0.01b*	1.03±0.41b	2.73±0.11a	2.46±0.12a
12	Eucalyptol[1]	Mnt	1.00±0.01b	1.68±0.10a	0.52±0.01c*	1.01±0.17b	2.44±0.10a	2.20±0.19a
13	(Z)-linalool oxide[1]	Mnt	1.00±0.01b	1.55±0.14a	1.69±0.14a	0.89±0.48a	nd	2.19±0.63a
14	(E)-linalool oxide[1]	Mnt	1.00±0.32a	nd	nd	0.70±0.34a	nd	nd
15	Linalool[1]	Mnt	1.00±0.34b	2.11±0.09a	0.39±0.01b*	1.04±0.12b	2.52±0.51a	3.04±0.15a
16	(Z)-limonene	Mnt	1.00±0.43a	0.56±0.14a	0.88±0.09a	0.92±0.46a	0.47±0.01a	1.00±0.06a
17	(E)-limonene	Mnt	1.00±0.40a	0.83±0.06a	1.27±0.12a	0.82±0.24a	0.63±0.05b	1.37±0.02a
18	β-citronellal[1]	Mnt	1.00±0.18a	3.74±1.33a	1.06±0.16a	1.53±1.05a	3.00±1.32a	1.94±1.05a
20	Terpinen-4-ol[1]	Mnt	1.00±0.38b	4.02±0.62a	0.65±0.03b*	1.04±0.5b	4.33±0.31a	3.33±0.05a
21	α-terpineol[1]	Mnt	1.00±0.56a	1.66±0.14a	0.35±0.03a*	1.04±0.04b	2.36±0.19a	2.47±0.21a
22	Dihydrocarvone[1]	Mnt	1.00±0.26b	1.78±0.03a	0.83±0.02b*	1.11±0.01b	1.63±0.22b	2.3±0.01a
23	β-citronellol[1]	Mnt	1.00±0.15b	2.00±0.12a	0.80±0.07b*	1.12±0.27b	2.23±0.16a	2.37±0.04a
24	Carvone[1]	Mnt	1.00±0.03b	1.27±0.02a	1.08±0.08ab	1.05±0.03c	1.60±0.02b	1.75±0.01a
25	Perillaldehyde[1]	Mnt	1.00±0.11b	1.28±0.02a	0.35±0.03c*	1.17±0.07a	1.53±0.24a	1.52±0.13a
26	(Z)-carvyl acetate[1]	Mnt	1.00±0.50a	1.35±0.01a	0.86±0.24a	1.23±0.2ab	0.30±0.10b	1.54±0.34a
27	Citronellyl	Mnt	1.00±0.17a	1.27±0.03a	0.66±0.02b*	0.88±0.34a	1.02±0.17a	1.45±0.02a
28	Sesquiterpene 1[1]	Sqt	1.00±0.15b	2.79±0.44a	1.11±0.01b*	1.28±0.55a	2.02±0.28a	3.23±0.61a
29	α-copaene[1]	Sqt	1.00±0.35b	2.38±0.10a	0.98±0.08b*	0.74±0.09b	2.30±0.12a	2.79±0.39a
30	Sesquiterpene 2[1]	Sqt	1.00±0.05a*	0.45±0.01c*	0.84±0.03b*	0.15±0.01b	0.23±0.01b	0.49±0.11a
31	Sesquiterpene 3[1]	Sqt	1.00±0.53b	2.43±0.07a	0.51±0.04b*	1.10±0.28b	2.21±0.11a	1.81±0.04a
32	β-caryophyllene[1]	Sqt	1.00±0.03a	0.65±0.02b	0.90±0.02a	0.24±0.05b	0.53±0.04a	0.73±0.11a
33	Sesquiterpene 4[1]	Sqt	1.00±0.08a*	0.36±0.02b	0.84±0.02a*	0.11±0.01b	0.13±0.01b	0.32±0.03a
34	α-humulene[1]	Sqt	1.00±0.01a*	0.72±0.03b	0.87±0.07ab	0.23±0.04b	0.48±0ab	0.72±0.13a
35	Sesquiterpene 5[1]	Sqt	1.00±0.02a*	0.41±0.02c*	0.87±0.01b*	0.12±0.03b	0.13±0.01b	0.29±0.02a
36	Valencene[1]	Sqt	1.00±0.01a*	0.54±0.01b	0.92±0.04a*	0.16±0.02b	0.20±0.01b	0.37±0.02a
37	Sesquiterpene 6[1]	Sqt	1.00±0.04a*	0.40±0.02b	0.93±0.01a*	0.11±0.02b	0.13±0b	0.32±0.02a
38	Sesquiterpene 7[1]	Sqt	1.00±0.02a*	0.34±0.01c*	0.86±0.02b*	0.10±0.02b	0.11±0.02b	0.28±0.01a
39	Sesquiterpene 8[1]	Sqt	1.00±0.01a*	0.37±0.02c*	0.83±0.01b*	0.10±0.02b	0.14±0.02b	0.24±0.01a
40	Nootkatone[2]	Sqt	nd	1.63±0.16a	1.00±0.02b*	nd	0.25±0.03a	0.21±0.05a
41	MHO	Nor	1.00±0.08a*	0.48±0.10b	0.65±0.05b*	8.51±0.46a	2.75±0.08b	2.75±0.43b
42	β-cyclocitral[1]	Nor	1.00±0.44b	2.09±0.01a	0.58±0.01b*	1.11±0.02b	2.25±0.01a	2.60±0.48a
43	Neral[1]	Nor	1.00±0.55a	1.57±0.06a	0.20±0.01b*	1.10±0.05b	2.19±0.11a	2.18±0.38a
45	Geranial[1]	Nor	1.00±0.48a	1.71±0.06a	0.21±0.02b*	1.06±0.01b	2.34±0.17a	2.46±0.42a
46	Neryl acetate[1]	Nor	1.00±0.55b	2.80±0.14a	0.62±0.02b*	0.94±0.21b	2.36±0.25a	2.95±0.31a
47	Geranyl acetate[1]	Nor	1.00±0.45b	2.77±0.11a	0.54±0.01b*	1.01±0.05b	2.71±0.21a	3.15±0.27a
48	Geranylacetone[1]	Nor	1.00±0.03a*	0.52±0.16b	0.64±0.05ab	19.51±0.77a	5.40±0.61b	7.47±1.31b
49	α-ionone[1]	Nor	1.00±0.17a*	0.51±0.02b	0.53±0.03b*	0.62±0.13a	0.07±0.01b	0.22±0.03b
50	β-ionone[1]	Nor	1.00±0.06a*	0.28±0.01c*	0.47±0.04b	0.68±0.02a	0.13±0.01c	0.50±0.04b

Table Supplementary 4. Relative levels (fold change) of VOCs detected in the pulp of Navel and Kirkwood fruits at harvest, 28 d at 2 °C (cold storage) and 5 d at 20 °C (shelf-life).

			Navel			Kirkwood		
Code	VOCs	Family	Harvest	4w 2 °C	+5d 20 °C	Harvest	4w 2 °C	+5d 20 °C
51	Ethanol[1]	Alc	1.00±0.55b	0.29±0.02b	3.07±0.08a	1.22±0.09b	0.85±0.09b	3.97±0.63a
52	1-penten-3-ol[1]	Alc	1.00±0.10b	nd	1.48±0.08a	1.03±0.10a	nd	nd
56	Acetaldehyde[1]	Ald	1.00±0.17a	0.63±0.06b	1.10±0.04a*	1.06±0.41a	0.73±0.01a	0.89±0.01a
57	Pentanal[1]	Ald	1.00±0.12a	0.42±0.03b	1.36±0.11a*	0.94±0.17a	0.35±0.01b	0.67±0.15a
58	(E)-2-pentenal[1]	Ald	1.00±0.29a	0.18±0.04b	1.25±0.02a*	0.92±0.03a	0.12±0.04b	0.74±0.09a
59	(Z)-3-hexenal[1]	Ald	1.00±0.16a	0.15±0.03b	1.11±0.01a*	1.00±0.52a	0.12±0.04a	0.49±0.01a
60	Hexanal[1]	Ald	1.00±0.11a	0.22±0.02b	1.16±0.08a*	1.00±0.45a	0.22±0.03a	0.75±0.09a
61	(E)-2-hexenal[1]	Ald	1.00±0.07a	0.34±0.01b	1.40±0.20a*	0.99±0.06a	0.22±0.07b	0.7±0.2ab
62	Heptanal[1]	Ald	1.00±0.04a	0.40±0.06b	0.94±0.04a*	0.98±0.22a	0.35±0.02b	0.51±0.09a
63	(E)-2-heptenal[1]	Ald	1.00±0.01a	0.35±0.03b	1.05±0.06a*	0.92±0.16a	nd	0.53±0.06a
64	Octanal[1]	Ald	1.00±0.19a	1.25±0.05a	0.24±0.01b*	1.01±0.16b	2.43±0.03a	2.69±0.20a
65	(E)-2-octenal[1]	Ald	1.00±0.11a	0.45±0.01b	0.86±0.07a*	0.98±0.18a	0.40±0.01b	0.62±0.05a
66	Nonanal[1]	Ald	1.00±0.23b	1.83±0.14a	0.45±0.02b*	1.03±0.12b	2.97±0.13a	2.96±0.11a
67	(E)-2-nonenal[1]	Ald	1.00±0.06a	0.56±0.04c	0.75±0.02b	1.04±0.34a	0.55±0.03a	0.77±0.12a
68	Decanal[1]	Ald	1.00±0.25b	2.45±0.15a	0.47±0.01b*	1.05±0.07b	4.23±0.26a	3.79±0.45a
69	Undecanal[1]	Ald	1.00±0.29b	2.14±0.05a	0.55±0.07b*	1.06±0.04b	3.61±0.59a	2.96±0.55a
70	(E,E)-2,4-	Ald	1.00±0.4b	2.45±0.16a	0.34±0.03b*	1.25±0.15b	4.11±0.14a	3.84±1.06a
71	Dodecanal[1]	Ald	1.00±0.23b	2.51±0.05a	0.53±0.09b*	1.01±0.01b	4.03±0.22a	3.47±0.77a
72	1-penten-3-one[1]	Ket	1.00±0.12b	0.33±0.04c	1.57±0.08a*	0.93±0.22a	0.24±0.01b	0.81±0.08a
73	1-octen-3-one[1]	Ket	1.00±0.08a	0.39±0.08b	0.87±0.04a*	0.99±0.13a	0.33±0.01b	0.60±0.05b
74	Ethyl acetate[1]	Est	1.00±0.17b	0.95±0.10b	5.34±0.69a	0.90±0.10a	1.05±0.13a	4.16±1.71a
75	Ethy propionate[1]	Est	1.00±0.87b	0.67±0.13b	10.66±0.18a	0.99±0.72b	3.52±0.81a	5.96±1.39a
76	Ethyl butanoate[1]	Est	1.00±0.20b	2.10±0.28b	5.75±0.33a*	0.92±0.03a	0.77±0.09a	1.49±0.33a
77	Ethyl 2-	Est	1.00±0.73b	0.28±0.08b	24.02±1.19a	0.87±0.59b	0.69±0.15b	13.47±2.43a
78	Methyl hexanoate[1]	Est	1.00±0.4a	1.38±0.06a	1.23±0.03a*	0.98±0.14a	0.46±0.05b	0.21±0.04b
79	Ethyl caproate[1]	Est	1.00±0.35b	0.76±0.01b	3.76±0.10a*	0.98±0.26a	0.41±0.02a	0.87±0.11a
80	Hexyl acetate[1]	Est	1.00±0.59a	1.89±0.08a	0.28±0.02b*	0.88±0.38a	1.60±0.17a	1.81±0.33a
81	Ethyl heptanoate[1]	Est	1.00±0.04b	2.32±0.29a	2.81±0.04a*	1.18±0.51a	1.47±0.13a	1.82±0.06a
82	Methyl octanoate[1]	Est	1.00±0.41a	1.74±0.02a	1.61±0.01a*	1.05±0.15a	0.79±0ab	0.55±0.05b
83	Ethyl octanoate[1]	Est	1.00±0.3b	0.94±0.01b	4.55±0.21a*	1.01±0.03b	0.87±0.10b	1.91±0.08a
84	Octyl acetate[1]	Est	1.00±0.44b	3.84±0.33a	0.62±0.01b*	0.95±0.29b	2.20±0.25a	3.12±0.15a
85	Ethyl nonanoate[2]	Est	nd	nd	1.00±0.02a*	nd	nd	0.26±0.03a
86	Hexyl hexanoate[1]	Est	1.00±0.92a	0.38±0.02a	0.07±0.01a*	0.82±0.11a	0.53±0.05b	1.01±0.08a
87	Ethyl decanoate[1]	Est	1.00±0.13b	nd	14.70±0.15a	0.98±0.19b	1.19±0.09b	11.86±0.55a
88	Decyl acetate[1]	Est	1.00±0.32a	1.16±0.09a	0.46±0.04a*	0.89±0.26a	0.53±0.07a	0.80±0.08a
89	2-methylfuran[1]	Fur	1.00±0.35a	0.65±0.27a	1.22±0.05a	0.85±0.44a	nd	1.04±0.15a
90	3-methylfuran[1]	Fur	1.00±0.39a	1.06±0.15a	1.43±0.19a*	0.95±0.21a	0.52±0.09a	0.79±0.03a
91	2-ethylfuran[1]	Fur	1.00±0.33a	0.22±0.06a	1.07±0.12a	1.05±0.23a	0.24±0.05a	0.81±0.28a
92	2-pentylfuran[1]	Fur	1.00±0.12a	nd	1.08±0.01a	1.00±0.17a	nd	nd

Data are normalized to the mean values in Navel harvested fruits, unless otherwise indicated. Mean corresponding to n = 2 values. Lower letters indicate significant differences between postharvest stagesby one-way ANOVA ($p<0.05$). Asterisks indicate significant differences between varieties by t-test ($p<0.05$). Mt hd: Monoterpene hydrocarbon; Mnt; Monoterpenes; Sqt: Sesquiterpene; Nor: Norcarotenoid; Alc: Alcohol; Ald: Aldehyde; Ket: Ketone; Est: Ester; Fur: Furane; Ac: acid; Ar: Aromatic hydrocarbon. MHO; 6-methyl-5-hepten-2-one. [1]Data normalized with the mean of Navel at harvest. [2]Data normalized with the mean of Navel shelf-life.

Table Supplementary 5. Relative levels (fold change) of VOCs detected in the pulp of Valencia and Ruby fruits at harvest, 28 d at 2 °C (cold storage) and 5 d at 20 °C (shelf-life).

			Valencia			Ruby		
Code	VOCs	Family	Harvest	4w 2 °C	+5d 20 °C	Harvest	4w 2 °C	+5d 20 °C
1	α-pinene[1]	Mt hd	0.77±0.01a*	0.69±0.3a	0.60±0.01a	0.96±0.01a	0.94±0.12a	1.15±0.32a
2	Camphene[1]	Mt hd	0.72±0.01a*	0.72±0.14a	0.73±0.04a	0.90±0.02a	0.86±0.02a	0.98±0.22a
3	Myrcene[1]	Mt hd	0.83±0.02a	0.86±0.01a	0.78±0.01*	1.11±0.01b	1.06±0.08b	1.41±0.04a
4	β-pinene[1]	Mt hd	0.76±0.04a	0.84±0.08a	0.77±0.06a	0.85±0.08a	0.74±0.14a	0.70±0.10a
5	α-phellandrene[1]	Mt hd	0.89±0.11a	0.81±0.02a	0.75±0.01a*	1.11±0.13a	0.96±0.08b	1.40±0.05a
6	3-carene[1]	Mt hd	0.26±0.02a*	0.28±0.07a	0.40±0.01a	0.45±0.01a	0.33±0.03b	0.53±0.07a
7	α-terpinene[1]	Mt hd	1.34±0.38a	0.89±0.08a	0.94±0.04a	1.17±0.17a	0.82±0.09a	0.94±0.02a
8	p-cymene[1]	Mt hd	0.99±0.05a	1.07±0.16a	0.90±0.05a*	1.12±0.06a	1.19±0.28a	1.48±0.03a
9	Limonene[1]	Mt hd	0.98±0.01a*	0.96±0.01a	0.92±0.02b*	1.07±0.01a	1.02±0.03a	1.11±0.02a
10	γ-terpinene[1]	Mt hd	0.89±0.08a	0.85±0.05a	0.86±0.01a*	1.06±0.12a	0.72±0.08a	0.76±0.02a
11	Terpinolene[1]	Mt hd	0.90±0.19a	0.88±0.03a	0.82±0.03a*	1.53±0.32a	0.93±0.06a	1.27±0.11a
12	Eucalyptol[1]	Mnt	0.52±0.11a*	0.38±0.01a	0.44±0.03a*	0.99±0.10a	0.74±0.07a	1.07±0.07a
13	(Z)-linalool oxide[1]	Mnt	nd	nd	nd	2.47±1.41a	nd	1.64±0.15a
14	(E)-linalool oxide[1]	Mnt	2.68±0.18a	nd	nd	0.87±1.23a	nd	nd
15	Linalool[1]	Mnt	0.56±0.07a*	0.39±0.01b	0.34±0.02b*	2.01±0.25a	0.88±0.02b	1.34±0.06b
16	(Z)-limonene	Mnt	0.54±0.04a	0.30±0.02b	0.30±0.02b*	0.56±0.04a	0.44±0.13a	0.43±0.01a
17	(E)-limonene	Mnt	0.68±0.02a	0.38±0.04a	0.51±0.13a	0.62±0.01a	0.44±0.04b	0.52±0.03a
18	β-citronellal[1]	Mnt	0.52±0.39a	1.11±0.13a	1.09±0.01a	0.69±0.3ab	nd	1.13±0.24a
19	Terpinen-4-ol[1]	Mnt	0.90±0.02a	0.63±0.1b	0.63±0.01b	1.67±0.33a	0.61±0.01b	0.71±0.07b
20	α-terpineol[1]	Mnt	0.62±0.11a	0.31±0.01b	0.26±0.02b*	1.09±0.16a	0.55±0.01b	1.23±0.01a
21	Dihydrocarvone[1]	Mnt	0.68±0.14a	0.67±0.06a	0.49±0.01a	1.06±0.02a	0.71±0.01b	1.05±0.09a
22	β-citronellol[1]	Mnt	0.89±0.03a*	0.72±0.01a	0.91±0.18a	1.27±0.05a	0.86±0.09b	1.27±0.01a
23	(E)-carveol[3]	Mnt	1.00±0.25a	nd	nd	1.43±0.41a	nd	nd
24	Carvone[1]	Mnt	1.11±0.13a	1.21±0.05a	1.24±0.02a	1.69±0.03a	1.17±0.07b	1.69±0.06a
25	Perillaldehyde[1]	Mnt	0.58±0.11a	0.51±0.04a	0.31±0.02a	1.47±0.04a	0.61±0.11b	0.81±0.04b
26	(Z)-carvyl acetate[1]	Mnt	1.75±0.02a	2.11±0.28a	0.82±0.03b	2.06±0.16a	1.97±0.15a	1.14±0.11b
27	Citronellyl	Mnt	2.81±0.40a	2.50±0.60a	0.54±0.03b	6.22±0.32a	4.10±0.39b	5.77±0.08a
28	Sesquiterpene 1[1]	Sqt	3.41±0.13a*	2.28±0.43b	1.39±0.01c*	1.49±0.07a	1.31±0.18a	1.79±0.08a
29	α-copaene[1]	Sqt	3.55±0.12a*	2.53±0.74a	1.58±0.06b*	1.52±0.01b	1.13±0.05c	2.19±0.11a
30	Sesquiterpene 2[1]	Sqt	0.50±0.04a	0.35±0.03b	0.27±0.02b*	0.46±0.03a	0.37±0.02a	0.38±0.02a
31	Sesquiterpene 3[1]	Sqt	0.80±0.13a	0.46±0.05b	0.32±0.01b*	0.94±0.03a	0.32±0.05c	0.56±0.02b
32	β-caryophyllene[1]	Sqt	0.26±0.01a*	0.16±0.02b	0.15±0.01b	0.19±0.01a	0.09±0.01b	0.18±0.01a
33	Sesquiterpene 4[1]	Sqt	0.14±0.01a*	nd	0.08±0.01b	0.09±0.01a	nd	0.10±0.01b
34	α-humulene[1]	Sqt	0.79±0.06a*	0.46±0.09b	0.37±0.01	0.46±0.01a	0.29±0.03b	0.45±0.03a
35	Sesquiterpene 5[1]	Sqt	0.15±0.01a*	0.05±0.01b	0.06±0.01b	0.08±0.01a	0.03±0.01b	0.09±0.01a
36	Valencene[1]	Sqt	0.19±0.02a*	0.08±0.01b	0.10±0.01b	0.11±0.01a	0.05±0.01b	0.14±0.02a
37	Sesquiterpene 6[1]	Sqt	0.17±0.03a*	0.06±0.01b	0.09±0.01b	0.08±0.01b	0.04±0.01c	0.11±0.01a
38	Sesquiterpene 7[1]	Sqt	0.12±0.01a*	0.03±0.01c	0.07±0.01b	0.06±0.01b	0.03±0.01c	0.09±0.01a
39	Sesquiterpene 8[1]	Sqt	0.13±0.01a*	0.04±0.01b	0.06±0.01b	0.07±0.01a	0.03±0.01b	0.08±0.01a
40	Nootkatone[2]	Sqt	nd	0.17±0.02a	0.16±0.01a	nd	0.09±0.01b	0.16±0.01a
41	MHO[1]	Nor	0.94±0.15a*	0.73±0.09a	0.78±0.07a*	7.44±0.23a	4.30±0.31c	5.31±0.12b
42	Nerol[1]	Nor	1.00±0.08a	nd	nd	4.77±2.63a	nd	nd
43	β-cyclocitral[1]	Nor	0.32±0.07a	0.15±0.01b	0.17±0.04ab	0.63±0.11a	0.26±0.02b	0.35±0.02a
44	Neral[1]	Nor	0.49±0.08a*	0.20±0.08b	0.18±0.01b*	1.34±0.12a	0.36±0.04c	0.67±0.07b
45	Geranial[1]	Nor	0.36±0.01a*	0.16±0.02b	0.17±0.02b*	1.38±0.17a	0.37±0.02c	0.80±0.04b
46	Neryl acetate[1]	Nor	1.13±0.15a	0.94±0.39a	0.29±0.05a	1.53±0.01a	0.60±0.07c	1.18±0.07b
47	Geranyl acetate[1]	Nor	1.08±0.01a	0.93±0.23a	0.20±0.03b*	1.00±0.03a	0.35±0.08b	0.94±0.07a
48	Geranylacetone[1]	Nor	0.99±0.09a*	0.50±0.08b	0.58±0.05b*	12.43±2.54a	4.70±0.08b	3.81±0.17b

Table Supplementary 5. Continuation.

			Valencia			Ruby		
Code	VOCs	Family	Harvest	4w 2 °C	+5d 20 °C	Harvest	4w 2 °C	+5d 20 °C
49	α-ionone[1]	Alc	0.57±0.07a	0.32±0.01b	0.13±0.01c*	0.96±0.01a	0.94±0.12a	1.15±0.32a
50	β-ionone[1]	Alc	0.56±0.04a	0.21±0.01b	0.11±0.01c*	0.90±0.02a	0.86±0.02a	0.98±0.22a
51	Ethanol[1]	Ald	0.47±0.17c	2.04±0.22b	4.98±0.09a*	1.11±0.01b	1.06±0.08b	1.41±0.04a
52	1-penten-3-ol[1]	Ald	0.91±0.12a	nd	nd	0.85±0.08a	0.74±0.14a	0.70±0.10a
53	1-hexanol[3]	Ald	1.00±0.11a	nd	nd	1.11±0.13a	0.96±0.08b	1.40±0.05a
54	1-octen-3-ol[3]	Ald	1.00±0.09a	nd	nd	0.45±0.01a	0.33±0.03b	0.53±0.07a
55	1-octanol[3]	Ald	1.00±0.06a	nd	nd	1.17±0.17a	0.82±0.09a	0.94±0.02a
56	Acetaldehyde[1]	Ald	0.72±0.16b	0.72±0.02b	1.23±0.02a*	1.12±0.06a	1.19±0.28a	1.48±0.03a
57	Pentanal[1]	Ald	1.15±0.10a	0.88±0.09a	1.15±0.11a	1.07±0.01a	1.02±0.03a	1.11±0.02a
58	(E)-2-pentenal[1]	Ald	0.59±0.03a	0.19±0.01c	0.37±0.04b	1.06±0.12a	0.72±0.08a	0.76±0.02a
59	(Z)-3-hexenal[1]	Ald	0.54±0.05a	0.21±0.01b	0.35±0.04b	1.53±0.32a	0.93±0.06a	1.27±0.11a
60	Hexanal[1]	Ald	0.81±0.04a	0.25±0.06b	0.69±0.04a*	0.99±0.10a	0.74±0.07a	1.07±0.07a
61	(E)-2-hexenal[1]	Ald	0.85±0.1a	0.23±0.02b	0.44±0.05b	2.47±1.41a	nd	1.64±0.15a
62	Heptanal[1]	Ald	0.69±0.01a	0.68±0.05a	0.74±0.09a	0.87±1.23a	nd	nd
63	(E)-2-heptenal[1]	Ald	0.94±0.03a*	1.09±0.14a	0.94±0.04a	2.01±0.25a	0.88±0.02b	1.34±0.06b
64	Octanal[1]	Ald	0.65±0.10a*	0.24±0.01c*	0.33±0.03b*	0.56±0.04a	0.44±0.13a	0.43±0.01a
65	(E)-2-octenal[1]	Ald	0.69±0.06a*	0.79±0.06a	0.72±0.07a	0.62±0.01a	0.44±0.04b	0.52±0.03a
66	Nonanal[1]	Ald	0.42±0.05a*	0.35±0.02a	0.38±0.01a*	0.69±0.3ab	nd	1.13±0.24a
67	(E)-2-nonenal[1]	Ket	0.78±0.05a*	0.75±0.04a	0.55±0.04b	1.67±0.33a	0.61±0.01b	0.71±0.07b
68	Decanal[1]	Ket	0.62±0.07a*	0.46±0.02a	0.49±0.03a*	1.09±0.16a	0.55±0.01b	1.23±0.01a
69	Undecanal[1]	Est	0.32±0.01a*	0.29±0.03a	0.27±0.04a*	1.06±0.02a	0.71±0.01b	1.05±0.09a
70	(E,E)-2,4-	Est	0.52±0.08a*	0.22±0.03b	0.24±0.04b*	1.27±0.05a	0.86±0.09b	1.27±0.01a
71	Dodecanal[1]	Est	0.72±0.07a*	0.70±0.1ab	0.41±0.01*	1.43±0.41a	nd	nd
72	1-penten-3-one[1]	Est	0.94±0.03a*	0.23±0.01c	0.43±0.06b	1.69±0.03a	1.17±0.07b	1.69±0.06a
73	1-octen-3-one[1]	Est	1.32±0.05a*	1.15±0.06a	1.65±0.30a	1.47±0.04a	0.61±0.11b	0.81±0.04b
74	Ethyl acetate[1]	Est	0.84±0.04b	0.74±0.08b	4.25±0.09a*	2.06±0.16a	1.97±0.15a	1.14±0.11b
75	Ethy propionate[1]	Est	0.93±0.09b	3.98±0.10b	16.59±2.13a	6.22±0.32a	4.10±0.39b	5.77±0.08a
76	Ethy butanoate[1]	Est	0.40±0.02b*	0.36±0.01b	7.00±0.53a*	1.49±0.07a	1.31±0.18a	1.79±0.08a
77	Ethy 2-	Est	3.81±0.46b*	1.82±0.29c*	69.61±0.16a	1.52±0.01b	1.13±0.05c	2.19±0.11a
78	Methyl	Est	0.19±0.01b	0.14±0.02b	0.40±0.02a	0.46±0.03a	0.37±0.02a	0.38±0.02a
79	Ethy caproate[1]	Est	0.29±0.01b*	0.12±0.01c	1.51±0.09a	0.94±0.03a	0.32±0.05c	0.56±0.02b
81	Ethyl heptanoate[1]	Est	nd	nd	3.57±0.05a*	0.19±0.01a	0.09±0.01b	0.18±0.01a
82	Methyl octanoate[1]	Est	0.22±0.04b	nd	0.41±0.01a*	0.09±0.01a	nd	0.10±0.01b
83	Ethy octanoate[1]	Est	0.27±0.02b*	0.12±0.01b	1.62±0.08a*	0.46±0.01a	0.29±0.03b	0.45±0.03a
84	Octyl acetate[1]	Est	1.51±0.25a	0.97±0.18a	0.28±0.04b*	0.08±0.01a	0.03±0.01b	0.09±0.01a
87	Ethyl decanoate[1]	Fur	1.04±0.07b	nd	5.85±0.01a*	0.11±0.01a	0.05±0.01b	0.14±0.02a
88	Decyl acetate[1]	Fur	2.63±0.09a*	1.97±0.38a	0.64±0.04b*	0.08±0.01b	0.04±0.01c	0.11±0.01a
89	2-methylfuran[1]	Fur	0.71±0.08a	nd	nd	0.06±0.01b	0.03±0.01c	0.09±0.01a
90	3-methylfuran[1]	Fur	0.96±0.11b	nd	1.44±0.09a*	0.07±0.01a	0.03±0.01b	0.08±0.01a
91	2-ethylfuran[1]	Fur	0.64±0.08a	0.31±0.07b	0.45±0.02ab	nd	0.09±0.01b	0.16±0.01a
92	2-pentylfuran[1]	Fur	nd	nd	0.96±0.1a*	7.44±0.23a	4.30±0.31c	5.31±0.12b

Data are normalized to the mean values in Navel harvested fruits, unless otherwise indicated. Mean corresponding to n = 2 values. Lower letters indicate significant differences between postharvest stagesby one-way ANOVA ($p<0.05$). Asterisks indicate significant differences between varieties by t-test ($p<0.05$). Mt hd: Monoterpene hydrocarbon; Mnt; Monoterpenes; Sqt: Sesquiterpene; Nor: Norcarotenoid; Alc: Alcohol; Ald: Aldehyde; Ket: Ketone; Est: Ester; Fur: Furane; Ac: acid; Ar: Aromatic hydrocarbon. [1]Data normalized with the mean of Navel at harvest. [2]Data normalized with the mean of Navel shelf-life. [3]Data normalized with the mean of Valencia at harvest

3.5. CAPÍTULO 5

Juices and By-products of Red-fleshed Sweet Oranges: Assessment of Bioactive and Nutritional Compounds

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Abstract

The content of nutrients and bioactive compounds, and antioxidant capacity were assessed in the juices from two red-fleshed oranges, Cara Cara and Kirkwood, and compared with that of a standard Navel orange. Two juice extraction procedures, hand-squeezing and industrial, and two treatments, pasteurization (85 °C/30s) and high-pressure homogenization (HPH, 150 MPa/55 °C/1min) were evaluated. For most of the nutrients and bioactive compounds, the hand and industrial juice squeezing rendered similar extraction efficiency. Individual composition of carot-enoids in the juices were differentially affected by the extraction procedure and the treatments, but the red-fleshed orange juices contained between 3- to 6-times higher total carotenoids than the standard Navel juices, being phytoene and phytofluene the main carotenoids. The industrial and treated juices of both red-fleshed oranges contained 20-30% higher amounts of tocopherols but about 20% lower levels of vitamin C than Navel juices. Navel juices exhibited higher hydrophilic antioxidant capacity, while the red-fleshed orange juices showed an improved lipophilic antioxi-dant capacity. The main distinctive characteristic of industrial juice by-product of the red-fleshed oranges was a higher content in carotenoids (x10) and singlet oxygen antioxidant capacity (x1.5-2) than Navel by-product.

Keywords: red-fleshed orange juice; pasteurization; high-pressure homogenization; juice quality; bioactive compounds; antioxidant capacity.

1. Introduction

Citrus are one of the main horticultural crops worldwide in terms of production and economic value. Spain produces over 50% of total citrus fruits in EU and is the main exporter for fresh consumption. Other producer countries as Brazil assign the 90% of its production to the juice industry, while in Spain about 80% of the total production is marketed as fresh and nearly 20% is being processed [1,2].

Citrus fruits and juices are widely consumed worldwide and are a natural source of micro- and macronutrients such as minerals, sugars, organic acids, vitamins C, E and folates and other bioactive phytochemicals such as carotenoids and phenolic compounds (including flavonoids and coumarins), among others [3-5]. Citrus fruit and juice consumption has been related to numerous beneficial effects on health mainly attributed to the balanced content in nutrients and bioactive compounds [3, 6-9].

Among the bioactive compounds present in citrus fruits and juices, carotenoids also determine their characteristic pigmentation [10-11]. Carotenoids are very efficient quenchers of singlet oxygen and scavengers of other ROS [12- 14]. The carotenoid profile in the pulp and juice of standard blond sweet oranges can vary among varieties [15-17] but, in general, the β,β-xanthophylls content represents over 90% of total carotenoids, and linear carotenes are at low concentrations [18-22]. Within β,β-xanthophylls, violaxanthin is the predominant carotenoid, followed by lower amounts of lutein, anteraxanthin, zeaxanthin, β-cryptoxanthin [19,23].

Accumulation of lycopene is an unusual characteristic that has been reported in the pulp of different species of Citrus and confers a bright pink/reddish coloration. To date a limited number of red-fleshed orange varieties have been described: Shara [24], Cara Cara [25] and Hong Anliu [26]. Among them, Cara Cara (CC), a spontaneous mutation from the blond-fleshed orange Washington Navel, has been the best characterized [20,21,27-29]. Recently, a new red-fleshed orange, referred to as Kirkwood (K), which is a spontaneous mutation from Palmer Navel, was characterized for the first time [22, 30]. The pulp of both red-fleshed varieties, CC and K, are characterized by a higher carotenoids concentration compared to standard orange-coloured Navel varieties. The main feature of the carotenoid composition of CC and K is the outstanding amount of the colorless phytoene and phytofluene, low to moderate contents of lycopene and β-carotene, and reduced levels of violaxanthin compared

to standard blond oranges [20-22, 28-30]. Because of their exceptional coloration, carotenoids have been the most evaluated compounds in these red-fleshed oranges. Nonetheless, other studies have also assayed the concentration of other relevant compounds both in pulp and juice. Zacarías-García et al. [31] reported that the content of vitamin C, tocopherols, flavonoids, sugars and organic acids in K pulp was essentially similar to that found in the standard variety Foios Navel. Nevertheless, contrasting results have been obtained in CC. Some studies found virtually the same content of vitamin C and flavonoids [32], whereas others reported minor amounts of vitamin C and total flavanones compared to standard Navel oranges [21,29].

In vitro and in vivo studies have investigated the potential health-related effects of the CC juice intake in comparison to the juice from standard varieties, reporting improved responses in antioxidant, inflammatory, and other relevant clinical parameters for the red-fleshed orange [33-35].

Citrus juice industry has developed several processing technologies to increase product shelf-life, ensure microbiological safety and preserve organoleptic attributes, nutrients, and bioactive phytochemicals of juices. However, juice processing and treatment technologies can affect both sensory and nutritional quality, and the concentration of bioactive compounds in a variable degree. Traditionally, pasteurization has been used to assure microbiological safety and to inactivate enzymes as pectin methylesterase (PME), responsible of loss of turbidity, consistency, and gelation of orange juices [36,37]. In particular, pasteurization of red-fleshed orange juice from CC did not influence the total carotenoids and lycopene content [29, 38], whereas β,β-xanthophylls levels were significantly affected [29]. On the other hand, high-pressure homogenization (HPH) is considered a promising alternative juice processing treatment for the commercialization of high quality, healthy and safe citrus juices [39]. HPH is considered a non-thermal technology where the fluid goes through a minute gap, which not only homogenizes the fluid but also increases the fluid's temperature. This process reduces and homogenizes the particle size while inactivating microorganisms by minimally modifying the sensory and nutritional characteristics [39,40]. The effect of HPH treatment has been recently evaluated in citrus juices and it seems a sustainable option to obtain juices with improved nutritional properties [39,41].

During the juice production process only approximately half of the total weight of the orange fruit is transformed into juice, generating a large amount of waste [42]. The peculiar characteristics of citrus processing residues involve considerable limitations for their management due both to economic and environmental factors [43]. The waste of orange juice industry is composed mainly by peel (flavedo and albedo), pulp and seeds, and is rich in fiber, essential oils, pectin and antioxidants [44]. Citrus wastes have traditionally been used for animal feed and biorefinery industries. However, as innovative and more sustainable technologies are being developed, new uses for citrus by-products are emerging as added-value products for cosmetics, antifungals, biofuels, fertilizers, among others [42-44].

This study aimed at evaluating the suitability for juice production of two red-fleshed orange varieties, CC and K, in comparison to a standard orange variety Navel (N). To that end, we studied the effect of two juice extraction methods, freshly hand-squeezed and industrial, and two treatments, high-pressure homogenization and pasteurization, on the content of bioactive compounds (with special interest in carotenoids), compounds related to organoleptic quality and the antioxidant capacity. Finally, we investigated the composition of main bioactives and antioxidant capacity of the orange by-product of each variety generated during the industrial juice extraction process.

Materials and methods

2.1. Plant material

Fruits of the three orange varieties (Citrus sinensis L. Osbeck) used in this study belong to the Navel group: two red-fleshed, Cara Cara (CC) and Kirkwood (K), and one standard Navel, Navel Foios (N). The standard N is a mid-season variety being one of the most cultivated varieties in Spain and characterized by the intense orange color. CC and K are bud mutations characterized by the red coloration of the pulp due to an altered carotenoid composition [22, 27, 28]. Fruit of the red-fleshed varieties CC and K were harvested in commercial orchards from Ayamonte (Huelva, Andalucía, Spain) showing a maximum and minimum average of temperature during the month of harvest (January) of 15.7 °C and 6.1 °C, respectively. On the other hand, fruit of the blond-fleshed orange variety Navel were harvested in commer-cial orchards from Picassent (Valencia, Comunidad Valenciana, Spain) with a maximum and minimum average temperature during the month of harvest of 15 °C and 3.5 °C, re-spectively. (https://www.meteoblue.com/; https://www.aemet.es). Fruits of all varieties were harvested at commercial maturity following the criteria described by Lado et al. [11]. Figure 1 illustrates the differences in pigmentation between pulp and juice of the blond-fleshed N and the red-fleshed CC and K oranges.

Figure 1. Appearance of pulp and freshly hand-squeezed juice of the sweet orange Navel, and the red-fleshed Cara Cara and Kirkwood oranges.

2.2. Experimental design

Immediately after harvesting, fruits were delivered to the laboratory, cold stored until processed in the next 3 days. For juice extraction, between 150 and 160 kg of fruits of each

variety were used. The fresh hand-squeezed and the industrial juice, and the treated (high-pressure homogenized and pasteurized) juices were obtained according to the experimental design showed in Figure 2.

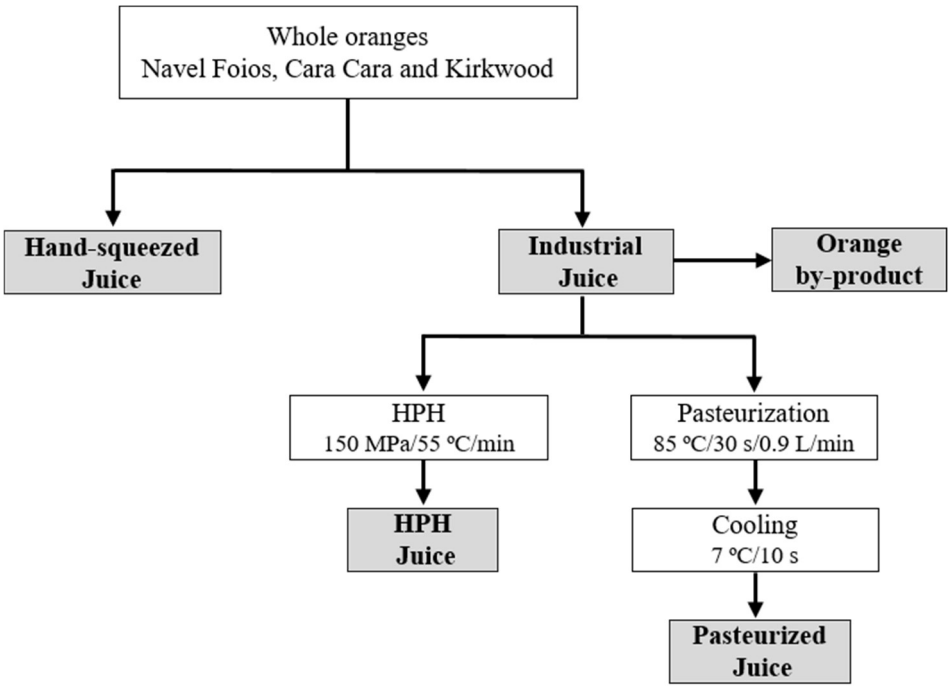

Figure 2. Flow diagram of the experimental design. HPH: high-pressure homogenization.

Hand-squeezed juice (HS): This juice (approximately 10 L) was obtained by hand squeezing of the fruits through a household electric hand reamer (Citromatic MPZ22, Braun, Barcelona, Spain) and subsequent filtered with a steel sieve of 2 mm to remove pulp.

Industrial juice: This juice was obtained through an industrial extractor (Exzel, Luzzysa; Valencia, Spain) and sieved in a paddle finisher (0.4 mm mesh diameter, model EPF 06, Luzzysa, Valencia, Spain). The industrial juice was split into two batches of approximately 25 L each for processing: one batch was pasteurized and the other high-pressure homogenized.

Pasteurized juice: The pasteurization was performed at 85 °C during 30 s using a plate heat exchanger and cooled at 7 °C in the outlet section [39].

High-pressure homogenized juice (HPH): The industrial juice was processed at 150 MPa reaching a temperature of 55 °C for 1 min. Homogenization was performed by a continuous system (NS3015H model, GEA Niro Soavi S.p.A., Parma, Italy) [39].

Orange by-product: The orange by-product consisted in peel (flavedo and albedo) and pulp remaining after industrial juice extraction. A sample of approximately 1 kg of each orange variety by-product was freeze-dried using a LYOBETA 6 PL (Telstar, Ter-rassa, Barcelona) for preservation and then, stored at -80 °C until analyses. After freeze-drying, all orange by-products lost about 80% of their initial fresh weight.

2.2. Maturity index and juice color

Total soluble solids (TSS, °BRIX) and total titratable acidity (TA, mg of citric ac-id/100 mL of juice) were determined by using a digital refractometer PAL-BX/ACID1 (ATAGO, Japan). Maturity index (MI) was calculated as the TSS/TA ratio.

The juice color index, expressed as the ratio a/b, was analyzed using a CM-3500d spectrophotometer (Konica Minolta, INC., Japan). Color data were measured using HunterLab scale, which define the color in a three-dimensional space. The coordinate L indicates lightness and a and b green-red and blue-yellow coordinates, respectively. L is an approximate measurement of luminosity, taking values within the range 0–100. The Hunter parameters a (assigns positive values for the reddish colors and negative values for the greenish ones) and b (assigns positive values to the yellowish colors and nega-tive values to the bluish ones) were calculated and expressed as the ratio a/b, a classic relationship for color measurements in citrus fruits [45].

2.3. Carotenoids determination

Carotenoids in the orange juice of the different varieties were extracted and ana-lysed essentially as described Rodrigo et al. [20] with slight modifications. Briefly, 2 mL of orange juice was placed in screw-capped polypropylene tubes (15 mL), 3 mL of ex-traction solution composed of methanol:acetone:dichloromethane (25:25:50 v/v/v) was added and the sample was sonicated for 5 minutes in an ultrasonic water bath at room temperature (XUBA3, Grant Instruments, Cambridge, England), centrifuged at 4500 g for 5 minutes at 4°C (Labofuge 400R centrifuge, Thermo Scientific, Heraeus, Germany), and finally the organic phase was recovered. The aqueous phase was re-extracted with 1.5 mL of dichloromethane (HPLC grade, Sharlau, Barcelona, Spain), until the organic phase was colorless. The extracts were saponified in methanolic KOH (12%, w/v) for 90 minutes, at room temperature and under nitrogen atmosphere in darkness. After the saponification, 3 mL of 50 mM Tris-HCl pH 7.5 with 1 M

NaCl and 3 mL of dichloro-methane were added, stirred, and centrifuged at 4500 rpm for 5 minutes at 4°C. The aqueous phase was discarded. This step was repeated (3 mL of Tris-NaCl buffer solu-tion) until the discarded aqueous phase was neutral. The extracts were dried and kept -20 °C until further analysis.

Carotenoids extraction and analysis was carried out from 0.25 g of freeze-ground dry orange by-product as essentially described Zacarías-García et al. [22,31] for pulp and peel orange.

Carotenoid composition was analyzed by high-performance liquid chromatog-raphy (HPLC) with a Waters liquid chromatography system equipped with a 600E pump, coupled to a 2998 photodiode array detector (PAD) and Empower3 software (Waters, Barcelona, Spain). A C30 carotenoid column (250 x 4.6 mm, 5 μm) coupled to a C30 guard column (20 x 4.0 mm, 5 μm) (YMC, Tecknochroma, Barcelona, Spain) was used. Chromatographic conditions are described in Zacarías-García et al. [22,31]. The carotenoids were identified by absorbance spectra and retention time, peaks integrated at their individual maximal wavelength, and their contents were calculated using the appropriate calibration curves, as described elsewhere [22,31]. Two independent repli-cates of each sample were analysed, and the results are expressed as mean ± standard deviation.

2.5. Vitamin C determination

L-Ascorbic acid was extracted from 2 mL of orange juice and 0.2 g orange by-product and determined essentially as described in Alòs et al. [46] using a Waters Acquity Arc HPLC system (Waters, Barcelona, Spain) equipped with a DAD, Empower 3 software and an Ultrabase C18 column. Two independent replicates of each sample were analysed, and the results are expressed as mean ±standard deviation.

2.6. Tocopherols determination

Tocopherols extraction from orange juices and orange by-products was carried out essentially as described in Rey et al. [47] with slight modifications. Two mL of juice or 0.25 g of lyophilized orange by-product was extracted with 1.5 mL of methanol and 4 mL of dichloromethane, vortexed and sonicated for 5 minutes. The samples were cen-trifuged at 3500 rpm for 10 minutes at 4°C. The organic phase was recovered and ex-tracted again with 2 mL dichloromethane.

Tocopherol content was determined by HPLC (Waters Acquity Arc system) cou-pled to a Waters 2475 FLR fluorescence detector and a YMC C30 column (150 × 4.6 mm, 3 μm) (Teknokroma, Barcelona, Spain), at room temperature. Chromatographic condi-tions used are described in Rey et al. [47]. The identification and quantification of to-copherols was achieved by comparison with the retention times of δ-, γ- and α-tocopherol standards (Sigma-Aldrich, Barcelona, Spain). Total tocopherol content was calculated as the sum of the different tocopherol isoforms. Two independent repli-cates of each sample were analysed, and the results are expressed as mean ±standard deviation.

2.7. Analysis of total phenolic and flavonoids

Total phenolic and flavonoids content were determined as described in Zaca-rías-García et al. [31] for orange pulps. Total phenolic was expressed as mg of gallic acid equivalents (GAE) per 100 mL of juice or 100 g of dry weight. Total flavonoids were analysed in the orange by-products and expressed as mg of hesperidin equiva-lents (HesE) per 100 g of dry weight. Determinations were performed per triplicate in each extract.

2.8. Flavonoid analysis by HPLC-DAD

Extraction of flavonoids from 2 mL orange juice was performed according to the procedure described in Zacarías-García et al. [31]. Flavonoids composition was deter-mined by HPLC-DAD (Waters Acquity Arc system). Separation of flavonoids was car-ried out with a XBridgeTM BEH C18 column (4.6 x 150 mm, 2.5 μm Column XP). Chromatographic conditions are described in Zacarías-García et al. [31]. Identification and quantification of the different flavonoids was achieved by comparison with the retention times and peak areas of authentic standards of hesperidin, narirutin, nar-ingin, eriocitrin, dydimin and rutin (Sigma-Aldrich, Barcelona, Spain). Two independ-ent replicates of each sample were analysed, and the results are expressed as mean ± standard deviation.

2.9. Sugars and organic acids determination

Sugars and organic acids were extracted and determined essentially as described in Zacarías-García et al. [31]. Sugars and organic acids content was measured by HPLC-DAD (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index detector. For glucose, fructose, citric acid, malic acid, quinic acid and succinic acid separation and determination was employed a HyperREZTM XP Carbohydrate H+ 8μm column (Thermo

Fisher Scientific). For sucrose determination, a Pb column was used (Hi-Plex Pb, 300 x 7.7 mm, Agilent Technologies). Chromatographic conditions used are described in Pérez-Través et al. [48]. The identification and quantification of sugars and organic acids were carried out by comparison with the retention times of authentic standards (Sigma-Aldrich, Barcelona, Spain). Two independent replicates of each sample were analysed, and the results are expressed as mean ±standard deviation.

2.10. Determination of antioxidant capacity

Three different methods were performed for the antioxidant capacity of the orange juices and by-products. The hydrophilic antioxidant capacity (HAC) of juices (2 mL) and orange by-product (0.25 g DW) was determined using DPPH free radical assay (2,2-diphenyl-1-picrylhydrazyl) (Rey et al., 2020), FRAP (ferric reducing antioxidant power) [31] and radical ABTS (2,2'-azino-di-(3-ethylbenzthiazoline sulfonate)) [31]. The lipophilic antioxidant capacity (LAC) of orange juices and by-products was also determined by ABTS [31]. Each sample was analyzed in two independent assays and samples were run in triplicate in each plate.

2.11. Singlet oxygen absorption capacity (SOAC)

The singlet oxygen absorption capacity (SOAC) of orange by-products was deter-mined according to the procedure previously described [31,49,50]. The relative SOAC value for each sample was calculated with the following formula (1):

(t1/2 sample − t1/2 blank)/(t1/2 α-toc − t1/2 blank) × ([α-toc, g/L]/[sample, g/L]) (1)

2.12. Retinol equivalents

The capacity pro-vitamin A expressed as retinol equivalents (RE) was calculated following the equation of Sanchez-Moreno et al. [51]

RE= μg β-carotene/6 + μg α-carotene/12 + μg β-cryptoxanthin/12 (2)

2.13. Statistical analysis

Results are the mean of two independent replicates ± standard deviation (SD). One-way ANOVA was carried out and Tukey's test (significance level $p \leq 0.05$) was used for mean comparisons among varieties and treatments. Principal component analysis (PCA) was set up using the compounds concentration and antioxidant values. Analysis was made using the XLSTAT Software version 2019.3.2 (Addinsoft, Paris, France). The correlation matrix between

bioactive compounds concentration and antioxidant ca-pacity was carried in RStudio (version 1.3.1093, RStudio Team, PBC, Boston, MA, United States) using the function "cormat" and visualized using the function "cor-rplot" of the package "ggplot2" [47].

3. Results and Discussion

This study aimed at evaluating the potential of red-fleshed orange varieties for juice production and how juice processing affects main bioactive and quality compounds. To that end, hand-squeezed (HS), industrial pasteurized (85 °C/30s) and non-thermal high-pressure homogenization (HPH) (150 MPa/55 °C/1 min) juices were prepared from fruits of the red-fleshed orange (Citrus sinensis) varieties Cara Cara (CC) and Kirkwood (K), and from fruits of the standard orange variety Navel (N). The juice yield for the three varieties ranged from 42-45% (w/v). The industrial orange juice ex-traction generates great amounts of fruit wastes of a high potential interest for food/feed, pharmaceutical and cosmetic sectors or for bio-transformation processes. Thus, to investigate the potential value of red-fleshed orange by-products in compari-son to that of the traditional variety N, a characterization of the bioactive compounds was also carried out.

3.1. Maturity index and orange juice color

The maturity index is a reference quality parameter in citrus fruits and is determined by the ratio between the content of soluble solids (ºBrix) and acidity [11]. Juices of CC presented lower ºBrix levels than N and K fruits (Table 1). However, no significant differences in acidity and in the maturity index were observed between the three varieties (Table 1). Therefore, our results showed that the red-fleshed oranges CC and K reached similar maturity index than the standard N at mature stage. Other works re-ported that fruits of Navel orange presented slightly higher levels of ºBrix and maturity index compared to CC [20,21] but these differences are more likely attributed to variability in the ripening stage at harvest and/or the environmental and agronomic conditions [11].

Table 1. Soluble solids (SS), titratable acidity (TA) maturity index (MI) of Navel, Cara Cara and Kirkwood oranges.

Parameters	Navel	Cara Cara	Kirkwood
SS (ºBrix)	11.45 ± 0.07a	9.97 ± 0.06b	11.17 ± 0.06a
TA (mg/CA 100 ml)	0.82 ± 0.05a	0.70 ± 0.06a	0.91 ± 0.06a
MI (SS/TA)	14.08 ± 0.94a	14.36 ± 1.04a	12.35 ± 0.87a

Lowercase letters indicate significant differences between varieties by one-way ANOVA ($p<0.05$).

The color represents one of the most important features of citrus fruit quality and it is a crucial issue for consumer's acceptance. Moreover, the utility of the Hunter color parameters (L and a/b) to classify different orange juices according to the industrial processing requirements has been previously reported [52]. The CC and K fruits stand out by the reddish coloration of the pulp, while fruits of N present the typical orange tone of traditional oranges [20-22,28]. The visual inspection of CC and K juices showed a more intense orange-reddish coloration than in N juices regardless the extraction procedure (hand-squeezed or industrial) and the treatment applied (HPH or pasteurization). This color perception corresponded to significantly higher a/b ratio and lower L values of CC and K juices compared to N (Table 2). It was noted that HS juice of K exhibited slightly higher colour index than CC. The method of juice extraction exerts a notable effect on the a/b ratio. Thus, the HS juice of all varieties displayed higher a/b ratio and lower L values than the corresponding industrial juice (Table 2). However, no differences were detected between the industrial and the processed juices, or between the treatments for any variety (Table 2). The carotenoids responsible for the color of common orange juice are the xanthophylls, mainly the isomers of violaxanthin, antheraxanthin, zeaxanthin and lutein [18], while the juice color of the red-fleshed oranges CC and K is strongly influenced by the concentration of lycopene [20-22,29]. Strikingly, the industrial juice of the three varieties evaluated displayed lower color index (a/b) but higher lightness (L) compared to their respective HS juices (Table 2).

Nevertheless, the industrial juices contained either similar or higher total carote-noids content than the HS juices as reported in next section. These results might be explained by the modification of the pulp structure and the particle size, since industrial extraction produces a greater mechanical stress and contributes to a higher dispersion of the particles resulting in lower pigmentation [53]. Stinco et al. [52] correlated the smaller particle size of the industrial orange juices with brighter (higher L) and more yellowish (lower a/b) color than HS juices.

Table 2. Color coordinate L and the ratio a/b of HS (hand-squeezed), industrial, HPH (high-pressure homogenized) and pasteurized juices of Navel, Cara Cara and Kirkwood oranges.

	Navel		Cara Cara		Kirkwood	
Juice	*L*	*a/b*	*L*	*a/b*	*L*	*a/b*
HS	45.01±0.05Ba	0.15±0.03Ac	40.48±0.01Bb	0.32±0.02Ab	40.97±0.33Bb	0.37±0.01Aa
Industrial	55.50±0.04Aa	0.03±0.01Bb	47.12±0.07Ac	0.24±0.01Ba	51.21±0.38Ab	0.21±0.01Ba
HPH	56.10±0.06Aa	-0.01±0.01Bb	49.78±0.30Ab	0.19±0.04Ba	49.40±0.09Ab	0.24±0.02Ba
Pasteurized	56.25±0.04Aa	0.02±0.01Bb	49.19±0.04Ab	0.22±0.01Ba	49.40±0.09Ab	0.20±0.01Ba

Uppercase letters indicate significant differences between different type of juices for the same variety and lower-case letters indicate significant differences between varieties for the same type of juice by one-way ANOVA ($p<0.05$).

Therefore, the lighter color detected for the industrial juices compared to those obtained by hand extraction agrees with previous studies [52]. According to these results, it could be concluded that the main differences in color between the HS and the industrial juices are more likely related to the extraction procedure than to the treatments, as it was previously proposed [52].

3.2. Carotenoids

The concentration of individual and total carotenoids of HS, industrial, HPH and pasteurized juices of N, CC and K are shown in Table 3. The total content of carotenoids in both red-fleshed orange juices ranged from nearly 25 to 35 μg/mL while in N was approximately between 6 and 9 μg/mL, Thus, the HS (3.4- and 4.0-fold), industrial (4.0- and 3.4-fold), HPH (5.4- and 5.8-fold) and pasteurized (5.7- and 5.2-fold) juices of CC and K contained more carotenoids than the corresponding juices of N, respectively. These differences in the total carotenoids among the juices of the red-fleshed and the ordinary orange varieties are in accordance with previous studies [20,21,29,34]. The carotenoid profile of N juices is primarily composed by β,β-xanthophylls, which provide the typical yellow-orange coloration. In the juice of this variety, the major carotenoid was violaxanthin (4.25 μg/mL) that represents 43-54% of the total carotenoids content, and other relevant β,β-xanthophylls were antheraxanthin (8-13%) (1.00-0.52 μg/mL), and β-cryptoxanthin (8-11%) (0.91-0.65 μg/mL) while low concentrations (less than 0.5 μg/mL) of zeaxanthin and the colorless carotenes phytoene and phytofluene were detected. Luteoxanthin and mutatoxanthin were also detected since the natural acidity of the juice promotes the rearrangement of 5,6-epoxy carotenoids, such as violaxanthin and antheraxanthin, to their respective 5,8-epoxy carotenoids [54]. The carotenoid profile of N juices is in consonance with that described for other Navel varieties [18,19,20,21,29]. On the other hand, the carotenoid composition of both red-fleshed orange

juices was completely different to that of N and resembles that described for CC juice in previous analysis [20,21,29] and pulp of K [22,31]. The juice of CC and K oranges was characterized by the high levels of phytoene (14.99-23.12 μg/mL and 18.99-22.14 μg/mL, respectively) and phytofluene (3.48-5.59 μg/mL and 3.99-4.89 μg/mL, respectively), accounting for 70-80% of total carotenoids (Table 3). The concentration of lycopene in the different juices varied between 1.30-2.30 μg/mL and 1.49-2.82 μg/mL in CC and K, respectively. It is also relevant to mention the reduction (40-50%) in the concentration of violaxanthin found in HS and industrial juices of CC and K compared to N. Interestingly, these differences were less relevant in HPH and pasteur-ized juices (Table 3). Besides, β-carotene was detected at low levels in CC (0.23-0.47 μg/mL) and K juices (0.29-0.45 μg/mL), while it was not detected in N. On the other hand, provitamin A capacity, expressed as retinol equivalents (RE), was determined by the precursors detected in orange juices: β-carotene, which shows the highest vitamin A activity, and β-cryptoxanthin which is assumed to have half provitamin A activity than β-carotene [52]. Traditionally, β-cryptoxanthin has been considered the main source of provitamin A in citrus juices since levels of β-carotene are very low or not detected. Nonetheless, the RE determined in CC and K juices was nearly twice than that of N due to the higher concentration of β-carotene (Table 3). Therefore, CC and K represent a better source of provitamin A than the traditional Navel oranges.

Even though the different juices of each variety presented similar qualitative composition of carotenoids, the method of juice extraction and treatment affected unevenly the content of carotenoids, depending on the orange genotype (Table 3). The industrial juice of N contained 18% more total carotenoids than the HS juice because of the larger content in phytoene, β-cryptoxanthin, luteoxanthin and mutatoxanthin. Phytofluene was not detected in HS juice of N, however, the industrial extraction favors its extractability although at very low levels (Table 3). Similarly, the industrial juice of CC presented 32% more total carotenoids compared to the HS, due to the amounts of phytoene (34% more) and nearby the double concentration of lycopene. Contrastingly, the content and composition of carotenoids in K juices hardly varied between the two extraction methods. The industrial extraction has been shown to increase the content of carotenoids in the orange juice since a more powerful squeezing of the fruits enhances the carotenoids release from the membranes of the pulp vesicles to the juice [55,56,57]. On the contrary, Stinco et al. [52] described that the industrial processing exerts a

negative impact on the content of carotenoids in the juice. These discrepancies may be attributed to the used of different orange varieties, and the impact of the processing conditions, which can have a significant influence on the carotenoid content.

Regarding the effects of the industrial juice treatments, HPH and pasteurization produced a notable reduction of the total carotenoid content in N juices, especially in β,β-xanthophylls, as β-cryptoxanthin, antheraxanthin, violaxanthin and luteoxanthin, with around a 30% reduction respect to the industrial juice (Table 3). It has been classically described that thermal treatments negatively affect the concentration of carotenoids in orange juices since high temperatures during thermal processing might cause the instability of the polyene chain of the carotenoids, resulting in their degradation by isomerization, oxidation and cleavage [29,41,58,59]. Our results agree with those of Etzbach et al. [60] which observed a significant reduction of violaxanthin in orange juice treated by conventional pasteurization (90 °C/30s). Nevertheless, in that study no changes in non-epoxy carotenoids such as lutein, zeaxanthin and β-cryptoxanthin nor in monoepoxides as anteraxanthin were detected, suggesting that diepoxy carotenoids such as violaxanthin and luteoxanthin were more susceptible toward thermal degradation. On the other hand, in HPH and pasteurized juices of CC, the concentration of vi-olaxanthin was also significantly reduced while the levels of β-cryptoxanthin and ze-axanthin increased. Furthermore, the total carotenoids content remained relatively stable since the predominant carotenoids correspond to linear carotenes which were not affected by the treatments. Variable observations concerning the effects of high-pressure treatments, high-pressure processing (HPP) and HPH, and pasteuriza-tion on the carotenoid content in orange juice are reported in the literature. In this sense, discrepancies might be attributed to the different processing conditions, such as juice extraction procedure, temperature, and equipment [37]. De Ancos et al. [21] showed that the HPP treatment at 200 and 400 MPa had a negative effect on the content of vioxalanthin and lycopene in Navel and CC juices, respectively. Sentandreu et al. [39] observed a significant decrease in total carotenoids in HPH-treated Navel juices.

Table 3. Carotenoid content and composition (μg/mL) of HS (hand-squeezed), industrial, HPH (high-pressure homogenized) and pasteurized juices of Navel, Cara Cara and Kirkwood oranges.

	Navel				Cara Cara				Kirkwood			
Carotenoids	HS	Industrial	HPH	Pasteur.	HS	Industrial	HPH	Pasteur.	HS	Industrial	HPH	Pasteur.
Phytoene	0.14±0.03Bb	0.38±0.02Ac	0.24±0.01ABb	0.15±0.05Bc	14.99±2.37Ba	23.12±0.68Aa	20.44±1.15Aa	20.52±0.20Aa	18.00±0.85Ba	18.35±0.22Bb	22.14±0.60Aa	19.97±0.04Bb
Phytoflluene	N.D.	0.09±0.01Ac	0.07±0.02Ab	0.06±0.01Ac	3.48±0.61Ca	5.02±0.19ABa	4.58±0.24Ba	5.59±0.01Aa	4.23±0.10BCa	3.99±0.01Cb	4.89±0.18Aa	4.50±0.01ABb
ζ-carotene	0.07±0.02Ab	0.08±0.01Ac	0.05±0.01Ab	0.07±0.01Ac	0.24±0.05Ba	0.26±0.03Ba	0.23±0.02Ba	0.34±0.01Aa	0.27±0.01Aa	0.17±0.01Cb	0.22±0.01Ba	0.28±0.01Ab
Neurosporene	N.D.	N.D.	N.D.	N.D.	0.06±0.02Ba	0.22±0.01Aa	0.08±0.06Ba	0.09±0.01Ba	0.07±0.03Aa	0.18±0.01Ab	0.11±0.05Aa	0.08±0.03Aa
Lycopene	N.D.	N.D.	N.D.	N.D.	1.30±0.27Ba	2.29±0.18Aa	1.95±0.03ABb	2.30±0.03Aa	1.49±0.52Ba	1.56±0.06Bb	2.82±0.24Aa	2.39±0.19ABa
Lutein	0.29±0.06Aa	0.35±0.02b	0.30±0.03Aa	0.30±0.06Aa	0.12±0.02Cb	0.18±0.01Bc	0.17±0.01Bb	0.24±0.01Aa	0.22±0.01Ba	0.54±0.02Aa	0.23±0.03Bab	0.21±0.01Ba
β-carotene	N.D.	N.D.	N.D.	N.D.	0.23±0.05Ca	0.31±0.01BCa	0.38±0.03ABb	0.47±0.03Aa	0.29±0.02Ca	0.34±0.02BCa	0.45±0.02Aa	0.37±0.01Bb
β-crypto.	0.65±0.14Aa	0.91±0.05Aa	0.69±0.05Ab	0.66±0.01Ac	0.55±0.11BCa	0.52±0.01Cc	0.73±0.02Bab	0.96±0.01Aa	0.64±0.04Ca	0.67±0.02BCb	0.85±0.04Aa	0.79±0.04ABb
Zeaxanthin	0.14±0.04Aa	0.18±0.01Aa	0.16±0.01Ab	0.15±0.04A	0.12±0.04Cb	0.15±0.01Ca	0.21±0.01Ba	0.29±0.02Aa	0.26±0.09Aa	0.26±0.05Aa	0.20±0.02Aa	0.30±0.05Aa
Antheraxanthin	1.00±0.13Aa	0.75±0.02ABa	0.63±0.04Bb	0.52±0.03Bc	0.47±0.08Bb	0.50±0.08Bb	0.59±0.05Bb	1.10±0.01Aa	1.25±0.03Aa	0.66±0.01Cb	0.95±0.01Ba	0.69±0.02Cb
Violaxanthin	4.25±0.31Aa	4.25±0.45Aa	2.84±0.06Ba	2.70±0.08Ba	2.80±0.48ABb	3.36±0.10Aa	2.49±0.15Ba	2.30±0.32Bab	2.41±0.04Ab	2.32±0.08Ab	2.04±0.03Bb	1.93±0.02Bb
Luteoxanthin	0.62±0.06Ca	1.44±0.09Aa	0.80±0.20BCa	1.14±0.01Ba	0.29±0.10Cb	0.65±0.01B	0.62±0.01B	0.87±0.03Aab	0.20±0.06Cb	0.77±0.05A	0.48±0.01Bb	0.69±0.07Ab
Mutatoxanthin	0.11±0.01Ca	0.52±0.07Aa	0.28±0.01Ba	0.45±0.01Aa	0.10±0.01Ca	0.25±0.01Bb	0.25±0.02Ba	0.41±0.03Aa	0.06±0.01Cb	0.45±0.01Aa	0.33±0.05Ba	0.44±0.01Aa
Total carot.	7.28±0.54Bb	8.94±0.24Ac	6.05±0.42Bb	6.21±0.12Bc	24.76±4.20Ba	36.88±0.82Aa	32.73±1.84Aa	35.47±0.12Aa	29.38±0.48Ca	30.27±0.25Cb	35.67±0.17Aa	32.64±0.28Bb
RE	0.05±0.01Ab	0.08±0.01Ab	0.06±0.0.4Ab	0.06±0.01A	0.09±0.02Ba	0.10±0.01Ba	0.12±0.01ABa	0.16±0.02Aa	0.10±0.03Aa	0.11±0.01Aa	0.15±0.02Aa	0.13±0.02Aa

Uppercase letters indicate significant differences between different type of juices for the same variety and lowercase letters indicate significant differences between varieties for the same type of juice by one-way ANOVA ($p<0.05$). β-crypto: β-cryptoxanthin. N.D: not detected. RE: retinol equivalents.

However, Lu et al. [38] did not observe changes in the content of carotenoids in CC juice after thermal treatment. The current study is the first evaluating the effect of pro-cessing on the juice from the red-fleshed orange K. We found that total carotenoids in HPH and pasteurized juices of K increased 15% and 8%, respectively, compared to the industrial juice (Table 3). These effects were associated with a higher concentration of phytoene and phytofluene (9-19%) and of lycopene content (45%) (Table 3). It is worth mentioning that these carotenes are greatly hydrophobic and, HPH and pasteurization treatments may severely disrupt suborganellar cell structures where carotenes are ac-cumulated [61,62] and then enhancing their release to the juice. Nevertheless, other studies in CC and the yellow-pigmented orange variety, Pinalate, reported that HPH and pasteurization decreased the content of colorless carotenes and lycopene [29,41,62]. Therefore, it is likely that the effect of juice processing treatment on carotenoids may be related to the cellular deposition structures where the carotenoids accumulated in the chromoplasts which may varies depending on their specific composition and contents [61,62].

In general, the industrial extraction increased or maintained the concentration of carotenoids of the juice compared to the hand-squeezing. Therefore, the industrial procedure may be considered a good option to optimize carotenoid content in the juice of the red-fleshed varieties without no detrimental effects. Additionally, HPH and pasteurization treatments have similar effects on carotenoids levels in industrial juices of these varieties, suggesting that temperature, rather than high pressures, is the main factor influencing carotenoid concentration in the juice [39,51,63]. The thermal stability of carotenoids is associated with their chemical structure, solubility, and hydrophobicity [64]. Hence, the variability of the results obtained by the HPH and pasteurization treatments might be explained by the differences in the matrix composition and in the structures of carotenoids storage between the orange varieties [21,22,29,61].

3.3. Vitamin C

All the juices of N orange presented 10-20% higher vitamin C concentration than those of CC and K (Figure 3A). This result is in good agreement with previous studies [21,38,65] suggesting that the genotype modulates the concentration of vitamin C [46]. However, recent studies accounted that the pulp of mature fruits of N and K contains similar values of vitamin

C [31] that may indicate the vitamin C extractability from pulp of red-fleshed oranges during juice processing is lower compared to standard oranges. Moreover, other factors such as ripening stage of the fruit and the growing conditions may also determinants of the vitamin C content and may result in differences between standard and red pulp oranges. In general, the extraction method and treatments applied to the industrial juices did not substantially affect the content of vitamin C of the three varieties although some minor changes were detected. The industrial ex-traction in CC juice slightly incremented (6%) the vitamin C levels compared with the HS juice while no differences between the extraction methods were detected in N and K. Gil-Izquierdo et al. [55] reported that Navel orange juice produced by commercial squeezing contained 25% more vitamin C than domestic squeezing. The HPH treat-ment increased the content of vitamin C in N juice, while it decreased in the CC and K juices compared to the industrial fresh juice. Similarly, other authors reported that high-pressure treatments exert variable effects in the concentration of vitamin C in orange juices [66,67] while others claimed that these types of treatments did not modify the concentration of this vitamin [21,59]. It has been suggested that only high-pressure treatments that entail a substantial temperature increment negatively affect vitamin C content due to its degradation or thermal oxidation [66]. However, pasteurization, which involve higher temperatures than HPH, did not reduce the concentration of vitamin C [55,68,69]. Taken together all these results, it is reasonable to suggest that discrepancies in the stability of vitamin C observed in the different studies depends on the combination of multiple factors, as juice extraction and the conditions of the treatments (temperature, pressure, time, etc.) [21].

3.5. Tocopherols

The study of accumulation and biosynthesis of tocopherols in sweet oranges has received little attention to date [47,70,71]. To our knowledge only a previous study has evaluated the concentration of tocopherols in red-fleshed oranges [31] and this is the first work analysing tocopherols in juices of red pulp varieties. α-Tocopherol was the only isoform identified in all juices with levels ranging from 61-129 μg/100 mL (Figure 3B). These values are very similar to those described for orange juices of standard varieties [70,71]. The analysis of tocopherols content showed that all juices of the red-fleshed varieties, except the hand-squeezed juice of CC, contained concentrations around 20-30% higher than those of N (Figure 3B).

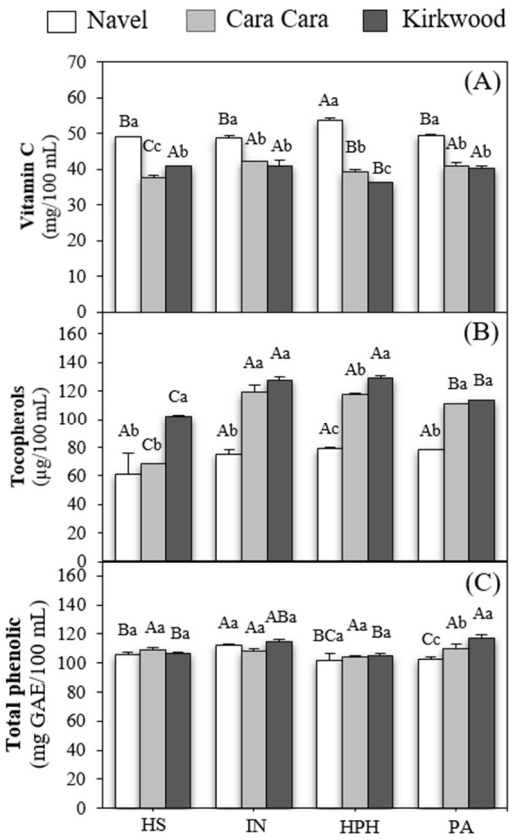

Figure 3. Content of vitamin C (A), tocopherols (B) and total phenolics (C) in hand-squeezed (HS), industrial (IN), HPH and pasteurized (PA) juices of N, CC and K orange. Uppercase letters indicate significant differences between different type of juices for the same variety and lowercase letters indicate significant differences between varieties for the same type of juice by one-way ANOVA ($p<0.05$).

However, previous data showed similar concentrations of tocopherols in the pulp of mature fruits of K and N [31]. The industrial extraction compared to HS juices in-creased 43% and 20% the content of tocopherols in CC and K juices, respectively, but not in N (Figure 3B). These data suggest that juice extraction, and specifically the industrial extraction, might enhance the extractability of tocopherols from the pulp vesicles to the juice matrix in the red-fleshed oranges. Pasteurization slightly reduced the levels of tocopherols in CC and K compared to the industrial and HPH juices (Figure 3B). Minor information is available on the stability of tocopherols during pasteurization and high-pressure processing in orange juices. Even though tocopherols are considered heat-sensitive compounds, contrasting results about their stability in different food matrix might be found. Cilla et al. [72] showed that HPP/400 MPa did not affect the concentration of α-tocopherol in milk-fruit beverages but strikingly, heat treatment (90 °C/30s) significantly increased its content. Instead, other works detected a

significant reduction of α-tocopherol in human milk after pasteurization [73]. Overall, processing conditions as well as food matrix characteristics appears to be crucial factors for tocopherols stability after thermal treatments.

3.4. Phenolics and flavonoids

The content of total phenolics experienced minor differences between varieties and treatments (Figure 3C). Only pasteurized juices displayed differences in total phenolics content between varieties in the following order K>CC>N. In agreement with our results, Brasili et al. [29] and De Ancos et al. [21] found similar total phenolic content in the juice of Navel compared to CC by Folin-Ciocalteu assay. Similarly, in a previous study we did not detect differences in neither level of phenolics nor flavonoids in the pulp of freshly harvested K and N fruits [31]. Additionally, our results agree with those of Bai et al. [56] that reported no differences in total phenolics between HS and industrial juices. Thus, a disparity on the effect of pasteurization on the levels of phenolics in orange juices is apparent. For example, Brasili et al. [29] described no effect of heat treatment on Navel and CC juices, while Bai et al. [56] supported that pasteurization increased total phenolic in orange juices.

The profile of flavonoids of N, CC and K juices was analyzed by HPLC-DAD (Table 4). In general, all juices showed similar flavonoid composition. The flavanone glycosides hesperidin (56-70% of the total) and narirutin (17-29% of the total) were the major flavonoids found in all juices, accounting both as much as 85-89% of total flavonoids (Table 4) consistent with previously data for other orange juices [74]. Other less abundant flavonoids detected in all juices were rutin, eriocitrin, naringin and dydimin. The concentration of total and individual flavonoids was similar between the juices of standard and the red-fleshed orange (Table 4). Previous studies indicated that Navel juices contained slightly higher flavonoids content than CC [29,32]. However, other studies did not find significant differences between the pulp [31] and juice [21] of blond and red-fleshed oranges. These discrepancies might be attributed to the influence of the maturity stage or the by environmental and growing conditions in the levels of these phytochemical. The comparison of the two juice extraction methods revealed that the hand-squeezing provided more flavonoids than the industrial extraction in the three varieties, although differences were only statistically significant in N and CC juices (Table 4).

This difference was mainly associated with the higher concentration of hesperidin in the HS juices. Regarding the processing juice treatments, neither the pasteurization nor the HPH treatment led to remarkable changes in the flavonoid content, in agreement with other citrus juice after HPH [39,59,63] and pasteurization [29,38,55,56,69].

Table 4. Flavonoid content and composition (mg/100 mL) of HS (hand-squeezed), industrial, HPH (high-pressure homogenized) and pasteurized juices of Navel, Cara Cara and Kirkwood oranges.

	Navel				Cara Cara				Kirkwood			
Flavonoids	HS	Industrial	HPH	Pasteurized	HS	Industrial	HPH	Pasteurized	HS	Industrial	HPH	Pasteurized
Rutin	1.20±0.06Ba	1.46±0.01Aa	1.53±0.06Aa	1.57±0.13Aa	1.09±0.03Ba	1.12±0.02Bb	1.35±0.02Ab	1.22±0.08ABa	1.11±0.04Aa	1.17±0.13Aab	1.08±0.01Ac	1.12±0.01Ab
Eriocitrin	0.36±0.01Aa	0.40±0.01Aa	0.41±0.01Ab	0.43±0.04Aa	0.40±0.05Ba	0.50±0.01ABa	0.57±0.02Aa	0.41±0.03Ba	0.48±0.03Aa	0.49±0.06Aa	0.53±0.01Aa	0.39±0.01Ba
Narirutin	5.72±0.19Aa	5.42±0.01Aa	5.57±0.18Aa	5.81±0.55Aa	4.14±0.08Bb	4.05±0.03Bb	4.81±0.07Ab	4.49±0.33ABb	4.01±0.20Bb	4.52±0.54ABa	4.15±0.03ABc	4.27±0.01Ab
Naringin	0.08±0.01Aa	0.10±0.01Aa	0.09±0.01Aa	0.09±0.01Aa	0.09±0.01Aa	0.08±0.01Aa	0.10±0.01Aa	0.10±0.01Aa	0.09±0.01Aa	0.11±0.01Aa	0.10±0.01Aa	0.10±0.01Aa
Hesperidin	17.57±1.04Aa	10.88±0.23Ba	10.67±0.54Ba	12.29±1.15Ba	17.32±0.07Aa	11.92±0.01Ba	11.54±0.28Ba	13.68±1.14Ba	15.53±2.04Aa	11.51±2.91Aa	10.80±0.07Aa	11.30±0.84Aa
Dydimin	1.12±0.02Aa	0.63±0.04Ba	0.64±0.04Bb	0.69±0.06Bb	1.21±0.06Aa	0.74±0.03Ca	0.86±0.06Ca	1.04±0.01Ba	0.80±0.06Ab	0.66±0.07Aa	0.73±0.03Aab	0.69±0.03Ab
Total	26.05±1.33Aa	18.89±0.29Ba	18.91±0.84Ba	20.89±1.94Ba	24.25±0.29Aa	18.41±0.09Ba	19.23±0.45Ba	20.94±1.70AB	22.03±2.37Aa	18.46±3.73Aa	17.39±0.15Ab	17.87±0.88Aa

Uppercase letters indicate significant differences between different type of juices for the same variety and lowercase letters indicate significant differences between varieties for the same type of juice by one-way ANOVA ($p<0.05$).

3.5. Antioxidant capacity

Hydrophilic antioxidant capacity (HAC) of N, CC and K juices was evaluated by DPPH (Figure 4A), FRAP (Figure 4B) and ABTS (Figure 4C). The HAC showed similar results by the three methods assayed and the N juices exhibited higher HAC than CC and K. Besides, no differences between both red-fleshed varieties were found for any type of juice. Positive correlations between DPPH and FRAP ($r^2 = 0.99$), ABTS-H and DPPH ($r^2 = 0.84$) and ABTS-H and FRAP ($r^2 = 0.83$) were obtained (Figure 5). These find-ings were similar to previous results reporting an enhanced HAC in Navel juices in comparison with CC [21,29]. Nonetheless, Zacarías-García et al. [31] indicated that HAC of the pulp of N and K fruit was similar. The analysis of correlations carried out between the bioactive compounds and antioxidant capacity display high positive cor-relation values between vitamin C content and DPPH ($r^2 = 0.95$), FRAP ($r^2 = 0.95$) and ABTS-H ($r^2 = 0.86$) (Figure 5). On the contrary, total phenolic compounds (TP) and flavonoids (TF) that were also at high concentrations in the water-soluble fraction of orange juices, did not show significant correlations with any of the antioxidant assays. These results agree with data provided in other studies, which indicate that vitamin C is the major contributor to the hydrophilic antioxidant capacity of orange juices [29,55, 75,76].

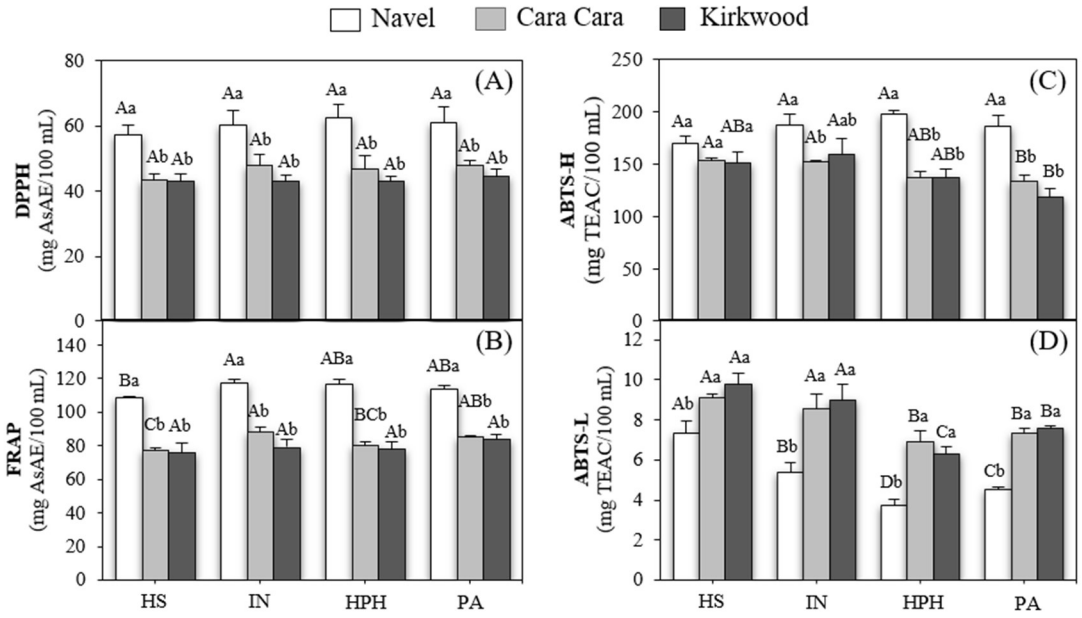

Figure 4. Hydrophilic antioxidant capacity assayed by DPPH (A), FRAP (B) and ABTS (ABTS-H) (C) and lipophilic antioxidant capacity assayed by ABTS (ABTS-L) (D) in hand-squeezed (HS), indus-trial (IN), HPH and pasteurized (PA) juices of N, CC and K orange. Uppercase letters indicate significant differences between different type of juices for the same variety and lowercase letters indicate significant differences between varieties for the same type of juice one-way ANOVA ($p<0.05$).

The lipophilic antioxidant capacity (LAC) was determined by ABTS (referred to as ABTS-L) (Figure 4D), since this method can determine the antioxidant capacity in the lipophilic fraction of the juice extracted with organic solvents [76]. The ABTS-L indicated that CC and K juices presented a remarkably major capacity than N juices (15-40%) (Figure 4D). The ABTS-L showed a good significant correlation with the concentration of total carotenoids ($r^2 = 0.65$) and the sum of phytoene and phytofluene ($r^2 = 0.68$), but lower than with the content of lycopene ($r^2 = 0.55$) (Figure 5). Even though lycopene has been described as the carotenoid with the highest antioxidant activity against ROS [12,77], the major lipophilic capacity of CC and K juices compared to N might be associated with the high levels of phytoene and phytofluene, which antioxidant properties have been also reported [13].

Therefore, the contribution of carotenoids to the LAC of juices depends not only on the total content, but also on the individual composition of carotenoids [75,76]. How-ever, other studies suggested that the contribution of carotenoids to the in vitro antioxidant capacity in orange juices is not relevant [21,75,78]. These discrepancies could be explained by the fact that the assays used to quantify this activity did not study independently the antioxidant capacity of the lipo- and hydrophilic fractions and then, most of results did not reflect the contribution of fat-soluble compounds [76,79,80]. On the other hand, despite the low correlation of tocopherols content with the lipophilic capacity ($r^2 = 0.33$), it cannot be excluded certain contribution of tocopherols to the antioxidant capacity of the juices.

The effect of the extraction method and treatment (HPH and pasteurization) on the antioxidant capacity of juices required an independent analysis. The hand and industrial squeezing demonstrated almost the same effectiveness in terms of antioxidant capacity [55]. The HAC of the juices of the three varieties was hardly affected by the treatments. Nevertheless, the LAC in the HPH and pasteurized juices of all varieties decreased in comparison to the corresponding industrial juice. Intriguingly, this reduction was not associated with a minor content of lipophilic compounds such as carotenoids and tocopherols in HPH and pasteurized juices (Table 3; Figure 3). Other authors also reported that thermal treatments might affect negatively to LAC [81]. Thus, our data suggest that the reduction in LAC in treated juices could be associated with a de-crease in the activity of other lipophilic compounds not analysed in this work. De An-cos et al. [21] observed that HPP/400 MPa treatment reduced the antioxidant capacity in hydrophilic extracts of Navel and CC juices.

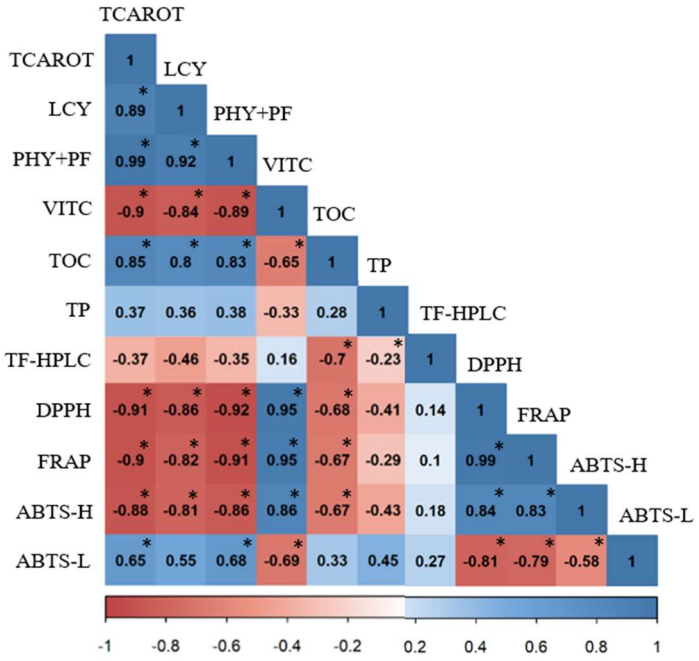

Figure 5: Matrix correlation between total carotenoids (TCAROT), vitamin C (VITC), tocopherols (TOC), total phenolics (TP), total flavonoids (TF), total flavonoids by HPLC (TF-HPLC) and antioxidant capacity (DPPH, FRAP, ABTS-H and ABTS-L) values obtained in the different juices of N, CC and K. Positive and negative correlations are shown in different shades of blue and red, respectively. Significant Pearson's correlation coefficient ($p<0.05$) is indicated with an asterisk.

In contrast, other authors did not detect differences in the antioxidant potential of orange juices after processing [55,59] or even observed an increase of the antioxidant capacity in CC juice [29]. The variability of results regarding the effects of thermal and non-thermal treatments on antioxidant capacity might be closely linked to the differences in the composition of the juices, processing conditions, type of extract (hydro- or lipophilic) and the antioxidant assay employed [37,82]. Together, the results obtained by the in vitro antioxidants assays, especially those of lipophilic, revealed promising perspectives of the antioxidant capacity of the red-fleshed varieties. Due to the limitations of the assays to determine the antioxidant capacity of the components of a complex food matrix, it would be of special interest to evaluate in future studies the biological effects of the juice from red-fleshed oranges by using in vivo systems, and to corroborate the contribution of the high content of carotenoids detected in these oranges to the antioxidant activity.

3.6. Sugars and organic acids

Sugars and organic acids are key compounds on the sensory properties of fruit juices [83]. Sucrose, glucose and fructose were the main sugars in all juices (Figure 6). Sucrose was the

most abundant (45.70-52.92 g/L) followed by glucose (25.48-37.04 g/L) and fructose (22.06-25.12 g/L), in a ratio near 2:1:1 (sucrose:glucose:fructose), which is a recognized parameter to identify genuine fresh orange juice from adulteration [58]. The concentrations of sugars detected in all juices were in the range described in the literature for orange juices [3,65]. The HS juice of the three varieties contained very similar concentration of sugars (Figure 6). The HPH and pasteurized juices of CC and K contained slightly higher sucrose than N (Figure 6A). The content of glucose was 20-30% higher in N juices compared to the red-fleshed varieties (Figure 6B) and the levels of fructose were higher in N and CC than in K juices (Figure 6C). Overall, the total content of sugars was approximately equivalent in the juices of the three varieties (Figure 6D). Some authors reported lower concentration of sugars in the juice of CC than those presented in this work [29,84]. Other studies obtained similar sugar concentrations in fruits of CC and K orange in comparison with N [29,31,65]. Moreover, HPH and pasteurization had no significant effect in the stability and composition of sugars in the juices of the three varieties, as reported previously [29,85].

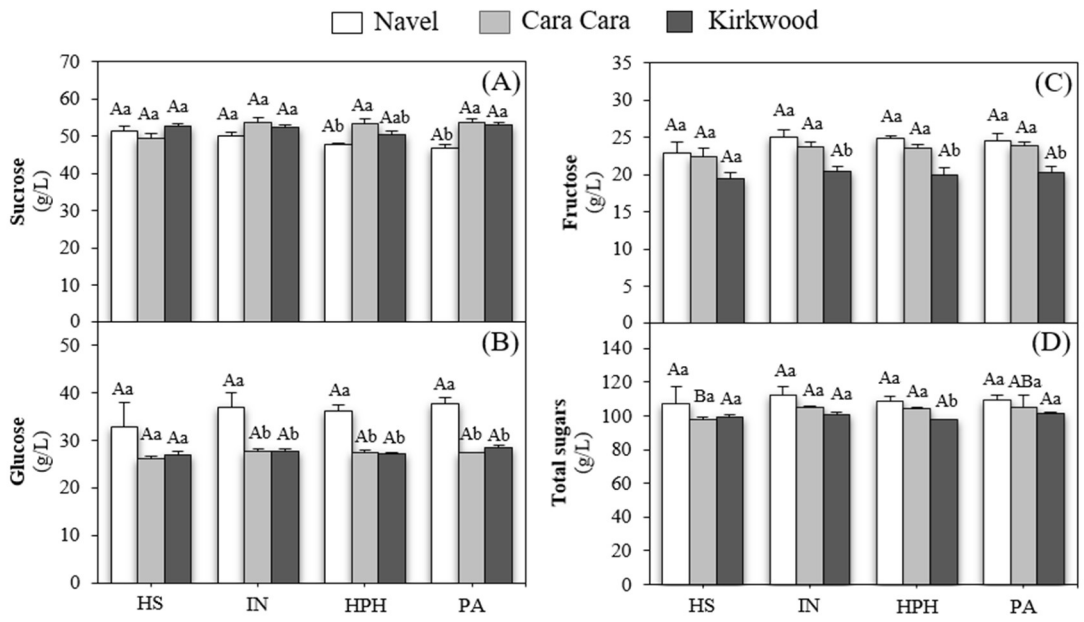

Figure 6. Content of sucrose (A), glucose (B), fructose (C) and total sugars (D) in hand-squeezed (HS), industrial (IN), HPH and pasteurized (PA) juices of N, CC and K orange. Uppercase letters indicate significant differences among different type of juices for the same variety and lowercase letters indicate significant differences among varieties for the same type of juice one-way ANOVA ($p<0.05$).

Overall, the juices from the red-fleshed varieties presented slightly higher sucrose and lower glucose concentration. Furthermore, the juices of N and CC contained larger amounts of fructose than K juices. Therefore, the differences found in the concentration of the individual sugars as in glucose, and especially in fructose, which has the highest sweetness perception between the sugars present in orange juice, might have an influence on the sweetness of the juice and their organoleptic quality.

The main organic acids detected in all orange juices in order of abundance were: citric acid (13.52-16.02 g/L), quinic acic (5.65-9.17 g/L), malic acid (2.66-5.68 g/L) and succinic acid (1.61-3.81 g/L) (Figure 7).

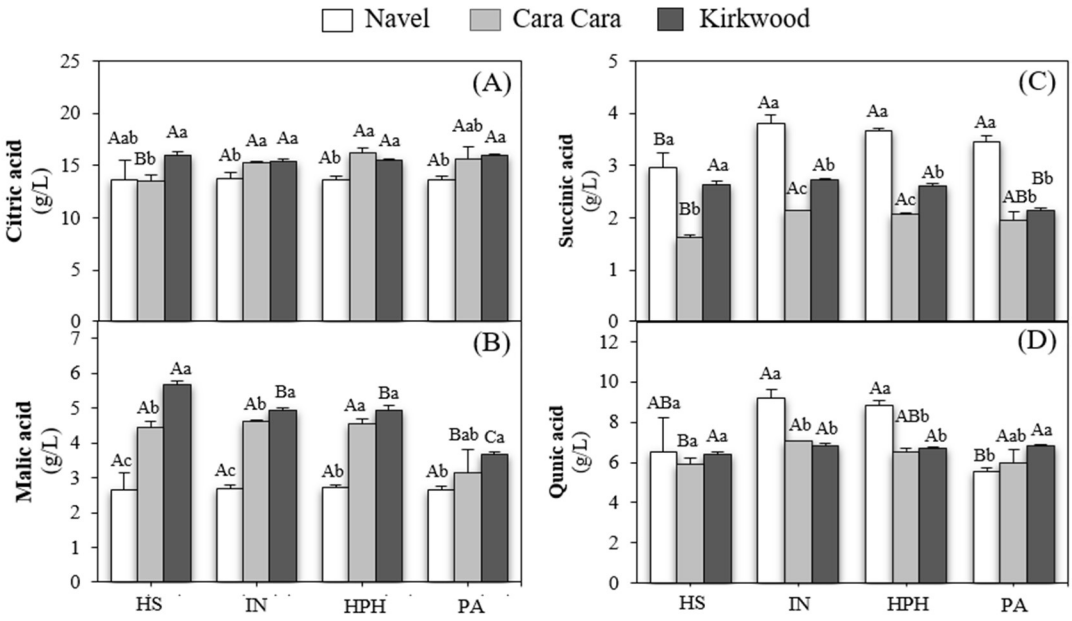

Figure 7. Content of citric acid (A), malic acid (B), succinic acid (C) and quinic acid (D) hand-squeezed (HS), industrial (IN), HPH and pasteurized (PA) juices of NF, CC and K orange. Uppercase letters indicate significant differences between different type of juices for the same variety and lowercase letters indicate significant differences between varieties for the same type of juice one-way ANOVA ($p<0.05$).

Between these organic acids, citric acid is the largest contributor to the acidity taste in orange juice and accounted for 46.8-58.5% of total organic acids in all juices. The ratio of citric acid to total organic acids found in our study is similar to that re-ported by Albertini et al. (2006) for other sweet oranges. In this work, consistent differences were observed in the concentration of individual acids between the juices of N and the two red-fleshed juices, which is a factor contributing to the characteristic acidity taste of orange juices and the organoleptic

quality [86]. Particularly, the composition of organic acids in CC and K juices in comparison with N juices was characterized by a slightly higher amount of citric acid (10-20%) (Figure 7A), a notably higher content of malic acid (Figure 7B) and lower levels of succinic acid (Figure 7C). Minor variations in the content of organic acids between the HS and the industrial juice were detected. Thus, the type of juice squeezing did not affect the concentration of organic acids. The HPH and pasteurization treatments barely affected the concentration of organic acids in all the varieties and only the pasteurization significantly reduced the content of malic acid in CC and K and around 40% that of quinic acid in N (Figure 7). These results agree with those described by other authors, which suggested that high pressures and thermal treatments did not substantially modify these compounds [29,85].

In general, the juices of both red-fleshed oranges presented a similar organic acids profile but concentrations of malic and succinic were higher and lower, respectively, than in the blond orange juices. Thus, these differences in total organic acids and their individual ratio could have an important effect on the sensory characteristics of the red-fleshed orange juices.

3.7. Multivariate analysis of chemical composition and antioxidant capacity in hand-squeezed, industrial, HPH and pasteurized juices

A principal component analysis (PCA) was performed to study the variability be-tween the varieties and the different type of juices, considering the concentration of all the compounds analyzed and the antioxidant capacity (Figure 8). The first two components (PCs) explained for the 78.52% of the total variance of the data. The PC1 (63.91%) discriminates samples according to the concentration of compounds and antioxidant values. Thus, for each variety the juices obtained by both extraction methods and treatments clustered together, indicating that minor differences were found between different type of juices. Furthermore, the juices of CC and K clustered very close, that means that the parameters considered in this study do not discriminate between juices of both red-fleshed oranges, but they clearly separate from those of N. In other terms, the juices of CC and K characterized, in order from highest to lowest variance contribution, by higher levels of carotenoids, citric acid, malic acid, tocopherols, ABTS-L, sucrose and total phenolic (Figure 8; Supplementary Table 1). Instead, the juices from N are distinguished by DPPH, FRAP, glucose, vitamin C, ABTS-H, succinic acid and fructose (Figure 8; Supplementary Table 1). On the other hand, the PC2 (14.61%) separates samples

principally by the content of total flavonoids by HPLC (TF-HPLC). This observation explained the fact that the HS juices, especially those of N and CC, grouped slightly separated regarding the other juices since their concentration of total flavonoids (TF-HPLC) was significantly higher. Considering the exhaustive characterization of the juices developed above and the results provided by the PCA, we might conclude that the concentration of carotenoids is the variable that contributes the most to the total variance and discriminates largely between the juices of N and the red-fleshed varieties.

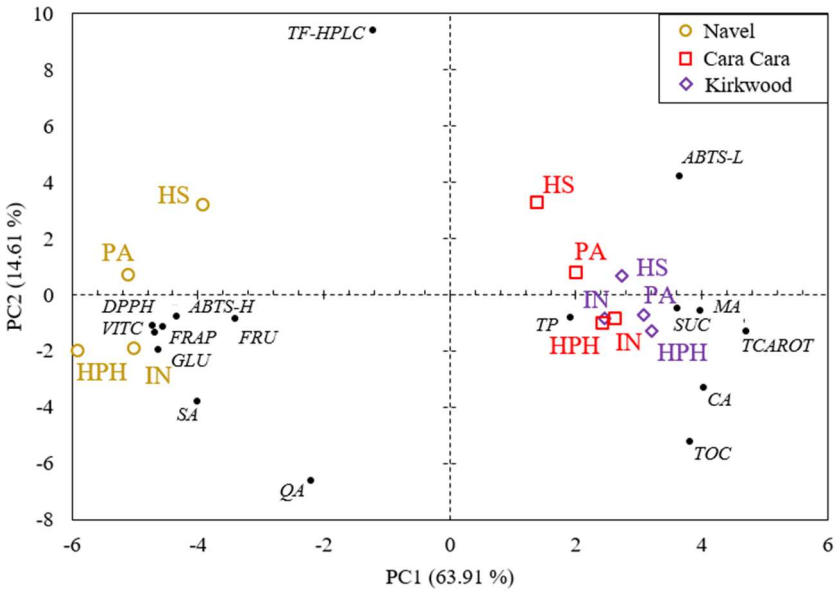

Figure 8. Principal component analysis (PCA) of total carotenoids (TCAROT), vitamin C (VITC), tocopherols (TOC), total phenolics (TP), total flavonoids by HPLC (TF-HPLC), sucrose (SUC), glucose (GLU), fructose (FRU), citric acid (CA), malic acid (MA), succinic acid (SA), quinic acid (QA) and antioxidant capacity (DPPH, FRAP, ABTS-H and ABTS-L) in hand-squeezed (HS), industrial (IN), high-pressure homogenized (HPH) and pasteurized (PA) juices of N (yellow circles), CC (red squares) and K (purple diamonds) orange.

3.7. Composition of bioactive compounds and antioxidant capacity of Navel Foios, Cara Cara and Kirkwood industrial juice by-product

In the present work, we carried out a comprehensive analysis of the composition of main bioactive compounds and antioxidant capacity of waste generated during the industrial juice squeezing of the standard variety N and the red-fleshed orange CC and K (Figure 9). We examined the composition of carotenoids and the contents of vitamin C, tocopherols, total phenolics and flavonoids, as well as the hydrophilic (DDPH, FRAP and ABTS-H) and lipophilic (ABTS-L and SOAC) antioxidant capacity of the lyophilized waste generated from

the three variety. The total concentration of carotenoids in the by-product of N was 132.22 μg/g DW, while both red-fleshed varieties presented a concentration 10-fold higher, 1323.81 μg/g DW and 1344.56 μg/g DW in CC and K, respectively (Table 5).

In addition, the carotenoid composition of the by-products of the three varieties was markedly different. The carotenoid profile of each variety resembled that described for the corresponding juice (section 3.2) and pulp [15,20-22,29,31]. The by-product from N is characterized by a large content of β,β-xanthophylls, mainly violaxanthin (47.15 μg/g DW) and luteoxanthin (18.67 μg/g DW), and the carotenes phytoene (29.72 μg/g DW) and phytofluene (16.39 μg/g DW). In contrast with the low proportion of the colorless phytoene and phytofluene in the pulp and juice of N oranges, these carotenes accounted for nearly 35% of total carotenoids in the by-product of N. Thus, it is likely that this proportion of colorless carotenes might come from the peel of the fruit (flavedo and albedo) that is a rich source of carotenoids [15,20,22].

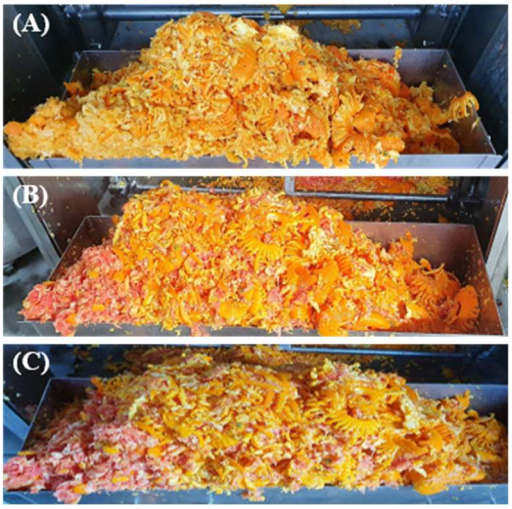

Figure 9. Image of the orange by-product of the standard variety Nave (A) and the red-fleshed varieties Cara Cara (B) and Kirkwood (C) generated during the industrial juice extraction.

The carotenoid content of the by-product of both red-fleshed oranges is mainly composed by large amounts of phytoene and phytofluene (87%) followed by lycopene (8-10%). β,β-Xanthophylls content was about the half than that found in N and ac-counted about 3.5% of total carotenoids. It is worth to mention that the concentration of phytoene and phytofluene in the by-product of both red-fleshed oranges was about 30- and 10-times, respectively, higher than in the by-product of the N orange (Table 5).

Table 5. Carotenoid content and composition (μg/g DW) of Navel, Cara Cara and Kirkwood orange by-products.

Parameters	Navel	Cara Cara	Kirkwood
Phytoene	29.72 ± 10.91b	998.09 ± 131.41a	988.94 ± 31.55a
Phytoflluene	16.39 ± 2.09b	164.01 ± 35.36a	166.72 ± 5.65a
ζ-carotene	4.91 ± 0.67a	3.57 ± 1.56ab	2.23 ± 0.14b
Neurosporene	N.D.	3.57 ± 0.07b	7.82 ± 0.27a
Lycopene	N.D.	104.36 ± 4.16b	123.25 ± 9.92a
Lutein	0.93 ± 0.05a	N.D.	0.57 ± 0.08b
β-carotene	N.D.	7.84 ± 2.30a	7.74 ± 0.85a
β-cryptoxanthin	5.73 ± 0.50a	4.09 ± 0.58a	3.27 ± 0.06b
Zeaxanthin	1.08 ± 0.13a	N.D.	1.39 ± 0.15a
Antheraxanthin	4.37 ± 6.18	N.D.	N.D.
Violaxanthin	47.15 ± 1.58a	30.70 ± 1.24b	33.43 ± 0.27b
Luteoxanthin	18.67 ± 0.64a	7.12 ± 0.38b	7.09 ± 0.34b
Mutatoxanthin	3.30 ± 0.14a	0.46 ± 0.21c	2.09 ± 0.13b
Total carotenoids	132.22 ± 8.73b	1323.81 ± 184.42a	1344.56 ± 47.29a
RE	0.96 ± 0.12b	1.66 ± 0.19a	1.56 ± 0.17a

Lowercase letters indicate significant differences between orange by-products of each variety by one-way ANOVA ($p<0.05$). N.D: not detected. R.E: retinol equivalents.

In addition, it is interesting to mention that β-carotene was also detected in the by-product of the red-fleshed oranges (0.6% of the total content) which determined a 50% increase in the value of retinol equivalents respect to the N by-product (Table 5).

Important amounts of other bioactive compounds were also detected in the orange by-products of the three varieties (Table 6). The concentration of vitamin C found in N (151.87 mg/100g DW) and CC (141.83 mg/100g DW) was significantly higher than in K (99.90 mg/100g DW). The levels of tocopherols in the three varieties ranged 128.60-148.16 μg/g DW, being CC the richest (Table 6). The isoforms α- and γ-tocopherol were identified in the by-product of all samples, being α-tocopherol the main in the three varieties, accounting for 86-88% of total tocopherols. The tocopherol composition observed in the by-product is similar to that described for the flavedo of mature orange fruits [47]. In the pulp of sweet orange, γ-tocopherol is barely detectable, and concentrations of total tocopherols are very low (≤ 2%) [31,47]. Hence, it is reasonable to assume that the content of tocopherols in the orange by-product mainly comes from flavedo tissue. The concentration of total phenolic was significantly higher in N (2174.56 mg GAE/100g DW) than in the red-fleshed CC (1901.64 mg GAE/100g DW) and K (1886.40 mg GAE/100g DW). The content of flavonoids was similar

between N (1548.96 mg HesE/100g DW) and CC (1516.14 mg HesE/100g DW), and slightly lower in K (1436.82 mg GAE/100g DW). In general, it seems that the juice processing by-product of N contain larger amounts of water-soluble antioxidant compounds such as vitamin C, phenolic and flavonoids than CC and K. This is consistent with the fact that the evaluation of the HAC determined by the FRAP and ABTS-H showed higher values of N orange by-product than those of CC and K (Table 6). Contrary, ABTS-L showed higher capacity in the processing by-products of both red-fleshed varieties than in N (Table 6). Additionally, in order to quantify the quenching singlet oxygen capacity in these by-products, the SOAC analysis was performed (Figure 10D). The K and CC by-product showed between 1.5 and 2-times higher SOAC values than N, thus by-product of both red-fleshed varieties were more efficient singlet oxygen scavengers than N (Table 6). Carotenoids are recognized as efficient quenchers of singlet oxygen and other reactive oxygen species [87]. The relationship between the concentration of carotenoids and the capacity of quenching singlet oxygen (SOAC) in citrus fruits has been established [50] and our results corroborate those in which the pulp of the red-fleshed fruit had higher SOAC than the ordinary Navel orange [31].

Different studies have addressed the physico-chemical characterization of citrus waste and its importance for the revalorization in many applications, such as biofuel, biorefinery, compost, production, animal feed, essential oils and extraction of pectin and other compounds with biological activity [42,43,88,89].

In this regard, the by-products from the red-fleshed oranges CC and K represent a higher added-value by-product compared to that of N due to the large concentration of carotenoids, especially phytoene, phytofluene, lycopene (which is not present in standard blond oranges), tocopherols and their enhanced antioxidant capacity against singlet oxygen. Altogether, red-fleshed orange processing by-product is a promising source of bioactive compounds with high potential in the food and nutraceutical industry.

Table 6. Content of vitamin C, tocopherols, total phenolics, total flavonoids and antioxidant capacity (DPPH, FRAP, ABTS-H, ABTS-L and SOAC) of Navel, Cara Cara and Kirkwood orange by-products.

Parameters	Navel	Cara Cara	Kirkwood
Vitamin C (mg/100g DW)	151.87 ± 4.62a	141.83 ± 5.07a	99.90 ± 2.80b
Tocopherols (μg/g DW)	128.60 ± 4.69b	148.16 ± 0.92a	139.12 ± 5.97ab
Total phenolics (mg GAE/100g DW)	2174.56 ± 4.85a	1901.64 ± 75.51b	1886.40 ± 78.62b
Total flavonoids (mg HesE/100g DW)	1548.96 ± 42.98a	1516.14 ± 16.18a	1436.82 ± 11.92b
DPPH (mg AsAE/100g DW)	179.16 ± 5.90a	167.72 ± 6.15ab	154.09 ± 3.01b
FRAP (mg AsAE/100g DW)	224.04 ± 12.23a	203.91 ± 0.97b	188.01 ± 4.38c
ABTS-H (TEAC/100g DW)	1237.61 ± 52.42a	1048.44 ± 54.72b	1049.28 ± 42.93b
ABTS-L (TEAC/100g DW)	155.37 ± 1.30b	172.92 ± 1.60a	171.52 ± 2.10a
SOAC[a]	0.47 ± 0.02c	0.79 ± 0.04b	0.92 ± 0.04a

GAE: gallic acid equivalents. HesE: hesperidin equivalents. AsAE: ascorbic acid equivalents. TEAC: trolox equivalent antioxidant capacity. SOAC: singlet oxygen absorption capacity. Lowercase letters indicate significant differences between orange by-products of each variety by one-way ANOVA ($p<0.05$). [a]Relative SOAC values based on g/L unit.

4. Conclusions

In this study, we investigated the composition of nutrients and bioactive compounds, and antioxidant capacity in juices of two red-fleshed oranges, CC and K, in comparison with the standard blond Navel orange, obtained by two extraction methods and two different treatments. Since fruit of the common Navel and the red-fleshed varieties were harvested in different locations we cannot discard an effect of growing conditions on the composition of the fresh juices. Even so, for the three orange varieties, the hand and industrial juice squeezing showed similar efficiency extracting the compounds from orange fruits, with some exceptions. The treatments HPH (150 MPa/55 °C/1min) and pasteurization (85 °C/30s) rendered red-fleshed orange juices with similar composition than the corresponding fresh juices. The most remarkable difference in CC and K compared with N juice was the 3- to 6-times higher carotenoids content, being phytoene and phytofluene the main carotenoids. The HPH and pasteurization affected variably the carotenoids concentration of each variety, mainly due to the different composition of the juices. Vitamin C was substantially higher in Navel, while both red-fleshed varieties presented higher amounts of tocopherols. Additionally, vitamin C was the main contributor to the hydrophilic antioxidant capacity, whereas the higher lipophilic antioxidant capacity of CC and K could be associated with the large concentrations of carotenes. Finally, the orange by-product of the red-fleshed oranges generated during the industrial juice extraction is greatly enriched in carotenoids and its lipophilic fraction had high antioxidant capacity against singlet oxygen.

The increasing demand of consumers for healthy and environmentally friendly products leads the food industry to search for sustainable processes and foods with high added-value. In this context, juices from red-fleshed orange varieties, fresh hand-squeezed or industrial processed represent a rich source of dietary carotenoids and enhanced antioxidant capacity, without significant detrimental effects on other bioactive compounds. Therefore, the juices from red pulp oranges could provide in-creased benefits for human nutrition and health in comparison with standard orange juice. In addition, the orange by-products from red-fleshed oranges show higher potential for revalorization as functional ingredients for designing healthy foods or other nutraceutical industrial applications.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Percentage of contribution of variables to total variance of principal component 1 and 2.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

01. FAOSTAT statistical database, 2020. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy. https://www. https://www.fao.org/3/cb6492en/cb6492en.pdf

02. Ministerio de Agricultura, Pesca y Alimentación de España (MAPA), 2020. Avance de datos de Frutales Cítricos. https://www.mapa.gob.es/es/estadistica/temas/estadisticas-agrarias/agricultura/superficies-producciones-anuales-cultivos/

03. Rampersaud, G.C.; Valim, M.F. 100% citrus juice: Nutritional contribution, dietary benefits, and association with anthropometric measures. Crit. Rev. Food Sci. Nut. **2017**, 57(1), 129–140. https://doi.org/10.1080/10408398.2013.862611

04. Lu, X.; Zhao, C.; Shi, H.; Liao, Y.; Xu, F.; Du, H.; Xiao, H.; Zheng, J. Nutrients and bioactives in citrus fruits: Different citrus varieties, fruit parts, and growth stages. Crit. Rev. Food Sci. Nut. **2021.** https://doi.org/10.1080/10408398.2021.1969891

05. Saini, R. K.; Ranjit, A.; Sharma, K.; Prasad, P.; Shang, X.; Gowda, K.G.M.; Keum, Y.S. Bioactive compounds of citrus fruits: A review of composition and health benefits of carotenoids, flavonoids, limonoids, and terpenes. Antioxidants **2022**, 11(2), 239. https://doi.org/10.3390/antiox11020239

06. Turner, T.; Burri B.J. Potential nutritional benefits of current citrus consumption. Agriculture **2013**, 170-187. https://doi.org/10.3390/agriculture3010170

07. Lv, X.; Zhao, S.; Ning, Z.; Zeng, H.; Shu, Y.; Tao, O.; Xiao, C.; Lu, C.; Liu Y. Citrus fruits as a treasure trove of active natural metabolites that potentially provide benefits for human health. Chem. Cent. J. **2015**, 9, 68. https://doi.org/10.1186/s13065-015-0145-9

08. Cirmi, S.; Maugeri, A.; Ferlazzo, N.; Gangemi, S.; Calapai, G.; Schumacher, U.; Navarra, M. Anticancer potential of Citrus juices and their extracts: A systematic review of both preclinical and clinical studies. Front. Pharmacol. **2017**, 8, 420. https://doi.org/10.3389/fphar.2017.00420

09. Maugeri, A.A.; Cirmi, S.A.; Minciullo, P.L.B.; Gangemi, S.B.; Calapai, G.C.; Mollace, V.D.; Navarra M.A. Citrus fruits and inflammaging: A systematic review. Phytochem. Rev. **2019**, 18(4), 1025–1049. https://doi.org/10.1080/14786419.2019.1601196

10. Tadeo, F.R.; Terol, J.; Rodrigo, M.J.; Licciardelo, C.; Sadka, A. Fruit growth and development. In The Genus Citrus; Talón, M., Caruso, M., Gmitter, F., Eds.; Woodhead Publishing: Cambridge, UK, 2020; pp. 495–508.

11. Lado, J.; Rodrigo, M.J.; Zacarías, L. Maturity indicators and citrus fruit quality. Stewart Postharvest Rev. **2014**, 10, 1–6.

12. Stahl, W.; Sies, H. Antioxidant activity of carotenoids. Mol. Asp. Med. **2003**, 24, 345–351. https://doi.org/10.1016/s0098-2997(03)00030-x

13. Martínez, A.; Stinco, C.M.; Meléndez-Martínez, A.J. Free radical scavenging properties of phytofluene and phytoene isomers as compared to lycopene: A combined experimental and theoretical study. J. Phys. Chem. **2014**, 118, 9819–9825. https://doi.org/10.1021/jp503227j

14. Barros, M.P; Rodrigo, M.J.; Zacarias, L. J. Agric. Food Chem. **2018,** 66(23), 5733-5740. https://doi.org/10.1021/acs.jafc.8b00866

15. Alquézar, B.; Rodrigo, M.J.; Zacarías, L. Carotenoid biosynthesis and their regulation in citrus fruits. Tree For. Sci. Biotech. **2008**, 2(1), 23-37.

16. Kato, M. Mechanism of carotenoids accumulation in Citrus fruit. J. Jpn. Soc. Hortic. Sci. **2012**, 219–233. https://doi.org/10.2503/jjshs1.81.219

17. Ikoma, Y.; Matsumoto, H.; Kato, M. Diversity in the carotenoid profiles and the expression of genes related to carotenoid accumulation among citrus genotypes. Breed. Sci. **2006**, 66, 139–147, https://doi.org/10.1270/jsbbs.66.139.17

18. Meléndez-Martínez, A.J.; Vicario, I. M.; Heredia, F.J. Carotenoids, color, and ascorbic acid content of a novel frozen-marketed orange juice. J. Agric. Food Chem. **2007**, 55(4), 1347–1355. https://doi.org/10.1021/jf063025b.

19. Meléndez-Martínez, A.J.; Vicario, I.M.; Heredia, F.J. Review: Analysis of carotenoids in orange juice. J. Food Compos. Anal. **2007**, 20, 638–649. https://doi.org/10.1016/j.jfca.2007.04.006.

20. Rodrigo, M.J.; Cilla, A.; Barberá, R.; Zacarías, L. Carotenoid bioaccessibility in pulp and fresh juice from carotenoid-rich sweet oranges and mandarins. Food Funct. **2015**, 6(6), 1950–1959. https://doi.org/10.1039/c5fo00258c

21. De Ancos, B.; Rodrigo, M.J; Sánchez-Moreno, C.; Cano, M.P; Zacarías, L. Effect of high-pressure processing applied as pretreatment on carotenoids, flavonoids and vitamin C in juice of the sweet oranges 'Navel' and the red-fleshed 'Cara Cara'. Int. Food Res. J. **2020**, 132, 109105. https://doi.org/10.1016/j.foodres.2020.109105

22. Zacarías-García, J.; Cronje, P.J.; Gianfranco, D.; Rodrigo, M.J.; Zacarías, L. A comprehensive analysis of carotenoids metabolism in two red-fleshed mutants of Navel and Valencia sweet oranges (Citrus sinensis). Front. Plant Sci. **2022**, 13,1034204. https://doi.org/https://doi.org/10.3389/fpls.2022.1034204

23. Giuffrida, D.; Cacciola, F.; Mapelli-Brahm, P.; Stinco, C.M.; Dugo, P.; Oteri, M.; Mondello, L.; Meléndez-Martínez A.J. Free carotenoids and carotenoids esters composition in Spanish orange and mandarin juices from diverse varieties. Food Chem. **2019**, 300:125139. https://doi.org/10.1016/j.foodchem.2019.125139

24. Monselise, S.P.; Halvey, A.H. Detection of lycopene in pink orange fruit. Science **1961**, 133, 1478. https://doi.org/10.1126/science.133.3463.1478

25. Saunt, J. Citrus Varieties of the World. Sinclair International Ltd: Norwich, England, 2000.

26. Liu, Q.; Xu, J.; Liu, Y.; Zhao, X.; Deng, X.; Guo, L., Gu, J. A novel bud mutation that confers abnormal patterns of lycopene accumulation in sweet orange fruit (Citrus sinensis L. Osbeck). J. Exp. Bot. **2007**, 58, 4161-4171. https://doi.org/10.1093/jxb/erm273

27. Lee, H.S. Characterization of carotenoids in juice of red navel orange (Cara Cara). J. Agric. Food Chem. **2001**, 49, 2563-2568. https://doi.org/10.1021/jf001313g

28. Alquézar, B.; Rodrigo, M.J.; Zacarías, L. Regulation of carotenoid biosynthesis during fruit maturation in the red-fleshed orange mutant Cara Cara. Phytochem. **2008**, 69(10). https://doi.org/10.1016/j.phytochem.2008.04.020

29. Brasili, E.; Seixas Chaves, D.F.; Xavier, A.A.O.; Mercadante, A.Z.; Hassimotto, N.M.A.; Lajolo, F.M. Effect of pasteurization on flavonoids and carotenoids in Citrus sinensis (L.)

Osbeck cv. 'Cara Cara' and 'Bahia' juices. J. Agric. Food Chem. **2017**, 65, 1371–1377. https://doi.org/10.1021/acs.jafc.6b05401

30. Lu, P.J.; Wang, C.Y.; Yin, T.T.; Zhong, S.L.; Grierson, D.; Chen, K.S.; Xu, C.J. Cytological and molecular characterization of carotenoid accumulation in normal and high-lycopene mutant oranges. Sci. Rep. **2017**, 7, 1-15. https://doi.org/10.1038/s41598-017-00898-y
31. Zacarías-García, J.; Pérez-Través, L.; Gil, J.V.; Rodrigo, M.J.; Zacarías, L. Bioactive compounds, nutritional quality and antioxidant capacity of the red-fleshed Kirkwood Navel and Ruby Valencia oranges. Antioxidants **2022**, 11, 1905. https://doi.org/10.3390/antiox11101905
32. De Ancos, B.; Cilla, A.; Barberá, R.; Sánchez-Moreno, C.; Cano, M.P. Influence of orange cultivar and mandarin postharvest storage on polyphenols, ascorbic acid and antioxidant activity during gastrointestinal digestion. Food Chem. **225**, 114–124. https://doi.org/10.1016/j.foodchem.2016.12.098
33. Silveira, J.Q.; Dourado, G.K.Z.S.; Cesar, T.B. Red-fleshed sweet orange juice improves the risk factors for metabolic syndrome. Int. J. Food Sci. Nutr. **2015**, 66(7), 830–836. https://doi.org/10.3109/09637486.2015.1093610
34. Cilla, A.; Rodrigo, M.J.; Zacarías, L.; de Ancos, B.; Sánchez-Moreno, C.; Barberá, R.; Alegría A. Protective effect of bioaccessible fractions of citrus fruit pulps against H2O2-induced oxidative stress in Caco-2 cells. Food Res. Int. **2018**, 103, 335–344. https://doi.org/10.1016/j.foodres.2017.10.066
35. De Oliveira, R.B.; Cadavid, C.O.M.; Carmona, L.; Peña, L.; de Paula Oliveira, R. Pasteurized orange juice rich in carotenoids protects Caenorhabditis elegans against oxidative stress and β-amyloid toxicity through direct and indirect mechanisms. Oxid. Med. Cell. Longev. **2019**, 5046280. https://doi.org/10.1155/2019/5046280
36. Carbonell, J.V.; Navarro, J.L.; Izquierdo, L., Sentandreu, E. Influence of high-pressure homogenization and pulp reduction on residual pectinmethylesterase activity, cloud stability and acceptability of Lane Late orange juice: A study to obtain high quality orange juice with extended shelf life. J. Food Eng. **2013**, 119, 696-700. https://doi.org/10.1016/j.jfoodeng.2013.06.04
37. Galaverna, G.; Dall'Asta, C. (2014). Production processes of orange juice and effects on antioxidant components. In Processing and Impact on Antioxidants in Beverages. Preedy V.R., Eds.; Elsevier: Amsterdam, 2014; (pp. 203–214).
38. Lu, Q.; Peng, Y.; Zhu, C.; Pan, S. Effect of thermal treatment on carotenoids, flavonoids and ascorbic acid in juice of orange cv. Cara Cara. Food Chem. **2018**, 265, 39–48. https://doi.org/10.1016/j.foodchem.2018.05.072
39. Sentandreu, E.; Stinco, C.M.; Vicario, I.M.; Mapelli-Brahm, P.; Navarro, J.L.; Meléndez-Martínez A.J. High-pressure homogenization as compared to pasteurization as a sustainable approach to obtain mandarin juices with improved bioaccessibility of carotenoids and flavonoids. J. Clean. Prod. **2020**, 262. https://doi.org/10.1016/j.jclepro.2020.121325
40. Martínez-Monteagudo, S.I.; Yan, B.; Balasubramaniam, V.M. Engineering process characterization of high-pressure homogenization from laboratory to industrial scale. Food Eng. Rev. **2017**, 9, 143-169. https://doi.org/10.1007/s12393-016-9151-5

41. Stinco, C.M.; Sentandreu, E.; Mapelli-Brahm, P.; Navarro, J.L.; Vicario, I.M.; Meléndez-Martínez, A.J. Influence of high-pressure homogenization and pasteurization on the in vitro bioaccessibility of carotenoids and flavonoids in orange juice. Food Chem. **2020**, 331:127259. https://https://doi.org/ 10.1016/j.foodchem.2020.127259

42. Cypriano, D. Z.; da Silva, L.L.; Tasic, L. High value-added products from the orange juice industry waste. Waste Manage. **2018**, 79, 71–78. https://doi.org/10.1016/j.wasman.2018.07.028

43. Zema, D.A.; Calabrò, P.S.; Folino, A.; Tamburino, V.; Zappia, G.; Zimbone S.M. Valorisation of citrus processing waste: A review. Waste Manage. **2018**, 80, 252–273. https://doi.org/10.1016/j.wasman.2018.09.024

44. Mahato, N.; Sharma, K.; Sinha, M.; Cho, M.H. Citrus waste derived nutra-/pharmaceuticals for health benefits: Current trends and future perspectives. J. Funct. Foods **2018**, 40, 307–316. https://doi.org/10.1016/j.jff.2017.11.015

45. Stewart, I.; Wheaton, T.A. Carotenoids in Citrus: their accumulation induced by ethylene. J. Agric. Food Chem. **1972**, 20, 448–449, https://doi.org/10.1021/jf60180a024

46. Alôs, E.; Rodrigo, M.J.; Zacarías, L. Differential transcriptional regulation of L-ascorbic acid content in peel and pulp of citrus fruits during development and maturation. Planta **2014**, 239(5), 1113–1128. https://doi.org/10.1007/s00425-014-2044-z.

47. Rey, F.; Rodrigo, M.J.; Zacarías, L. Regulation of tocopherol biosynthesis during fruit maturation of different Citrus species. Front. of Plant Sci. **2021**, 12, 743993. https://doi.org/10.3389/fpls.2021.743993

48. Pérez-Través, L.; Lopes, C.A.; González R.; Barrio, E.; Querol, A. Physiological and genomic characterisation of Saccharomyces cerevisiae hybrids with improved fermentation performance and mannoprotein release capacity. Int. J. Food Microbiol. **2015**, 205, 30-40. https://doi.org/10.1016/j.ijfoodmicro.2015.04.004

49. Takahashi, S.; Iwasaki-Kino, Y.; Aizawa, K.; Terao, J.; Mukai, K. Development of singlet oxygen absorption capacity (SOAC) assay method using a microplate reader. J. AOAC Int. **2016**, 99(1), 193–197. https://doi.org/10.5740/jaoacint.15-0165

50. Rey, F.; Zacarías, L.; Rodrigo, M.J. Carotenoids, vitamin c, and antioxidant capacity in the peel of mandarin fruit in relation to the susceptibility to chilling injury during postharvest cold storage. Antioxidants **2020**, 9(12), 1–21. https://doi.org/10.3390/antiox9121296

51. Sánchez-Moreno, C.; Plaza, L.; de Ancos, B.; Cano, M.P. Vitamin C, provitamin A carotenoids, and other carotenoids in high-pressurized orange juice during refrigerated storage. J. Agric. Food Chem. **2013**, 51(3), 647–653. https://doi.org/10.1021/jf020795o

52. Stinco, C.M.; Fernández-Vázquez, R.; Escudero-Gilete, M.L.; Heredia, F.J.; Meléndez-Martínez, A.J.; Vicario, I.M. Effect of orange juice's processing on the color, particle size, and bioaccessibility of carotenoids. J. Agric. Food Chem. **2012**, 60(6), 1447−1455. https://doi.org/10.1021/jf2043949

53. Arena, E.; Fallico, B.; Maccarone, E. Influence of carotenoids and pulps on the color modification of blood orange juice. J. Food Sci. **2000**, 65, 458-460. https://doi.org/10.1111/j.1365-2621.2000.tb16027.x

54. Meléndez-Martínez, A.J.; Britton, G.; Vicario, I.M.; Heredia, F.J. The complex carotenoid pattern of orange juices from concentrate. Food Chem. **2008**, 109, 546–53. https://doi.org/10.1016/j.foodchem.2008.01.003

55. Gil-Izquierdo, A.; Gil, M.I.; Ferreres, F. Effect of processing techniques at industrial scale on orange juice antioxidant and beneficial health compounds. J. Agric. Food Chem. **2002**, 50(18), 5107–5114. https://doi.org/10.1021/jf020162+

56. Bai, J.; Manthey, J.A.; Ford, B.L.; Luzio, G.; Cameron, R.G.; Narciso, J.; Baldwin, E.A. Effect of extraction, pasteurization and cold storage on flavonoids and other secondary metabolites in fresh orange juice. J. Sci. Food Agric. **2013**, 93(11), 2771–2781. https://doi.org/10.1002/jsfa.6097.

57. Li, Q.; Li, T.; Baldwin, E.A.; Manthey; J.A.; Plotto, A.; Zhang, Q.; Gao, W.; Bai, J.; Shan, Y. Extraction Method Affects Contents of Flavonoids and Carotenoids in Huanglongbing-Affected "Valencia" Orange Juice. Foods **2021**, 10, 783. https://doi.org/10.3390/foods10040783

58. Lee, H.S; Coates, G.A. Effect of thermal pasteurization on Valencia orange juice color and pigments. LWT, **2003**, 36, 153-156. https://doi.org/10.1016/S0023-6438(02)00087-7

59. Velázquez-Estrada, R.M.; Hernández-Herrero, M.M.; Rüfer, C.E.; Guamis-López, B.; Roig-Sagués, A.X. Influence of ultra high pressure homogenization processing on bioactive compounds and antioxidant activity of orange juice. Innov. Food Sci. Emerg. Technol. **2013**, 18, 89–94. https://doi.org/10.1016/j.ifset.2013.02.005

60. Etzbach, L.; Stolle, R.; Anheuser, K.; Herdegen, V.; Schieber; A.; Weber, F. Impact of different pasteurization techniques and subsequent ultrasonication on the in vitro bioaccessibility of carotenoids in Valencia orange (Citrus sinensis (l.) Osbeck) juice. Antioxidants **2020**, 9(6), 1–28. https://doi.org/10.3390/antiox9060534

61. Lado, J.; Cronje, P.; Alquézar, B.; Page, A.; Mazif, M.; Gómez-Cadenas, A.; Steag, A.D.; Rodrigo, M.J.; Zacarías, L. Fruit shading enhances peel color, carotenes accumulation and chromoplast differentiation in red grapefruit. Physiol. Plant. **2015**, 154, 469–484. https://doi.org/10.1111/ppl.12332

62. Mapelli-Brahm, P.; Stinco, C.M.; Rodrigo, M.J.; Zacarías, L.; Meléndez-Martínez, A.J. Effect of thermal treatments in orange juice on the bioaccessibility of phytoene and phytofluene in relation to changes in particle size and in plastids. J. Funct. Foods **2018**, 46, 38–47. https://doi.org/10.1016/j.jff.2018.04.044

63. Velázquez-Estrada, R.M.; Hernández-Herrero, M.M.; Guamis-Lopez, B.; Roig-Sagues, A. X. Influence of ultra-high-pressure homogenization on physicochemical and sensorial properties of orange juice in comparison with conventional thermal processing. Int. J. Food Sci. **2019**, 54, 1858-1864. https://doi.org/10.1111/ijfs.14089

64. Nguyen, M.; Francis, D.; Schwartz, S. Thermal isomerisation susceptibility of carotenoids in different tomato varieties. J. Sci. Food Agric. **2001**, 81(9), 910–917. https://doi.org/10.1002/jsfa.911

65. Kafkas, E.; Polatöz, S.; Koç, N.K. Quantification and comparison of sugars, carboxylic acids and vitamin C components of various citrus species using HPLC techniques. J. Agric. Sci. Technol. **2011**, 5, 175-180.

66. Sánchez-Moreno, C.; Plaza, L.; Elez-Martínez, P.; De Ancos, B.; Martín-Belloso, O.; Cano, M. P. Impact of high pressure and pulsed electric fields on bioactive compounds and antioxidant activity of orange juice in comparison with traditional thermal processing. J. Agric. Food Chem. **2005**, 53, 4403–4409. https://doi.org/10.1021/jf048839b

67. Escobedo-Avellaneda, Z.; Gutiérrez-Uribe, J.; Valdez-Fragoso, A.; Torres, J.A.; Welti-Chanes, J. High hydrostatic pressure combined with mild temperature for the preservation of comminuted orange: Effects on functional compounds and antioxidant activity. Food Bioproc. Tech. **2015**, 8(5), 1032–1044. https://doi.org/10.1007/ s11947-014-1465-9

68. Barba, F. J.; Esteve, M.J.; Frigola, A. Ascorbic acid is the only bioactive that is better preserved by high hydrostatic pressure than by thermal treatment of a vegetable beverage. J. Agric. Food Chem. **2010**, 58(18), 10070–10075. https://doi.org/10.1021/jf1019483

69. Aschoff, J.K.; Kaufmann, S.; Kalkan, O.; Neidhart, S.; Carle, R.; Schweiggert, R.M. In vitro bioaccessibility of carotenoids, flavonoids, and vitamin C from differently processed oranges and orange juices [Citrus sinensis (L.) Osbeck]. J. Agric. Food Chem. **2015**, 63(2), 578–587. https://doi.org/10.1021/jf505297t

70. Barba, F.J.; Esteve, M.J.; Frígola, A. Determination of vitamins E (α-, γ- and δ-tocopherol) and D (cholecalciferol and ergocalciferol) by liquid chromatography in milk, fruit juice and vegetable beverage. Eur. Food Res. Technol. **2011**, 232, 829–836. https://doi.org/10.1007/s00217-011-1450-8

71. Stinco, C.M.; Pumilia, G.; Giuffrida, D.; Dugo, G.; Meléndez-Martínez, A.J.; Vicario, I.M. Bioaccessibility of carotenoids, vitamin A and α-tocopherol, from commercial milk-fruit juice beverages: Contribution to the recommended daily intake. J. Food Compos. Anal. **2019**, 78, 24–32. https://doi.org/10.1016/j.jfca.2019.01.019

72. Cilla, A.; Alegría, A.; de Ancos, B.; Sánchez-Moreno, C.; Cano, M.P.; Plaza, L.; Clemente, G.; Lagarda, M.J.; Barbera, R. Bioaccessibility of tocopherols, carotenoids, and ascorbic acid from milk- and soy-based fruit beverages: influence of food matrix and processing. J. Agric. Food Chem. **2012**, 60, 7282– 7290. https://doi.org/10.1021/jf301165r

73. Romeu-Nadal, M.; Castellote, A.I.; Gayà, A.; López-Sabater, M.C. Effect of pasteurisation on ascorbic acid, dehydroascorbic acid, tocopherols and fatty acids in pooled mature human milk. Food Chem. 2**008**, 107(1), 434–438. https://doi.org/10.1016/j.foodchem.2007.06.060

74. Peterson, J.J.; Dwyer, J.T.; Beecher, G.R.; Bhagwat, S.A.; Gebhardt, S.E.; Haytowitz, D.B.; Holden, J.M. Flavanones in oranges, tangerines (mandarins), tangors, and tangelos: a compilation and review of the data from the analytical literature. J. Food Compos. Anal. **2006,** 19, 66–73. https://doi.org/10.1016/j.jfca.2005.12.006

75. Sánchez-Moreno, C.; Plaza, L.; de Ancos, B.; Cano, M.P. Quantitative bioactive compounds assessment and their relative contribution to the antioxidant capacity of commercial orange juices. J. Sci. Food Agric. **2003**, 83(5), 430–439. https://doi.org/10.1002/jsfa.1392

76. Zacarías-García, J.; Rey, F.; Gil, J.V.; Rodrigo, M.J.; Zacarías L. Antioxidant capacity in fruit of Citrus cultivars with marked differences in pulp coloration: Contribution of

carotenoids and vitamin C. Food Sci. Technol. Int. **2021**, 27(3), 210–222. https://doi.org/10.1177/1082013220944018.

77. Müller, L.; Fröhlich, K.; Böhm, V. Comparative antioxidant activities of carotenoids measured by ferric reducing antioxidant power (FRAP) ABTS bleaching assay (αTEAC) DPPH assay and peroxyl radical scavenging assay. Food Chem. **2011**, 129, 139-148. https://doi.org/10.1016/j.foodchem.2011.04.045

78. De Ancos, B.; Sgroppo, S.; Plaza, L.; Cano, M.P. Possible nutritional and health-related value promotion in orange juice preserved by high-pressure treatment. J. Sci. Food Agric. **2002**, 82, 790–796. https://doi.org/10.1002/jsfa.1093

79. Arnao, M.B.; Cano, A.; Acosta, M. The hydrophilic and lipophilic contribution to total antioxidant activity. Food Chem. **2001**, 73, 239-244. https://doi.org/10.1016/S0308-8146(00)00324-1.

80. Arnao, M.B.; Cano, A. Lipophilic and hydrophilic antioxidant activity and vitamin c content of commercial orange juices: correlation with organoleptic parameters. CYTA – J. Food, **2004**, 4(3), 185-189. https://doi.org/10.1080/11358120409487759

81. Escudero-López, B.; Cerrillo, I.; Gil-Izquierdo, A.; Hornero-Méndez, D., Herrero-Martín, G., Berná, G., Medina, S., Ferreres, F., Martín, F., Fernández-Pachón, M.S. Effect of thermal processing on the profile of bioactive compounds and antioxidant capacity of fermented orange juice. Int. J. Food Sci. Nutr. **2016**, 67(7), 779–788. https://doi.org/10.1080/09637486.2016.1204428

82. Rodríguez-Roque, M. J., De Ancos, B., Sánchez-Moreno, C., Cano, M. P., Elez-Martínez, P., Martín-Belloso, O. Impact of food matrix and processing on the in vitro bioaccessibility of vitamin C, phenolic compounds, and hydrophilic antioxidant activity from fruit juice-based beverages. Int. J. Food Sci. Nutr. **2015**, 14, 33–43. https://doi.org/10.1016/j.jff.2015.01.020

83. Asencio, A.D.; Serrano, M.; García-Martínez, S.; de Pretel, M.T. Organic acids, sugars, antioxidant activity, sensorial and other fruit characteristics of nine traditional Spanish citrus fruits. Eur. Food Res. Technol. **2018**, 244, 1497–1508. https://doi.org/10.1007/s00217-018-3064-x

84. Lu, Q.; Li, L.; Xue, S.; Yang, D. Wang, S. Stability of flavonoid, carotenoid, soluble sugar and vitamin C in 'Cara Cara' Juice during storage. Foods **2019**, 8(9), 417. https://doi.org/10.3390/foods8090417

85. Vervoort, L.; Van der Plancken, I.; Grauwet, T.; Timmermans, R. A.H.; Mastwijk, H.C.; Matser, A.M.; Hendrickx, M.E.; Van Loey, A. Comparing equivalent thermal, high pressure and pulsed electric field processes for mild pasteurization of orange juice Part II: impact on specific chemical and biochemical quality parameters. Innov. Food Sci. Emerg. Technol. **2011**, 12(4), 466–477. https://doi.org/10.1016/j.ifset.2011.06.003

86. Zheng, H.; Zhang, Q.; Quan, J.; Zheng, Q.; Xi, W. Determination of sugars, organic acids, aroma components, and carotenoids in grapefruit pulps. Food Chem. **2016**, 205, 112–121. https://doi.org/10.1016/j.foodchem.2016.03.007

87. Ouchi, A.; Aizawa, K.; Iwasaki, Y.; Inakuma, T.; Terao, J.; Nagaoka, S. I.; Mukai, K. Kinetic study of the quenching reaction of singlet oxygen by carotenoids and food

extracts in solution. Development of a singlet oxygen absorption capacity (SOAC) assay method. J. Agric. Food Chem. **2010**, 58(18), 9967–9978. https://doi.org/10.1021/jf101947a

88. Mamma, D.; Christakopoulos, P. Biotransformation of citrus by-products into value added products. Waste Biomass Valor **2014**, 5, 529–549. https://doi.org/10.1007/s12649-013-9250

89. Kaur, S.; Panesar, P.S.; Chopra, H.K. Citrus processing by-products: an overlooked repository of bioactive compounds. Crit. Rev. Food Sci. Nutr. **2021**, 63(1), 67-86. https://doi.org/10.1080/10408398.2021.1943647

Supplementary Material

Table Supplementary 1. Percentage of contribution (%) of variables to total variance of principal component 1 and 2. TCAROT: total carotenoids; VITC: vitamin C; TP: total phenolic; TF: total flavonoids by spectrophotometry; TF-HPLC: total flavonoids by HPLC; TOC: tocopherols; SUC: sucrose; GLU: glucose; FRU: fructose; CA: citric acid; MA: malic acid; SA: succinic acid; QA: quinic acid.

Variables	PC1	PC2
TCAROT	13.49	0.31
VITC	4.41	0.07
DPPH	4.75	0.06
FRAP	4.68	0.09
TP	0.79	0.03
TF	0.3	3.89
TF-HPLC	0.32	4.3
ABTS-H	4.03	0.03
ABTS-L	2.84	0.87
TOC	3.11	1.32
SUC	2.78	0.01
GLU	4.57	0.19
FRU	2.47	0.04
CA	3.48	0.53
MA	3.39	0.02
SA	3.44	0.71
QA	1.04	2.15
Total	**63.91**	**14.62**

En los últimos años existe un creciente interés en la Citricultura mundial por el desarrollo de nuevas variedades que permitan diferenciar la oferta entre países y que ofrezcan al consumidor frutos de excelente calidad y con valor añadido a lo largo de toda la campaña. En esta Tesis Doctoral se ha abordado la caracterización de dos nuevos mutantes de naranja de pulpa roja en dos fondos genéticos distintos: 'Kirkwood Navel' y 'Ruby Valencia', y su comparación con las variedades de naranja común Washington Navel (cv. Foios) y Valencia late (cv. Midknight), respectivamente. Para ello, se plantearon diferentes objetivos específicos que se distribuyen en los respectivos capítulos y que abordan, por un lado, elucidar los mecanismos bioquímicos y moleculares relacionados con la acumulación de licopeno en estos nuevos mutantes y, por otro, estudiar otros aspectos aplicados y de interés para la industria citrícola, como son sus características de calidad, su adecuación para la conservación y consumo en fresco, así como su potencial para la producción y procesado de zumo. Por ello, esta Tesis Doctoral se ha organizado en distintos capítulos, correspondientes a los diferentes objetivos y que han dado lugar a publicaciones independientes, y cuyos aspectos más relevantes se discuten a continuación.

4.1. Análisis del metabolismo de carotenoides de las naranjas de pulpa roja Kirkwood Navel y Ruby Valencia

Los análisis iniciales del contenido y composición de carotenoides en frutos de las dos nuevas variedades de naranja de pulpa roja se realizaron en muestras liofilizadas de piel y pulpa de frutos maduros procedentes de Citrusdal (Sudáfrica), producto de una colaboración con el Dr. Paul Cronje (Citrus Research Internacional, CRI; University of Stellenbosch). Estos primeros resultados mostraron que el contenido total de carotenoides era muy superior en ambos tejidos en los frutos de Kirkwood y de Ruby y, además, presentaban una composición muy inusual. La pulpa de Kirkwood y Ruby contenía concentraciones extremadamente altas de los primeros carotenos lineales de la ruta, fitoeno y fitoflueno, niveles moderados de licopeno y, además, menores concentraciones de violaxantina que los frutos de las variedades de referencia (Fig. 2, Capítulo 1). Posteriormente, la disponibilidad de ambas variedades de pulpa roja en parcelas experimentales de la Fundación ANECOOP SL (Museos, Valencia) nos permitió un acceso directo al material vegetal y realizar un seguimiento exhaustivo de las características del fruto a lo largo del desarrollo y maduración y, también, evaluar otras

características de las variedades, como su adaptación a las condiciones agronómicas y ambientales valencianas respecto a variedades tradicionales ampliamente cultivadas y adaptadas a estas condiciones.

El seguimiento del crecimiento y desarrollo del fruto de las dos variedades hasta su maduración comercial mostró que no parecen existir diferencias importantes en los parámetros generales del desarrollo, como cambios en el peso o tamaño, cambio de color externo o en otros parámetros de calidad interna (que se discuten más adelante en esta sección), siendo el color rojo de la pulpa la característica más destacada y distintiva en los frutos de las variedades Kirkwood y Ruby. El análisis detallado del contenido y composición de carotenoides a lo largo del desarrollo del fruto mostró que el color rojo en la pulpa y las diferencias en carotenoides en este tejido se manifiestan desde estados muy temprano del desarrollo (junio), inmediatamente después de la caída `fisiológica´ de frutos (conocida como 'June drop') que tiene lugar al inicio de la fase II del alargamiento celular (Bain, 1958). En la pulpa de los frutos de junio y julio ya se apreció una tonalidad rosada en comparación con la de los frutos de las naranjas comunes de coloración verde-amarilla (Fig. 3, Capitulo 1). Este fenotipo se confirmó posteriormente con el análisis de carotenoides, donde ya se detectó licopeno a muy bajas concentraciones, y la suma de fitoeno y fitoflueno representó en los frutos de junio y julio más del 96% de los carotenoides totales (Tabla 1 y 2, Capitulo 1). Esta característica demuestra que la mutación en Kirkwood y Ruby afecta claramente al metabolismo de carotenoides, y que la aparición del color rojo en la pulpa no está intrínsecamente asociada con la estimulación de la carotenogénesis que ocurre durante la maduración del fruto (Rodrigo et al., 2004; Alquézar et al., 2008a), sino que se manifiesta desde estados muy iniciales del desarrollo del fruto. La pigmentación de la pulpa y la temprana acumulación de carotenoides también se ha descrito en frutos de la naranja de pulpa roja Cara Cara (Alquézar et al., 2008b; Lu et al., 217) y en el limón rosado (Lana et al., 2020), pero no en variedades pigmentadas de pomelo o en otras mutaciones de naranja roja como Red Anliu (Liu et al., 2007; Alquézar et al., 2013). Estas evidencias sugieren que la naturaleza de la mutación que provoca la acumulación de licopeno y otros carotenos puede ser diferente entre las distintas variedades de cítricos que manifiestan este fenotipo, y que muy probablemente existe más de una causa que puede originar estas alteraciones.

El análisis del contenido y composición de carotenoides en el flavedo (piel) y la pulpa de los frutos de Kirkwood y Ruby y sus respectivas variedades de referencia a lo largo de la maduración, reveló claves importantes sobre la naturaleza de la mutación. Conforme avanza la maduración se incrementa la acumulación de licopeno, fitoeno y fitoflueno, que en estados avanzados (enero en Kirkwood y abril en Ruby) cuantifican entre el 82 y 85% de los carotenoides totales (Tabla 1 y 2, Capitulo 1). Al mismo tiempo, el contenido de la β,β-xantofila violaxantina es entre 2 y 3 veces inferior respecto a las naranjas Navel y Valencia de referencia, y en las naranjas tradicionales esta xantofila representa alrededor del 50% del contenido total de carotenoides (Tabla 1 y 2, Capitulo 1). Estos resultados sugieren un bloqueo parcial en la ciclación de licopeno a β-caroteno en los mutantes, lo que reduciría el flujo metabólico hacia las β,β-xantofilas y otros metabolitos finales de la ruta de carotenoides y, en consecuencia, generaría un cuello de botella a nivel del licopeno incrementando los niveles de los metabolitos de las etapas iniciales, como el fitoeno y fitoflueno. Además, la detección en la pulpa de Kirkwood y Ruby de los carotenoides intermediarios que no suelen detectarse en los extractos de las naranjas tradicionales como el neuroesporeno y, especialmente, δ-caroteno (Tabla 1 y 2, Capítulo 1), apoya la hipótesis de que la actividad licopeno β-ciclasa se encuentra parcialmente bloqueada en los mutantes. Esta hipótesis es compatible y explicaría los bajos niveles de ácido abscísico (ABA) y del conjugado ABA-GE (ABA-glucosil ester) en la pulpa de los frutos mutantes y que apenas aumentaron durante la maduración, en contraste con el incremento observado en las variedades Navel y Valencia (Fig. 7, Capítulo 1). Por lo tanto, en su conjunto estos resultados sugieren un bloqueo en la actividad licopeno β-ciclasa en la pulpa de los mutantes Kirkwood y Ruby.

Es interesante destacar que los contenidos en carotenoides en el flavedo (Tabla Suplementaria 5 y 6, Capítulo 1) y las hojas (Tabla 3, Capítulo 1) de Kirkwood y Ruby también están alterados respecto a las variedades de referencia, pero no de forma tan dramática como en la pulpa. Así, tanto el flavedo como las hojas de ambas variedades presentan mayores concentraciones de carotenoides, especialmente de fitoeno y, además, en las hojas también se detectaron niveles superiores de clorofilas respecto a las naranjas de referencia (Tabla 3, Capítulo 1). Esta observación sugiere que la mutación no solo afecta a los tejidos del fruto, sino que también se manifiesta en diferentes órganos/tejidos de la planta y posiblemente, en función de las características específicas y de la regulación de la carotenogénesis en cada tejido,

tendría efectos variables en el complemento de carotenoides. De hecho, es bien conocido que la acumulación de carotenoides y la expresión de los genes de biosíntesis es independiente y menos activa en la pulpa que en la piel de los frutos cítricos (Rodrigo et al., 2013; Ikoma et al., 2016), y sería lógico asumir que deficiencias parciales en la actividad licopeno ciclasa podrían tener consecuencias mucho más significativas en el metabolismo de carotenoides en la pulpa respecto a otros tejidos con una carotenogénesis más activa.

En favor de la hipótesis anterior, destaca la observación de la coloración roja del floema y/o el cambium en los tallos (ramas y tronco) de Kirkwood y Ruby (Fig. 6 y Tabla 4, Capítulo 1), que es una de las alteraciones fenotípicas más destacables y que se ha descrito por primera vez en cítricos en este trabajo. Sin embargo, esta excepcional característica solo ha sido reportada hasta la fecha en árboles cultivados en Sudáfrica. Esta coloración roja también se ha detectado en la base del cáliz y el eje vascular central del fruto maduro, y en haces vasculares del albedo, células más diferenciadas y vascularizadas (Fig. 2, Capítulo 1). El análisis de carotenoides en la corteza (que contenía el cambium y floema) y el xilema (madera) de los tallos de Ruby mostró concentraciones de carotenoides muy superiores en ambos tejidos del mutante en comparación con Valencia. Es de destacar la presencia de altas concentraciones de fitoeno y fitoflueno, trazas o bajas concentraciones de licopeno, y un menor contenido de xantofilas (Tabla 4, Capítulo 1). Esta particular alteración en el complemento de carotenoides es cualitativamente similar a la que se produce en la pulpa de los frutos de estos mutantes, y compatible con la hipótesis planteada del bloqueo en la actividad licopeno β-ciclasa, que limitaría el flujo natural de metabolitos en la ruta, como se demuestra por los bajos niveles de ABA y de sus catabolitos detectados en estos tejidos (Tabla 4, Capítulo 1). En Arabidopsis se ha demostrado que las células del floema en los haces vasculares son activas para la síntesis de ABA, posiblemente como señal hormonal de respuesta a estrés hídrico (Kuromori et al., 2014). Por lo tanto, nuestros resultados indicarían que las células del floema de los tallos de los cítricos son carotenogénicamente activas, seguramente para asegurar la síntesis de ABA y, de igual modo a lo observado en otros tejidos, el bloqueo en la ruta de biosíntesis implicaría la acumulación de licopeno y carotenos lineales. Este efecto parece que es más acusado en los tejidos más adultos, más lignificados y vascularizados que, probablemente, requieren una síntesis más activa de ABA y, por tanto, una síntesis más activa de carotenoides que se manifiesta con una coloración roja más intensa. Este particular fenotipo nos llevó a estudiar si

la acumulación de licopeno en el tallo también ocurre en otras variedades de cítricos que acumulan licopeno en el fruto. Para ello, se empleó el material vegetal disponible en el Banco de Germoplasma de Cítricos del IVIA (Moncada, Valencia) donde existen diferentes genotipos en las mismas condiciones ambientales y de cultivo. Los resultados fueron muy interesantes, observándose una correlación entre la intensidad de la coloración roja de los haces vasculares de los frutos y del tallo con el grado de acumulación de licopeno en la pulpa en las variedades de pomelo Star Ruby, Rio Red y Marsh (Fig. Suplementaria 5, Capítulo 1). Estas características no se habían descrito con anterioridad, a pesar de la elevada producción y comercialización de pomelos de pulpa roja en diferentes países desde hace décadas, e indican que el fenotipo de tallos rojos no es exclusivo de los mutantes de naranja, sino que también se produce en otras especies de cítricos que acumulan licopeno. De este conjunto de resultados podemos concluir que la mutación que afecta a la actividad licopeno β-ciclasa en los mutantes Kirkwood y Ruby se manifiesta en diferentes tejidos de la planta y, por lo tanto, no está asociada con la maduración del fruto y se inicia desde estados muy iniciales del desarrollo. Además, los resultados muestran que los tejidos que requieren de una síntesis activa de carotenoides muestran alteraciones similares en el contenido de éstos, aunque en función de intensidad carotenogénica de cada tejido se acumularía más o menos licopeno y, por tanto, tendría un efecto variable en el fenotipo.

Finalmente, el análisis transcriptómico de la expresión de 26 genes del metabolismo de carotenoides en la piel y la pulpa de los frutos de naranjas de las cuatro variedades estudiadas reveló que las alteraciones en el metabolismo de carotenoides en Kirkwood y Ruby no parecen estar asociadas a diferencias en la expresión de estos genes en comparación con las variedades comunes Navel y Valencia (Fig. 5 y 6, Fig. Suplementaria 2 y 3, Capítulo 1). En frutos de la naranja Cara Cara, donde la acumulación de carotenoides es similar a la de los mutantes estudiados en este trabajo (Alquézar et al., 2008) se ha sugerido una posible mayor activación de la ruta MEP y el incremento de precursores hacia la ruta de carotenoides como potencial mecanismo de la acumulación de carotenos iniciales y de licopeno. Sin embargo, posteriores estudios transcriptómicos de genes correspondientes de la ruta MEP, genes de biosíntesis de carotenoides y el uso de inhibidores enzimáticos de la actividad PDS y LCY no son totalemente consistentes con esta hipótesis (Lu et al., 2017). Otros trabajos en frutos de la naranja de pulpa roja Red Anliu (Lu et al., 2016), pomelos (Alquezar et al., 2013), pumelos (Promkaew et al.,

2002; Tatmala, et al., 2020) y limon rosado (Lana et al., 2020) han mostrado una menor expresión del gen *β-LCY2* junto con expresión preferencial del alelo no funcional *β-LCY2b*. Además, en la naranja Red Anliu se han descrito dos mutaciones en aminoácidos críticos para la correcta actividad del alelo *β-LCY2b* (Lu et al., 2016). Estas observaciones indican que alteración en la expresión y/o actividad del gen *β-LCY2* puede ser el principal mecanismo responsable de la acumulación de licopeno en estos mutantes. En frutos de otras especies, como papaya o sandía, se ha demostrado que alteraciones en la secuencia o en la expresión de este gen conducen a la acumulación de licopeno en la pulpa y al fenotipo de color rojo (Blas et al., 2010; Devitt et al., 2010; Zhang et al., 2020). En la pulpa de los frutos de Kirkwood y Ruby la expresión de los genes *β-LCY1*, *β-LCY2* (alelos *a* y *b*) no mostró diferencias sustanciales respecto a los frutos de las naranjas convencionales, aunque sí un ligero retraso en la inducción de su expresión en torno al momento del cambio de color externo del fruto (Fig. 5 y 6, Capítulo 1). Por lo tanto, no parece existir una correlación entre la expresión de los genes responsables de la β-ciclación del licopeno y la acumulación del carotenoide en las distintas fases del desarrollo del fruto. Para explorar otras alternativas que pudieran afectar a la actividad licopeno ciclasa, se secuenciaron los genes que codifican para *ε-LCY*, *β-LCY1* y *β-LCY2* en ambas variedades y no se detectaron cambios nucleotídicos o mutaciones entre los dos alelos identificados para cada gen (dada la heterozigosis de las naranjas) respecto a las secuencias de los genotipos de referencia. Así pues, se puede concluir que en las naranjas Kirkwood Navel y Ruby Valencia, la acumulación de licopeno y la deficiente actividad licopeno β-ciclasa que se deduce de los datos bioquímicos no está asociada a alteraciones en la expresión de los genes *LCYs* o a mutaciones en sus secuencias. El análisis de la expresión de genes relacionados con otros procesos del metabolismo de carotenoides que podrían conducir a alteraciones en su contenido, como alteraciones de las enzimas del catabolismo de carotenoides (CCDs), en proteínas asociadas con la acumulación (FIB y HSP21) o en la esterificación de xantofilas (PYP), tampoco mostraron diferencias consistentes respecto a los frutos de Navel y Valencia (Fig. 5 y 6, Capítulo 1). En definitiva, el conjunto de resultados indica que la mutación en las naranjas Kirkwood y Ruby que conduce a la acumulación de licopeno podría estar relacionada, por un lado, con modificaciones postranscripcionales o postraduccionales de la enzima licopeno β-ciclasa, o con factores todavía desconocidos y que son necesarios para la correcta actividad de esta enzima. Futuros estudios comparativos del transcriptoma global entre estas variedades

mediante estrategias de secuenciación masiva (RNAseq), puede conducir a identificar factores diferenciales implicados y/o responsables del fenotipo rojo. La disponibilidad de dos mutaciones independientes con características fenotípicas y bioquímicas similares representa una estrategia óptima para identificar los factores comunes potencialmente responsables de la acumulación de licopeno en los frutos de naranja.

4.2. Compuestos bioactivos, calidad nutricional y capacidad antioxidante de las naranjas de pulpa roja Kirkwood Navel y Ruby Valencia

Los frutos cítricos son una importante fuente de macro- y micronutrientes como azúcares, vitaminas (vitamina C, vitamina E, ácido folico), minerales y fibra dietética, entre otros (Lu et al, 2021). Además, acumulan otros fitoquímicos o compuestos bioactivos con propiedades relevantes para la salud. Entre estos compuestos destacan los carotenoides, flavonoides, y limonoides (Saini et al., 2022). La ingesta de estos compuestos puede proporcionar numerosos beneficios para la salud ya que se les han atribuido propiedades antioxidantes, antinflamatorias, anticancerígenas, cardiovasculares, neuroprotectoras, etc (Turner & Burri, 2013; Rampersaud & Valim, 2016; Maugeri et al., 2019). El contenido y composición de nutrientes y compuestos bioactivos en los frutos cítricos varía ampliamente entre las diferentes especies y variedades, tejidos y estados de maduración, determinando su calidad, valor nutricional y propiedades saludables (Lu et al., 2021).

En la actualidad hay disponibles un número limitado de estudios en los que se ha analizado la composición de nutrientes y compuestos bioactivos, aparte de los carotenoides, en naranjas de pulpa roja. En particular, se ha descrito el contenido de vitamina C y compuestos fenólicos en el zumo de Cara Cara (De Ancos et al., 2017, 2020; Brasili et al., 2018). Sin embargo, existe amplia información sobre la acumulación de diferentes compuestos con interés nutricional y organoléptico en la pulpa de naranjas ordinarias (Huang et al., 2007; Ladaniya, 2008; Bermejo & Cano, 2012; Alòs et al., 2014; Multari et al., 2020; Rey et al., 2021). Así, el objetivo del Capítulo 2 fue caracterizar la evolución de la calidad interna, color, contenido en vitamina C, tocoferoles (vitamina E), fenoles, flavonoides, azúcares, ácidos orgánicos y capacidad antioxidante en la pulpa de las variedades Kirkwood y Ruby a lo largo del desarrollo y maduración del fruto en comparación con las variedades comunes Navel y Valencia, respectivamente.

Los resultados obtenidos en el Capítulo 2 muestran que los frutos de las naranjas de pulpa roja y las variedades de referencia no presentan diferencias en cuanto a la tasa de crecimiento y evolución del color externo durante el desarrollo y maduración de los frutos (Tabla 1 y 2, Capítulo 2). Además, el aumento del contenido en sólidos solubles y la reducción de la acidez durante la maduración ocurrió de forma similar en las naranjas de pulpa roja en comparación con sus correspondientes variedades estándar , alcanzando índices de madurez similares en el momento de la recolección comercial (Tabla 1 y 2, Capítulo 2). Los resultados del Capítulo 1 mostraron que la inducción de los genes de biosíntesis de carotenoides durante la maduración en las variedades de pulpa roja, especialmente en Kirwkood, presentaban cierto retraso, respecto a la variedad de referencia (Fig. 4 y 5; Tabla Suplementaria 1, Capítulo 1). Además, estudios anteriores han observado que la maduración de la naranja Cara Cara se encuentra ligeramente atrasada, presentando niveles inferiores de ºBrix e índice de madurez, respecto a la naranja Navel de referencia (Brasili et al., 2017; De Ancos et al., 2017). Estas diferencias en cuanto a la maduración entre las variedades de pulpa roja y las variedades comunes pueden explicarse por el efecto de las condiciones climáticas y ambientales, y/o las condiciones de cultivo (Lado et al., 2014), o posiblemente a algún factor endógeno relacionado con el metabolismo alterado de carotenoides (como el ABA), todavía no caracterizado. Así, la principal característica fisiológica que diferencia las variedades estudiadas es la coloración interna de los frutos de Kirkwood y Ruby (Fig. 1, Tabla 1 y 2, Capítulo 2).

Los azúcares y ácidos orgánicos son los principales compuestos que determinan el sabor de los frutos cítricos y, por tanto, influyen de forma importante en su calidad organoléptica y en la aceptación por parte del consumidor (Albertini et al., 2006). Así, los tres azúcares mayoritarios y principales nutrientes en las naranjas, sacarosa, glucosa y fructosa, aumentaron progresivamente durante la maduración (Fig. 2, Capítulo 2). Los niveles de azúcares detectados están en el rango descrito para los frutos de naranja (Kafkas et al., 2011; Bermejo & Cano, 2012). La naranja Ruby mostró niveles inferiores de azúcares en diferentes estados del desarrollo, destacando una reducción en la concentración de azúcares en la pulpa del fruto maduro entre el 10-20% en comparación con la variedad Valencia (Fig. 2B-D, Capítulo 2). Por su parte, la concentración de ácido cítrico, ácido orgánico mayoritario en naranjas, disminuye de forma importante durante la maduración (Fig. 3, Capítulo 2), debido principalmente a un efecto de dilución causado por el crecimiento del fruto y el aumento del contenido de agua de

la pulpa (Bain et al., 1958) y por la activación de diferentes procesos relacionados con su catabolismo (Chen et al., 2013). En general, las dos variedades de Navel mostraron pocas diferencias entre ellas en cuanto al contenido de ácidos orgánicos, especialmente en el fruto maduro. En cambio, la naranja Ruby presentó un claro desequilibrio en el perfil de los ácidos orgánicos, con mayores niveles de ácido cítrico y málico a lo largo de todo el desarrollo del fruto (Fig. 3B y D, Capitulo 2). Sin embargo, estas diferencias en ácidos individuales no se traducen en cambios en el índice de madurez, probablemente debido a un equilibrio con otros metabolitos (Lado et al., 2018). No obstante, sería interesante evaluar en futuros estudios si estas diferencias en el perfil de ácidos orgánicos se asocian con diferencias en el sabor del fruto entre ambas variedades. El mutante de naranja Red Anliu se caracteriza por unos niveles de hasta cuatro veces inferiores de ácido cítrico respecto a la variedad parental (Liu et al., 2007). Estudios transcriptómicos y metabolómicos indican que esta característica se debe a una regulación negativa de diversos genes asociados al metabolismo del ácido cítrico (Yu et al., 2012). Sin embargo, esta situación no parece ser el caso de la variedad Ruby y, como se ha indicado en el capítulo anterior, la mutación que causa el fenotipo de Ruby no parece tener el mismo origen que en la naranja Red Anliu de origen chino.

El análisis de los diferentes compuestos relacionados con la calidad nutricional (Fig. 4, Capítulo 2), así como la capacidad antioxidante (Fig. 5, Capítulo 2), reveló que los perfiles a lo largo de la maduración siguieron esencialmente el mismo patrón de acumulación entre las variedades de pulpa roja y las naranjas comunes. Así, los niveles de vitamina C, tocoferoles, fenoles y flavonoides totales disminuyeron a lo largo del proceso de crecimiento y maduración de fruto, como se ha descrito para las naranjas comunes (Alòs et al., 2014; Nadi et al., 2019; Multari et al., 2020; Rey et al., 2021). Los resultados obtenidos en este estudio indican que la concentración de vitamina C es similar en Kirkwood y Ruby y sus respectivas variedades de referencia (Fig. 5, Capítulo 2). En cambio, otros trabajos han descrito que la naranja Cara Cara contiene niveles ligeramente inferiores de este compuesto respecto a otras naranjas Navel (De Ancos et al., 2017, 2020), por lo que no está totalmente claro si esta diferencia de vitamina C podría ser una característica común de las naranjas rojas o si, por el contrario, puede estar más relacionada con otros factores ambientales y/o agronómicos, o a variaciones en el estado de maduración del fruto entre los distintos trabajos.

En general, la información sobre el contenido y acumulación de tocoferoles en cítricos es limitada, y recientemente se ha descrito la regulación de su biosíntesis durante la maduración del fruto y se han estudiado las diferencias entre diferentes variedades y especies (Rey et al 2021). En esta Tesis Doctoral se ha estudiado por primera vez la acumulación de tocoferoles en variedades de naranja de pulpa roja mostrando que estas variedades contienen prácticamente los mismos niveles de tocoferoles que las tradicionales (Fig. 5C-D, Capítulo 2).

Por otra parte, no se observaron diferencias importantes en los niveles de fenoles y flavonoides totales en los frutos de las cuatro variedades, especialmente en fruto maduro (Fig. 5E-H, Capítulo 2). Respecto al nivel de compuestos fenólicos, otros trabajos han obtenido resultados variables, pero sin grandes diferencias entre la naranja Cara Cara y otras variedades de naranja Navel (Brasili et al. 2017; de Ancos et al., 2017, 2020). Asimismo, y de forma complementaria, se analizó el perfil de flavonoides individuales en fruto maduro, ya que son los compuestos fenólicos más relevantes y abundantes en frutos cítricos y para los que se han descrito importantes propiedades beneficiosas (Deng et al., 2021). El perfil de flavonoides observado en las diferentes variedades coincide con la composición descrita para otros frutos de naranja, en el que la hesperidina es el flavonoide mayoritario (alrededor del 80%) (Chen et al., 2015; De Ancos et al., 2020). Además, tanto la composición individual como el contenido total de flavonoides fueron muy similares entre las naranjas de pulpa roja y las variedades Navel y Valencia común. No obstante, otros estudios han detectado niveles de flavonoides significativamente más reducidos en Red Anliu y Cara Cara en comparación con las variedades de referencia Anliu y Seike Navel, respectivamente, y se ha sugerido que la acumulación de licopeno tiene un efecto directo o indirecto sobre la biosíntesis de flavonoides (Chen et al., 2015). Sin embargo, nuestros resultados no apoyan dicha hipótesis en las variedades Kirkwood y Ruby y, por tanto, serán necesarios estudios adicionales para esclarecer si existe una influencia de la mutación en el metabolismo de los compuestos fenólicos.

La actividad antioxidante de un compuesto se define como su habilidad para reaccionar con especies reactivas de oxígeno/nitrógeno, inhibir la peroxidación lipídica o prevenir el daño oxidativo de biomoléculas (Zou et al., 2016). Así, uno de los objetivos de este trabajo ha sido evaluar el potencial antioxidante *in vitro* de las variedades de pulpa roja en comparación con las dos variedades de naranja común de referencia, y explorar la contribución de los diferentes

componentes en la capacidad antioxidante de los extractos hidro- y liposoluble de las diferentes variedades (Fig. 5, Capítulo 2). La capacidad antioxidante de extractos hidrosolubles de pulpa (CAH), determinada por los métodos DDPH y FRAP, disminuyó en función del estado de maduración y de forma paralela a la evolución de la vitamina C, fenoles y flavonoides. Así, el contenido de la vitamina C correlacionó significativamente con la CAH analizada por DPPH ($r^2 = 0.79$) y FRAP ($r^2 = 0.67$), del mismo modo que los fenoles ($r^2 = 0.86$ y $r^2 = 0.65$) y flavonoides totales ($r^2 = 0.89$ y $r^2 = 0.72$) (Tabla 2). Estos resultados sugiere que la actividad de la vitamina C y los compuestos fenólicos contribuyen de manera notable a la CAH en extractos de pulpa de naranja (Sánchez-Moreno et al., 2003; Sicari et al., 2016; Huang et al., 2007; De Ancos et al., 2017, 2020). Por su parte, la CAH por ABTS (ABTS-H) mostró un patrón ascendente a lo largo de la maduración y no se correlacionó con ninguno de los compuestos analizados (Tabla 2). Estas diferencias entre metodologías pueden explicarse, en cierto modo, a que los ensayos DPPH y FRAP, y ABTS se llevaron a cabo en solventes diferentes y, por lo tanto, la capacidad de extracción de los diferentes compuestos y su solubilidad puede variar en función del solvente utilizado e influir de forma importante en su concentración final en el extracto y en la capacidad de reaccionar frente a radicales libres. Además, estos resultados subrayan que los diferentes radicales empleados en estas metodologías exhiben diferentes afinidades respecto a los compuestos presentes en las muestras (Apak et al., 2013). La comparativa entre variedades para la CAH es consistente con los resultados obtenidos para los diferentes compuestos de naturaleza hidrosoluble, ya que no se observan diferencias relevantes entre las variedades de pulpa roja y las respectivas variedades comunes (Fig. 5, Capítulo 2).

Tabla 2. Coeficientes de correlación de Pearson (r^2) entre la vitamina C (VIT C), tocoferoles (TOC), fenoles totales (TP), flavonoides totales (TF), DPPH, FRAP, ABTS-H y ABTS-L.

	VIT	TO	TP	TF	DP	FR	ABTS-	ABT
VIT C	-	0.85*	0.82	0.80*	0.79	0.67	-0.14	0.72*
TOC	0.85	-	0.91	0.92*	0.82	0.65	-0.07	0.85*
TP	0.82	0.91*	-	0.97*	0.86	0.65	-0.24	0.87*
TF	0.80	0.91*	0.97	-	0.88	0.72	-0.28	0.86*
DPPH	0.79	0.82*	0.86	0.89*	-	0.85	-0.39	0.79*
FRAP	0.67	0.65*	0.65	0.72*	0.85	-	-0.43*	0.63*
ABTS-	-	-0.07	-	-0.28	-	-	-	-0.02
ABTS-	0.72	0.85*	0.87	0.85*	0.79	0.63	-0.02	-

Los asteriscos indican correlaciones significativas a nivel de $p \leq 0.05$.

Por otro lado, la capacidad antioxidante liposoluble (CAL) analizada por ABTS (ABTS-L) mostró niveles entre un 20-25% superiores en los frutos de Kirkwood y Ruby respecto a Navel y Valencia, respectivamente. Así, la actividad ABTS-L mostró una alta correlación con los niveles de tocoferoles ($r^2 = 0.85$) (Tabla 2). Los tocoferoles han sido descritos como eficientes antioxidantes frente a ROS (Sies et al., 2017) y, por tanto, su contribución a la capacidad antioxidante de los frutos de naranja es de especial relevancia (Rey et al., 2021). A pesar de que hay ciertas discrepancias respecto a la capacidad de los carotenoides como antioxidantes frente a radicales libres, diferentes estudios han descrito cierta contribución a la capacidad antioxidante por el método ABTS de la fracción liposoluble en extractos de frutos cítricos (Arnao et al., 2001; Sánchez-Moreno et al., 2003; Rodríguez-Amaya, 2010). Por ende, es razonable sugerir que el elevado contenido en carotenoides presente en los extractos liposolubles de Kirkwood y Ruby contribuya de forma significativa a la mayor capacidad antioxidante ABTS-L observada en estas variedades.

Además, en un experimento independiente, hemos comparado la capacidad antioxidante frente al oxígeno singlete ($^1O_2$) o SOAC (singlet oxygen absorption capacity) de extractos de pulpa de fruto maduro de las cuatro variedades (Fig. 6, Capítulo 2). Este análisis reveló que los extractos de la pulpa de los frutos de Kirkwood y Ruby presentaban una capacidad antioxidante frente al $^1O_2$ entre 40% y 50% superior en comparación con las naranjas Navel y Valencia, respectivamente. Es ampliamente aceptado que el principal mecanismo de acción de los carotenoides como compuestos antioxidantes es el de secuestrar el $^1O_2$, disipando la energía excitatoria de la molécula en forma de calor (Forman et al., 2014). En este sentido, la capacidad antioxidante frente $^1O_2$ correlacionó significativamente con el contenido de carotenoides de la pulpa (Tabla Suplementaria 4, Capítulo 2). En particular, el licopeno se ha descrito como el compuesto más efectivo frente al $^1O_2$ (Stahl & Sies et al., 2004), mientras que el fitoeno y fitoflueno también se han descrito con actividad antioxidante frente a este radical, aunque en menor medida que el licopeno, debido al menor número de dobles enlaces conjugados (Martínez et al., 2014). Por tanto, los resultados indican que el mayor contenido y la especial composición de carotenoides que presentan las naranjas de pulpa roja proporciona una mayor capacidad antioxidante frente al $^1O_2$ respecto a las variedades de naranja común.

En conjunto, los resultados obtenidos en este capítulo proporcionan una clara evidencia de que las variedades de naranja Kirkwood y Ruby presentan una maduración interna y

composición muy similar en cuanto al contenido de azúcares, vitamina C, fenoles, flavonoides, tocoferoles, así como los niveles de capacidad antioxidante hidrosoluble en comparación con las variedades de naranja común Navel y Valencia, respectivamente. La principal característica que diferencia las naranjas de pulpa roja es la mayor capacidad antioxidante liposoluble y SOAC. Así, es razonable afirmar que la mutación responsable de la coloración roja de la pulpa y de la inusual composición de carotenoides en Kirkwood y Ruby no tiene efectos negativos en otras características de calidad del fruto e incrementa la capacidad antioxidante de los extractos liposolubles (ABTS y SOAC) de la pulpa.

4.3. Contribución de los carotenoides y la vitamina C a la capacidad antioxidante en diferentes variedades de cítricos con diversidad de color

La diversidad en la coloración, tanto interna como externa de los frutos cítricos, se debe a la diferencia en el contenido y composición de carotenoides entre las diferentes especies y variedades (Alquézar et al., 2008a; Ikoma et al., 2016). Esta diversidad varía desde el amarillo de limones y pomelos, diferentes tonalidades de naranja que caracterizan a las mandarinas y naranjas, así como mutantes que acumulan licopeno en la pulpa (Xu et al., 2006; Alquézar et al., 2008, 2013; Lana et al., 2020). Numerosos estudios indican que el consumo tanto en fresco como en zumo de frutos cítricos se relaciona con importantes beneficios para la salud (Lv et al., 2015; Rampersaud & Valim, 2016; de Oliveira et al., 2019; Miles & Calder, 2021) y hasta cierto punto, se ha relacionado con su capacidad antioxidante (Dhalaria et al., 2021). La actividad antioxidante de los frutos cítricos depende de una compleja combinación de compuestos de diversa naturaleza química, entre los que destacan la vitamina C y los carotenoides (Zou et al., 2016). La vitamina C o ácido L-ascórbico es la vitamina más abundante en los frutos cítricos y, entre otras funciones esenciales en el organismo, presenta una alta actividad antioxidante frente a radicales libres (Martí et al., 2009; Lykkesfeldt, 2020). Asimismo, los carotenoides, como se ha mencionado en el apartado anterior, son compuestos con una reconocida capacidad antioxidante, principalmente frente al oxígeno singlete y otras ROS (Stahl & Sies, 2004; Böhm et al., 2012). Así, de manera complementaria a los objetivos expuestos en esta Tesis Doctoral sobre la caracterización de las nuevas variedades de naranja de pulpa roja, y gracias a la disponibilidad en el laboratorio de muestras de pulpa de cítricos con una amplia diversidad genética en cuanto a la coloración y contenido y composición de

carotenoides, se planteó evaluar la capacidad antioxidante hidro- y liposoluble, y la contribución de los carotenoides y la vitamina C a la capacidad antioxidante en diferentes especies y variedades de frutos cítricos con una amplia variabilidad respecto al color de la pulpa. En concreto, se seleccionaron las siguientes variedades de cítricos (Fig. 1, Capítulo 3): pomelo Marsh o pomelo blanco y el pomelo de pulpa roja Star Ruby; la mandarina Clemenules con una pigmentación naranja pálida y la mandarina de color naranja intenso, Nadorcott (derivada de la mandarina Murcott) y, finalmente, dos de las variedades de naranja objeto de estudio en esta Tesis Doctoral, la naranja de pulpa roja Ruby Valencia y la variedad común de referencia Valencia late (cv. Midknight).

El análisis del contenido de carotenoides en la pulpa reveló una gran variabilidad en cuanto a su composición entre los seis genotipos, además de una evidente relación entre el contenido de carotenoides y el color de los frutos (Tabla 1 y 2, Capitulo 3). Considerando el contenido total de carotenoides las variedades se ordenaron como sigue: Ruby > Nadorcott > Star Ruby > Valencia > Clemenules > Marsh. El análisis detallado de la composición individual de carotenoides reveló resultados muy similares a los descritos en estudios anteriores para estas mismas variedades (Xu et al., 2006; Alquézar et al. 2008b, 2013; Fanciullino et al., 2008; Rodrigo et al., 2008; 2015; Giuffrida et al., 2019; Petry et al., 2019) (Tabla 2, Capitulo 3).

Los niveles de vitamina C de la pulpa presentaron también una gran variabilidad entre los seis genotipos (Fig. 2, Capítulo 3), que concuerdan con los niveles descritos para las diferentes especies del género *Citrus* (Cano et al., 2008; Martí et al., 2009; Kafkas et al., 2011; Alòs et al., 2014). Así, ambas naranjas presentaron los mayores niveles de vitamina C, seguidas por los pomelos y Clemenules, mientras que Nadorcott exhibe la menor concentración de esta vitamina (Fig. 2, Capítulo 3). Estos resultados indican que la concentración de vitamina C en cítricos es altamente dependiente del genotipo como se había descrito previamente (Alòs et al., 2014). La capacidad antioxidante hidrosoluble (CAH) determinada por DPPH y ABTS, correlacionó significativamente con los niveles de vitamina C (r^2 = 0.85 y r^2 = 0.91, respectivamente), lo que sugiere que la concentración de vitamina C está altamente relacionada con la CAH en los extractos de pulpa de naranjas, pomelos y mandarinas. De forma similar, otros estudios sugieren que la vitamina C es el compuesto con mayor contribución a la capacidad antioxidante, tanto en la pulpa como en el zumo de frutos cítricos (Sicari et al., 2016; Yoo & Moon, 2016; Sánchez-Moreno et al., 2002, 2003; Martí et al., 2009).

Por otro lado, el análisis de la capacidad antioxidante liposoluble (CAL) reveló importantes diferencias entre las tres especies (Fig. 4, Capítulo 3). Así, las variedades de naranja presentaron la mayor CAL, seguidas del pomelo rojo Star Ruby, y sin diferencias significativas entre las dos variedades mandarinas. Sin embargo, las diferencias reportadas en cuanto al contenido y composición de carotenoides entre las variedades no se tradujeron en diferencias comparables para la CAL de los extractos de pulpa medidas por ABTS (r^2 = 0.40). Así, por ejemplo, la variedad de naranja de pulpa roja Ruby presentó alrededor de 20 veces mayor concentración en carotenoides, pero sólo un 10% mayor CAL respecto a la naranja común Valencia, lo que sugiere la contribución de otros compuestos a dicha capacidad antioxidante.

La capacidad antioxidante total de un alimento es la combinación de la actividad de compuestos de diferente naturaleza: liposolubles como los carotenoides y tocoferoles (vitamina E), e hidrosoluble, como la vitamina C y polifenoles, entre otros (Zou et al., 2016; Patil et al., 2017). Así, la capacidad antioxidante total entendida como la suma de CAH y CAL por ABTS (datos no mostrados) es notablemente superior en las naranjas respecto a las otras variedades de cítricos. La contribución de la vitamina C a la capacidad antioxidante hidrosoluble por ABTS evaluada entre las seis variedades oscila entre el 18-35% (Fig. 5A, Capítulo 3). A pesar de la correlación significativa observada entre la vitamina C y CAH, la moderada contribución de la vitamina C puede explicarse en cierto modo por los siguientes motivos: 1) otros componentes presentes en el extracto, como por ejemplo los compuestos fenólicos, que contribuyen de manera importante a esta actividad, 2) la CAH determinada para las diferentes variedades no solo se debe a la actividad de los diferentes componentes individuales, sino también a la sinergia entre ellos y, por lo tanto, es posible que el ensayo antioxidante no esté estimando de manera eficiente la contribución relativa de los diferentes compuestos. Existe una gran controversia entre los diferentes estudios que han evaluado la capacidad antioxidante *in vitro* en frutos cítricos, ya que algunos autores estiman que la vitamina C es el componente con mayor contribución a la capacidad antioxidante en extractos hidrosolubles (Gardner et al., 2000; Abeysinghe et al., 2007; Xu et al., 2008; Yoo et al., 2004, Sánchez-Moreno et al., 2003; De Ancos et al., 2020), mientras otros han obtenido mayores contribuciones por parte de otros compuestos, especialmente flavonoides (Yoo & Moon, 2016; Shin, 2012; Sicari et al., 2016). Los resultados de esta Tesis Doctoral indican que existe una amplia variación en la contribución de la vitamina C y otros compuestos de naturaleza

hidrosoluble a la capacidad antioxidante entre las diferentes especies de cítricos e incluso entre variedades de la misma especie (Abeysinghe et al., 2007; Zou et al., 2106).

Por su parte, las variedades con mayor contenido en carotenoides presentaron una mayor contribución de estos compuestos a la capacidad antioxidante liposoluble: Ruby y Nadorcott (77%), Star Ruby (41%), Clemenules (20%) y Valencia (5%) (Fig. 5B, Capítulo 3). Sin embargo, este orden de contribución por parte de los carotenoides no coincide con el rango de CAL en las variedades (Fig. 4, Capítulo 3). Estos resultados sugieren que otros compuestos liposolubles, como por ejemplo los tocoferoles, junto con los carotenoides contribuirían de forma significativa a CAL en los extractis de pulpa de las diferentes variedades (Rodríguez-Amaya et al., 2010; Arnao et al., 2001; Sánchez-Moreno et al., 2003). De entre las seis variedades estudiadas, la naranja de pulpa roja Ruby exhibió la mayor CAL (Fig. 4, Capítulo 3). Destaca la relevante contribución a la capacidad antioxidante de la naranja Ruby no solo del licopeno, carotenoide con la mayor actividad frente a $ABTS^{\bullet+}$ (Müller et al., 2011), sino también del fitoeno y fitoflueno, compuestos que se encuentran en grandes concentraciones en esta variedad y para los cuáles han sido descritas propiedades antioxidantes (Müller et al., 2011; Martínez et al., 2014; Meléndez-Martínez et al., 2018). Por tanto, es probable que la contribución de los carotenoides a CAL no dependa solo de la concentración total, sino también de la composición particular de cada variedad (Sánchez-Moreno et al., 2003). En general, existen pocos trabajos en la literatura sobre la CAL de alimentos, ya que la mayoría de los estudios se han centrado en la capacidad antioxidante de extractos hidrosolubles. Asimismo, los trabajos publicados sobre la actividad antioxidante de los carotenoides en alimentos muestran evidentes discrepancias entre los resultados obtenidos. Algunos estudios han reportado correlaciones significativas entre el contenido de carotenoides y la capacidad antioxidante determinada en cítricos y otros frutos (Leonardi et al., 2000; Arnao et al., 2001; Sánchez-Moreno et al., 2003; Di Vaio et al., 2008), mientras que otros sugieren que los carotenoides no contribuyen a esta actividad (Gardner et al., 2000). El principal mecanismo de acción descrito para los carotenoides como antioxidantes es como 'quenchers' (secuestradores) del oxígeno singlete (Stahl & Sies, 2004), aunque también se ha descrito cierta capacidad para interactuar con radicales libres y desactivarlos (Böhm et al., 2012). Por tanto, teniendo en cuenta el mecanismo de acción de los carotenoides, las metodologías antioxidantes *in vitro* no

parecen determinar de manera apropiada la capacidad antioxidante real de los mismos (Rodríguez-Amaya, 2010).

En resumen, la naranja Ruby Valencia, con licopeno y concentraciones muy altas de los carotenos fitoeno y fitoflueno, y el híbrido de mandarina Nadorcott, con grandes cantidades β-criptoxantina, fueron las variedades de cítricos con mayores niveles de carotenoides entre los seis genotipos estudiados. Además, de entre las 3 especies de cítricos, los extractos de naranjas presentan el mayor contenido de vitamina C, y capacidad antioxidante hidro- y liposoluble. A pesar de que los ensayos de actividad antioxidante *in vitro* presentan ciertas limitaciones, como se ha mencionado anteriormente, las naranjas exhiben la mayor capacidad para reaccionar frente a radicales libres en comparación con las otras variedades comerciales de mandarinas y pomelos. Por tanto, sería de gran interés evaluar en futuros estudios los efectos de diferentes variedades/especies de cítricos en modelos in vivo y demostrar si las diferencias en cuanto a la composición se traducen en efectos biológicos diferentes.

4.4. Efectos de la conservación postcosecha a bajas temperaturas en las naranjas de pulpa roja Kirkwood Navel y Ruby Valencia

La conservación a bajas temperaturas es el sistema más efectivo y utilizado para la conservación postcosecha de los frutos cítricos y, así, reducir su tasa respiratoria, pérdida de agua e infecciones por patógenos y mantener la calidad interna del fruto (Zacarías et al., 2020). Además, la exportación de frutos cítricos a diferentes países, como EEUU, Japón o China, exige la aplicación de tratamientos cuarentenarios para la eliminación de las larvas de la mosca del Mediterraneo (Ceratitis capitata) u otras plagas en los que los frutos deben ser expuestos a temperaturas entre 0 y 2 °C por periodos relativamente prolongados (2 a 4 semanas). Sin embargo, y debido al origen subtropical de los cítricos, la exposición de los frutos de ciertas especies y variedades a bajas temperaturas puede presentar importantes limitaciones, entre las que destaca la aparición de lesiones, generalmente en la piel de los frutos y en ocasiones también en la pulpa, que se conocen como daños por frío (Lado et al., 2019; Strano et al., 2022). El concepto generalizado es que la sensibilidad a los daños por frío tiene un factor genético, pero es también altamente dependiente y modulable por factores exógenos relacionados con el patrón, las condiciones y zonas de cultivo, y especialmente las condiciones ambientales juegan un papel determinante en la tolerancia a las bajas temperaturas (Zacarias et al., 2020).

En general, la temperatura ambiental a la que están expuestos los frutos antes de la cosecha es un factor crítico y, además, la temperatura y el tiempo de conservación, los tratamientos y el manejo postcosecha inciden de forma decisiva en la susceptibilidad de los frutos a desarrollar lesiones en la piel y mantener adecuadamente la calidad de los frutos.

Por lo tanto, en el capítulo 4 de esta Tesis Doctoral se planteó como objetivo principal evaluar por primera vez la aptitud de las nuevas variedades de pulpa roja Kirkwood Navel y Ruby Valencia a la conservación postcosecha y compararlas con las dos variedades comunes Navel y Valencia, respectivamente. Para ello, se analizaron diferentes parámetros relacionados con la fisiología del fruto, así como cambios en el contenido de compuestos relacionados con la calidad organoléptica, compuestos bioactivos, capacidad antioxidante y compuestos volátiles durante la conservación a 2 °C durante 4 semanas, seguida de una simulación de vida comercial durante 5 días a 20 °C.

A lo largo de las 4 semanas de conservación a 2 °C el porcentaje de pérdida de agua de los frutos fue constante en las cuatro variedades, sin embargo, aumentó drásticamente durante la simulación de la vida comercial a 20 °C. La pérdida de peso durante la conservación postcosecha se debe principalmente a la transpiración, o proceso de difusión del agua a través de la superficie de los tejidos al medio ambiente, que aumenta en función de la temperatura de conservación (Cohen et al., 1994; Zacarías et al., 2020) (Fig. 1A, Capítulo 4). De igual modo, la firmeza de los frutos de las diferentes variedades se redujo significativamente durante la conservación en frío, pero sin cambios durante la vida comercial (Fig. 1B, Capítulo 4). Estos parámetros son importantes indicadores fisiológicos de la funcionalidad y estado de la piel de los frutos durante la conservación postcosecha y, en general, los frutos de las variedades de pulpa roja mostraron una evolución similar respecto a las variedades tradicionales.

Los daños por frío representan una alteración fisiológica de suma importancia debido a que afectan en gran medida a la calidad y apariencia externa del fruto y, por ende, a su comerciabilidad (Lado et al., 2019). En las condiciones ensayadas, los síntomas iniciales de daños por frío se manifestaron en las cuatro variedades a partir de la tercera semana de conservación a 2 °C (Fig. 1C, Capítulo 4). Sin embargo, tanto el índice de daños por frío (IDF) (Fig. 1C, Capítulo 4), que es un reflejo de la severidad de los mismos, como su incidencia, aumentaron significativamente en las diferentes variedades durante la vida comercial respecto

a la conservación en frío. Aunque, en general, los frutos de las cuatro variedades exhibieron bajo IDF (≤0.6) e incidencia (≤30% de frutos afectados), las dos variedades de pulpa roja mostraron, desde la aparición de los primeros síntomas, menor sensibilidad que los frutos de los genotipos tradicionales. Algunos estudios han mostrado una posible relación entre el contenido de carotenoides y sus propiedades antioxidantes, con la tolerancia a los daños por frío en frutos de mandarinas (Rey et al., 2020) y de pomelos rojos (Lado et al., 2015). Por lo tanto, el alto contenido de carotenoides en el flavedo de los frutos de Kirkwood y Ruby, con altas concentraciones de fitoeno y fitoflueno (Tabla Suplementaria 1 y 2, Capítulo 4), podría explicar la menor sensibilidad de estas variedades a desarrollar daños por frío. Así pues, estos parámetros indican una buena capacidad de conservación a 2 °C de los frutos de las dos variedades de pulpa roja.

El aumento de sólidos solubles y descenso de la acidez en los frutos de Navel provocaron un incremento significativo en el índice de madurez al final de la conservación, mientras que no se observaron cambios para las otras tres variedades en cuanto a los parámetros de maduración interna (Tabla 1, Capítulo 4). Estos resultados sugieren que la naranja Kirkwood podría tener una maduración más lenta que las Navel común, como se ha mencionado anteriormente y como refleja el mantenimiento de la acidez durante la conservación. Esta característica, sin embargo, no va en detrimento de la calidad de los frutos, ya que permite prolongar su almacenamiento, retrasando el desarrollo de posibles 'off-flavors' y, por tanto, mantener la calidad organoléptica. Además, tanto el color externo como el interno de las cuatro variedades no se vio alterado como consecuencia de las bajas temperaturas o posterior vida comercial (Tabla 1, Capítulo 4). Por lo tanto, estos parámetros también avalan la buena aptitud para la conservación refrigerada en estas variedades.

La conservación postcosecha tuvo un efecto similar en cuanto a los cambios en el contenido y composición de carotenoides entre ambas variedades de pulpa roja, que a su vez fueron distintos a los que ocurrieron en las variedades comunes. En particular, el contenido total de carotenoides disminuyó en Kirkwood y Ruby durante la conservación en frío y posteriormente, aumentó durante la vida comercial hasta alcanzar niveles similares a los del inicio de la conservación (Tabla 2 y 3, Capítulo 4). Entre los diferentes carotenoides detectados en las naranjas rojas, el fitoeno fue el que presentó los mayores cambios por la temperatura de conservación. Es interesante mencionar que los niveles de licopeno aumentaron

significativamente al final de la conservación respecto a las concentraciones en los frutos recién cosechados. El incremento de la concentración de fitoeno y licopeno al final de la vida comercial puede estar asociado con al aumento de la producción de etileno a consecuencia del cambio de temperatura de conservación de 2 °C a 20 °C (Lado et al., 2015) y que induciría la expresión de genes de biosíntesis de carotenoides en respuesta al etileno (Rodrigo et al., 2007). Por otro lado, la transferencia de los frutos de la variedad Navel a 20 °C produjo un aumento del 50% de los carotenoides totales (Tabla 2, Capítulo 4), mientras que la conservación postcosecha tuvo escaso efecto en el contenido de carotenoides de la variedad Valencia (Tabla 3, Capítulo 4). Estos resultados son consistentes con los observados en otros cítricos en los cuales el contenido de carotenoides se mantiene o reduce a bajas temperaturas, pero aumenta considerablemente a temperaturas de conservación entre 10-15 °C o superiores (Matsumoto et al., 2009, Carmona et al., 2012). En conjunto, los resultados obtenidos indican que la conservación a bajas temperaturas y posterior vida comercial, en las condiciones ensayadas en este trabajo, mantienen las diferencias en el contenido de carotenoides entre las variedades de pulpa roja y las variedades de referencia.

Por otro lado, tanto la conservación en frío como la posterior de vida comercial mantuvieron los niveles de otros compuestos, además de los carotenoides, relacionados con la calidad nutricional y propiedades saludables del fruto. En general, los cambios en los diferentes compuestos analizados fueron similares entre las variedades, pero también se detectaron algunos efectos particulares de variedad (Tabla 4 y 5, Capítulo 4). Así, la conservación postcosecha tuvo un efecto positivo (incremento) en cuanto a los niveles de azúcares y tocoferoles. En particular, los niveles de sacarosa aumentaron significativamente respecto a los frutos recién cosechados, aumentando así el dulzor de la pulpa y del zumo de los frutos y, por tanto, mejorando la calidad organoléptica de los mismos. Experimentos postcosecha con diferentes variedades cítricos han observado incrementos similares en el contenido de azúcares durante la conservación a bajas temperaturas por períodos moderados. Sin embargo, otros trabajos han descrito descensos significativos después de largos períodos de conservación, generando un impacto negativo en el sabor de los frutos (Habibi et al., 2020a; Zhang et al., 2022). Estos efectos pueden deberse al estado de maduración de los frutos en el momento de la conservación y al contenido en azúcares, además de a la tasa respiratoria, que puede acelerar su consumo en función de la senescencia de los frutos (Zacarias et al., 2020).

El descenso de ácidos orgánicos y la pérdida de acidez durante la conservación postcosecha es una consecuencia de la actividad respiratoria del fruto y ha sido descrita para frutos cítricos en diferentes trabajos (Echeverria & Ismail, 1987; Sun et al., 2012; Habbibi et al., 2020a). Se considera que la pérdida de acidez es un factor positivo para el sabor de los cítricos, sin embargo, un descenso muy acusado de la misma puede ocasionar una pérdida de sabor y de la calidad del fruto (Marcilla et al., 2009; Obenland et al., 2011). Los resultados de esta Tesis indican que los cambios ocurridos en los contenidos de los ácidos orgánicos en las condiciones de conservación ensayadas son muy variables y dependientes del genotipo, y en general, se mantienen relativamente estables, en particular el ácido cítrico que es el mayoritario, aunque en el caso particular del ácido quínico, su incremento durante la conservación parece ser una respuesta fisiológica común en las cuatro variedades de naranja (Tabla 4 y 5, Capítulo 4).

Los niveles de tocoferoles en el momento de la cosecha fueron similares entre las variedades rojas y tradicionales, confirmando los resultados obtenidos en el Capítulo 2. En cambio, durante la postcosecha, los tocoferoles aumentaron de manera muy notable en Kirkwood y Ruby hasta alcanzar concentraciones alrededor de 25-30% superiores respecto a Navel y Valencia, respectivamente (Tabla 4 y 5, Capítulo 4). Estos resultados sugieren una mayor capacidad para la biosíntesis de tocoferoles en ambas variedades de naranja de pulpa roja, como respuesta a la conservación en frío y posterior transferencia a 20 °C.

Por otra parte, los niveles de vitamina C y fenoles totales permanecieron estables en las diferentes variedades e incluso aumentaron ligeramente en Navel y Valencia (Tabla 4 y 5, Capítulo 4). De modo que la conservación hasta 4 semanas a 2 °C y 5 días a 20 °C mantiene los niveles de estos compuestos respecto al fruto fresco y, por tanto, sus propiedades nutricionales y de calidad (Lattanzio et al., 1994; Rapisarda et al., 2008). Trabajos previos sugieren que, en general, la conservación en frío produce cambios mínimos en la concentración de flavonoides en frutos cítricos (Lafuente et al., 2011; Zhang et al., 2022). Este trabajo muestra resultados similares, en el que los cambios en la composición individual de flavonoides fueron muy variables entre los genotipos, pero al final de la vida comercial presentaban concentraciones similares o incluso superiores a los frutos previos a la conservación (Tabla 6 y 7, Capítulo 2). Así, los frutos de las dos variedades de naranja tuvieron una respuesta a la conservación muy similar a las de las variedades ordinarias, sin efectos negativos en la concentración de estos compuestos bioactivos.

En capítulos anteriores se han descrito las diferencias en la capacidad antioxidante hidro- y liposoluble frente a diferentes radicales libres y oxígeno singlete entre los extractos de las naranjas de pulpa roja y las respectivas variedades de referencia, durante la maduración y en el fruto maduro comercial (Capítulo 2 y 3). En este Capítulo se demuestra que la capacidad antioxidante hidrosoluble (ABTS-H) (Fig. 2A y B, Capítulo 4), determinada por el método de ABTS, no varía durante la conservación a 2 °C y posterior vida comercial, y, además, no refleja diferencias entre variedades. Estos resultados no son sorprendentes, ya que como se ha comentado anteriormente, los principales compuestos antioxidantes de naturaleza hidrosoluble no se ven sustancialmente alterados por la conservación refrigerada. En cambio, la capacidad antioxidante liposoluble, también determinada por ABTS (ABTS-L) (Fig. 2C y D, Capítulo 4), así como la capacidad antioxidante frente al oxígeno singlete (SOAC) (Fig. 2E y F, Capítulo 4), en los extractos de pulpa de Kirkwood y Ruby fueron entre 20-30% y 50-60% superiores, respectivamente, en comparación con las variedades de naranja tradicionales. Además, la mayor capacidad antioxidante de la fracción lipofílica de las naranjas de pulpa roja se mantiene durante todo el almacenamiento postcosecha y, por lo tanto, los frutos de estas variedades pueden alcanzar el mercado y llegar a los consumidores con el mismo valor añadido que el fruto fresco.

En el análisis del contenido y composición de compuestos volátiles en la pulpa de los frutos durante la conservación postcosecha por HS-SPME/GC-MS se identificaron 92 compuestos diferentes (Tabla Suplementaria 3, Capítulo 4). De entre todos ellos, los más abundantes fueron los terpenos (mono- y sesquiterpenos), y aldehídos y ésteres alifáticos (Fig. 3 Capítulo 4). Estas familias de compuestos, clasificadas por su naturaleza química, son los principales responsables del aroma de las naranjas (Pérez-Cacho and Rouseff, 2008a,b). Además, se detectaron 10 compuestos descritos como norisoprenoides (C9-C13) cuyo origen biosintético es, principalmente, a partir de la fragmentación de determinados carotenoides (Lewinsohn et al.,2005a,b; Vogel et al., 2008). Los resultados indican que las principales diferencias en el perfil de compuestos volátiles entre las variedades de pulpa roja y comunes se deben a cambios en el contenido (cuantitativos) y no en la composición (cualitativos) (Fig. 3, Capítulo 4). Además, el análisis de componentes principales indica que el estado de conservación (cosecha, 4 semanas 2 °C y/o 5 días 20 °C) es el factor que explica la mayor variabilidad entre las muestras, y no el genotipo o la variedad (Fig. 4, Capítulo 4). En general, la conservación en frío y vida

comercial tuvo efectos importantes en el contenido relativo de los volátiles y, aunque las variedades de pulpa roja y sus respectivas variedades de referencia presentaron patrones de cambios similares, éstos dependieron mayoritariamente del genotipo. Los monoterpenos, norisoprenoides, aldehídos y ésteres fueron los compuestos que exhibieron los mayores cambios en la pulpa de las diferentes variedades (Fig. 4, Capítulo 4). Los niveles de monoterpenos aumentaron significativamente durante la conservación en ambas variedades de pulpa roja, especialmente en Kirkwood, respecto a las variedades Navel y Valencia de referencia (Fig. 4, Capítulo 4). Los niveles altos de monoterpenos (C10) parecen ser una característica metabólica particular en las naranjas de pulpa roja, ya que en el mutante rojo Red Anliu también se ha detectado niveles más elevados de estos compuestos en comparación con la variedad parental (Liu et al., 2019). Por el contrario, el contenido de sesquiterpenos (C15) es notablemente superior en la variedad tradicional Navel en comparación con Kirkwood en frutos en el momento de la cosecha y al final del periodo de conservación. En cambio, mientras que Ruby presenta niveles ligeramente inferiores de sesquiterpenos en el momento de la cosecha, la conservación no reveló diferencias significativas en estos compuestos respecto a los frutos de Valencia (Fig. 4, Capítulo 4).

Los norisoprenoides son compuestos volátiles para los que se ha descrito valores de umbral de percepción extremadamente bajos (Mahattanatawee et al., 2005). Entre estos, destacan los niveles muy superiores de 6-metil-5-hepten-2-ona (MHE), neral, geranial y geranilacetona en Kirkwood y Ruby en comparación con Navel y Valencia, respectivamente (Fig. 4, Tabla Suplementaria 4 y 5, Capítulo 4). Uno de los posibles mecanismos de biosíntesis de estos compuestos es a través de la degradación enzimática de los carotenos fitoeno, fitoflueno y licopeno (Lewinhson et al. 2005a,b; Vogel et al., 2008). Dado que la pulpa de las naranjas Kirkwood y Ruby acumulan elevadas concentraciones de estos compuestos, como se ha descrito en los diferentes capítulos de esta Tesis Doctoral, los resultados evidencian una clara relación entre los altos niveles de norisoprenoides y sus precursores carotenos. Así, el inusual contenido y composición de carotenoides en los frutos Kirkwood y Ruby no solo afecta al color de los mismos, sino también al perfil de compuestos volátiles y posiblemente al aroma.

Por otro lado, los aldehídos alifáticos fueron los compuestos que presentaron mayor variabilidad entre los genotipos en respuesta a la conservación refrigerada y posterior vida comercial, destacando la mayor concentración de aldehídos C8-C12 en Kirkwood y Ruby

respecto a las variedades de referencia (Fig. 4, Tabla Suplementaria 4 y 5, Capítulo 4). Otro cambio de especial relevancia en el perfil de compuestos de volátiles en estas variedades es el aumento del contenido de ésteres, especialmente aquellos con grupo etilo cuyo precursor es el etanol, que parece ser una característica común en las naranjas tras la conservación a bajas temperaturas (González-Mas et al., 2011) y que ha sido relacionado con la incidencia de los daños por frío (Obenland et al., 2003; Habibi et al., 2020b).

En general, se considera que en las plantas existen dos rutas compartimentalizadas para la biosíntesis de isoprenoides. Por un lado, la ruta del mevalonato (MVA), que se localiza en el citosol, produce precursores para la síntesis de sesquiterpenos, entre otros compuestos. En cambio, la ruta del metileritritol fosfato (MEP) opera en los plastidios y genera precursores para la síntesis de monoterpenos, diterpenos, carotenoides, tocoferoles, entre otros (Gutensohn et al., 2012). De este modo, la mutación responsable del inusual contenido y composición de carotenoides en la pulpa de Kirkwood y Ruby, asociada a un potencial bloqueo de la actividad licopeno ciclasa (Capítulo 1) y que tiene como consecuencia una estructura anómala de los plastidios, está alterando de algún modo a las diferentes rutas biosintéticas cuyos precursores proceden de la ruta MEP. Esto se ve sustentado por el mayor incremento en el contenido de tocoferoles y monoterpenos en las variedades de pulpa roja y que se manifiestan durante la conservación postcosecha. Además, el menor contenido de sesquiterpenos observado especialmente en Kirkwood, sugiere que el metabolismo de isoprenoides en su conjunto se encuentra claramente alterado en los frutos de ambos mutantes.

En conclusión, los resultados de este apartado demuestran que las naranjas de pulpa roja Kirkwood Navel y Ruby Valencia tiene una adecuada aptitud para la conservación postcosecha a bajas temperaturas, con ligeramente mejor tolerancia a los daños por frío y, en general, manteniendo los niveles de la mayoría de los componentes en relación con la calidad organoléptica y nutricional, y con características similares a las de los frutos de las variedades tradicionales. Además, los altos niveles de carotenoides y su particular composición, así como el valor añadido que proporcionan (mayor capacidad antioxidante liposoluble y SOAC) se mantienen durante todo el periodo de conservación, lo que haría que llegaran a los consumidores los frutos de estas variedades con sus propiedades distintivas intactas.

4.5. Zumos de naranja de pulpa roja y subproducto del zumo industrial: evaluación de compuestos bioactivos y nutricionales

El zumo de naranja es el más popular y consumido en el mundo con una industria que genera 29.700 millones de dólares anuales (Statista, 2022). Estudios en humanos han relacionado el consumo de zumo de naranja 100% con múltiples efectos positivos en la salud, como mejora en los marcadores de estrés oxidativo y procesos inflamatorios, así como reducción del colesterol (O'Neil et al., 2012; Silveira et al., 2015; Alhabeeb et al., 2022).

Hasta la fecha han sido pocos estudios los que han abordado el análisis del zumo de la naranja de pulpa roja (Cara Cara), así como los efectos de la pasteurización y tratamientos con altas presiones (Brasili et al., 2017; De Ancos et al., 2017, 2020). En esta Tesis se ha caracterizado por primera vez el zumo de la nueva variedad de naranja Kirkwood en comparación con el zumo de la variedad Cara Cara, que es el más estudiado hasta la fecha en zumos de naranja de pulpa roja, junto con una variedad convencional Navel (cv. Foios). Para ello, se ha llevado a cabo un estudio comparativo de los efectos del método de extracción del zumo, manual (exprimidor de tipo casero) e industrial, en el contenido de los principales compuestos bioactivos y nutricionales para cada una de las variedades. Además, para simular un procesado industrial de zumo y los efectos sobre la composición del mismo, se llevaron a cabo dos tratamientos diferentes en el zumo extraído mediante extractor industrial: un tratamiento térmico convencional de pasteurización (85 °C/30 segundos), y un tratamiento de homogeneización a altas presiones (HPH) (150 MPa/55 °C/min) (Fig. 2, Capítulo 5).

El color de los zumos es uno de sus principales atributos de calidad y tiene un papel determinante en la valoración por parte del consumidor. El análisis del color del zumo de las diferentes variedades reveló resultados interesantes (Tabla 2, Capítulo 5). Así, los zumos de ambas variedades de pulpa roja, Kirkwood y Cara Cara, presentaron mayor índice *a*/*b* y menor *L* que los correspondientes zumos de la naranja Navel. Este resultado no es sorprendente, ya que las naranjas Kirkwood y Cara Cara se caracterizan por una intensa coloración roja de su pulpa debido a la acumulación de licopeno. Así, esta diferencia de color respecto a las naranjas comunes se traslada también al zumo, como indica el mayor índice *a*/*b*, independientemente del sistema de extracción y/o tratamiento. Sin embargo, es interesante destacar el menor índice *a*/*b* y el mayor *L* en los zumos industriales de las tres variedades respecto a los zumos manuales

(Tabla 2, Capítulo 5). Estos resultados podrían estar relacionados con la modificación de la estructura de la pulpa y el tamaño de partícula, ya que la extracción industrial produce una mayor disrupción mecánica que la extracción manual, disminuyendo el tamaño de partícula y contribuyendo a una mayor dispersión de éstas, facilitando el paso de la luz y, por tanto, resultando en un índice *a*/*b* menor pero mayor luminosidad (*L*) (Arena et al., 2000; Stinco et al., 2012).

Contenido en compuestos bioactivos, azúcares, ácidos orgánicos y evaluación de la capacidad antioxidante

Las dos metodologías de extracción y los dos tratamientos utilizados produjeron efectos variables en función de las características de cada compuesto analizado. Además, y de forma general, las diferencias más relevantes en el contenido de los diferentes compuestos y en la capacidad antioxidante entre los diferentes zumos se deben principalmente a las características y a la composición particular de la variedad de origen, más que al efecto de la extracción y/o tratamiento, salvo algunas excepciones. A continuación, se describen los principales resultados en cuanto a la composición de los zumos de las dos variedades de pulpa roja en comparación con el zumo de la variedad de naranja común (Tabla 3).

El análisis del contenido y composición de carotenoides en los diferentes zumos reveló un perfil de carotenoides muy similar al descrito previamente para la pulpa correspondiente de Navel y Kirkwood (Capítulo 1-4), y para la pulpa y zumo de Cara Cara (Alquézar et al., 2008; Brasili et al., 2017; De Ancos et al., 2017, 2020; Lu et al., 2017). Así, los zumos de la naranja tradicional Navel se componen principalmente de β,β-xantofilas, siendo la violaxantina el carotenoide mayoritario (Tabla 3, Capítulo 5). Por su parte, los zumos de Kirkwood y Cara Cara presentaron una composición individual de carotenoides muy similar entre ellos y una concentración total entre 3 y 6 veces superior respecto al zumo de Navel (Tabla 3, Capítulo 5).

Tabla 3. Resumen de las principales diferencias entre los zumos de la variedad de naranja común Navel y los zumos de las naranjas de pulpa roja Cara Cara y Kirkwood. CAH, capacidad antioxidante hidrosoluble. CAL, capacidad antioxidante liposoluble.

Compuestos	Navel	Cara Cara y Kirkwood
Carotenoides	↑ Violaxantina	↑ Carotenoides totales (3-6x) ↑ Fitoeno y fitoflueno ↑ Licopeno ↑ β-Caroteno
Vitamina C	↑ 10-20%	
Tocoferoles		↑ 20-30%
Fenoles totales		=
Flavonoides		=
Azúcares	↑ Glucosa (20-30%)	↑ Sacarosa pasteurizados y HPH ↓ Fructosa Cara Cara
Ácidos orgánicos	↑ Ácido succínico	↑ Ácido cítrico y málico
CAH	↑ DPPH, FRAP y ABTS (10-30%)	
CAL		↑ ABTS (15-40%)

En cambio, y de modo similar a lo descrito para la pulpa en capítulos anteriores, el zumo manual e industrial de Kirkwood y Cara Cara contiene entre 40-50% menos violaxantina que los correspondientes zumos de Navel (Tabla 3, Capítulo 5). Otros estudios también han observado menores concentraciones de β,β-xantofilas en los zumos de Cara Cara respecto a las naranjas Navel (Rodrigo et al., 2015; De Ancos et al., 2020). Este efecto, tal como se ha discutido en el Capítulo 1, podría explicarse por el bloqueo parcial en la actividad licopeno ciclasa en los frutos de pulpa roja que limitaría el flujo metabólico hacia los productos finales de la ruta de carotenoides, y estas diferencias en el contenido de carotenoides de la pulpa se trasladarían también al zumo.

La vitamina C es el compuesto que más atención ha suscitado en los estudios de zumos de naranja por ser uno de los principales alimentos que más vitamina C aportan en nuestra dieta (Marti et al., 2009). Los diferentes zumos obtenidos a partir de las naranjas de pulpa roja Cara Cara y Kirkwood, en general, presentaron un contenido de vitamina C entre un 10-20% menor que Navel (Fig. 3A, Capítulo 5). De manera similar, trabajos anteriores han descrito que el zumo de Cara Cara contiene menores niveles de vitamina C respecto al zumo de Navel (Kafkas et al., 2011; De Ancos et al., 2020). Las causas de estas diferencias no se conocen con exactitud, pero podrían estar en relación con las características antioxidantes particulares de las naranjas de pulpa roja, o posiblemente con otras diferencias metabólicas no identificadas todavía.

En el Capítulo 2 y 4 se han descrito por primera vez la evolución del contenido de tocoferoles en la pulpa de las naranjas rojas Kirkwood y Ruby durante la maduración y conservación postcosecha, respectivamente. Así, en este capítulo (Capítulo 5) se evalúa por primera vez los niveles de tocoferoles en el zumo procedente de naranjas rojas. En particular, el α-tocoferol fue la única isoforma detectada en los diferentes zumos de cada variedad. Comparando los valores de tocoferoles obtenidos para las diferentes variedades, a excepción del zumo manual de Cara Cara, los zumos de las naranjas de pulpa roja presentan niveles entre un 20-30% superiores que los zumos de Navel (Fig. 3B, Capítulo 5). Estas diferencias, como se ha comentado anteriormente, es muy posible que puedan estar relacionadas con alteraciones del metabolismo de carotenoides, ya que los tocoferoles y los carotenoides comparten parcialmente la ruta de precursores y es razonable asumir que defectos en la biosíntesis de carotenoides en los frutos de pulpa roja también podrían afectar a sus precursores y, por ende, a la acumulación de tocoferoles.

El análisis de la composición de flavonoides individuales en el zumo de las tres variedades reveló un perfil muy similar al descrito para la pulpa en los Capítulos 2 y 4. Así, las flavanonas hesperidina (56-70%) y narirutina (17-29%) representan entre el 85-89% del contenido total de flavonoides en los diferentes zumos de naranja (Tabla 4, Capítulo 5). Este resultado es consistente con la composición de flavonoides tradicionalmente descrita en zumos de naranja (Velázquez-Estrada et al., 2013; De Ancos et al., 2020; Stinco et al., 2020). Además, los zumos de las variedades de pulpa roja Kirkwood y Cara Cara contienen niveles de flavonoides muy similares a los de la variedad común Navel y, por tanto, no se observa ningún efecto negativo de la mutación en el contenido de estos compuestos como se ha sugerido en otros trabajos (Chen et al., 2015), manteniendo así sus propiedades en el zumo procedente de naranjas rojas (Deng et al., 2021).

El análisis de la capacidad antioxidante mostró resultados consistentes entre los diferentes ensayos antioxidantes (Fig. 4, Capítulo 5) y también con otros resultados de esta Tesis Doctoral (Capítulo 2-4). Así, la capacidad antioxidante hidrosoluble (CAH), determinada por DPPH, FRAP y ABTS (ABTS-H), fue entre un 10-30% superior en los diferentes zumos de Navel que en los de las variedades de pulpa roja, independientemente del tipo de extracción o tratamiento (Fig. 4A-C, Capítulo 5). La CAH muestra una alta correlación significativa con los contenidos en vitamina C (Fig. 5, Capítulo 5). Así, entre los distintos compuestos hidrosolubles

analizados en este estudio, la vitamina C es el que exhibe mayor contribución a esta capacidad antioxidante de los diferentes zumos, ya que los fenoles y flavonoides no se correlacionaron significativamente con ninguno de los ensayos de evaluación de capacidad antioxidante (Fig. 5, Capítulo 5). Los resultados obtenidos en numerosos estudios también sugieren que la vitamina C es el principal compuesto que determina la capacidad antioxidante en extractos procedentes de zumos de naranja (Gil-Izquierdo et al., 2002; Sánchez-Moreno et al., 2003a, b).

Los diferentes zumos de las variedades de pulpa roja Cara Cara y Kirkwood presentan entre un 15-40% mayor capacidad antioxidante liposoluble (CAL), determinada por ABTS (ABTS-L), que los zumos procedentes de naranjas Navel convencionales (Fig. 4D, Capítulo 5). El análisis de correlaciones muestra que la CAL se correlacionó significativamente con los carotenoides en el siguiente orden: fitoeno + fitoflueno (r^2 = 0.68) > contenido total de carotenoides (r^2 = 0.65) > licopeno (r^2 = 0.55). Estos resultados son consistentes con lo anteriormente descrito para la capacidad antioxidante en la pulpa de las variedades Kirkwood y Ruby. De este modo, a pesar de que el licopeno es el carotenoide con mayor capacidad antioxidante frente a radicales libres como el $ABTS^{\bullet+}$ (Müller et al., 2011) y ROS (Stahl & Sies, 2003), las elevadas concentraciones de fitoeno y fitoflueno en los zumos de Kirkwood y Cara Cara son probablemente los principales componentes antioxidantes liposolubles en los zumos de estas variedades. Además, como se ha comentado en el Capítulo 3, estos resultados refuerzan el concepto de que la contribución de los carotenoides a la CAL de las naranjas de pulpa roja no solo depende de la concentración total de carotenoides sino también de su inusual composición de carotenoides.

Los zumos de Cara Cara y Kirkwood presentaron alrededor de un 20% menor contenido de glucosa (Fig. 6, Capítulo 5), mayores concentraciones de ácido cítrico y málico pero menores niveles de ácido succínico (Fig. 7, Capítulo 5) respecto a los zumos de Navel. Los niveles de azúcares y ácidos orgánicos, así como la relación entre ellos, determinan en gran medida el sabor de los zumos de naranja, por tanto, estas ligeras diferencias pueden tener un alto impacto en la calidad organoléptica de los zumos y la apreciación por parte del consumidor.

En conjunto, los zumos de las naranjas de pulpa roja Cara Cara y Kirkwood se caracterizan por un alto contenido en carotenoides, mayores niveles de tocoferoles, sacarosa, ácido cítrico, ácido málico y capacidad antioxidante liposoluble. Por el contrario, los zumos de la naranja

tradicional Navel contienen mayores niveles de vitamina C, glucosa y capacidad antioxidante hidrosoluble. Entre todos los parámetros analizados, el contenido de carotenoides totales es el factor que explica la mayor variabilidad de los zumos, indicando que es el principal parámetro que diferencia a los zumos de las naranjas de pulpa roja respecto a los zumos de naranja tradicional (Fig. 8, Capítulo 5).

Extracción manual versus extracción industrial

En este apartado se analizan las diferencias observadas entre los zumos obtenidos mediante una extracción manual e industrial en las tres variedades (Tabla 4) (ver diseño experimental; Fig. 2, Capítulo 5).

A pesar de que los diferentes zumos de cada variedad de naranja presentaron una composición cualitativa de carotenoides similar, el método de extracción influyó de manera desigual al contenido de carotenoides en función del genotipo. Así, la extracción industrial incrementó en un 18% y 32% el contenido total de carotenoides en el zumo de Navel y Cara Cara, respectivamente (Tabla 3, Capítulo 5). El aumento notable de carotenoides en el zumo de Cara Cara se debe principalmente al incremento en fitoeno, pero es especialmente interesante destacar que la extracción industrial incrementó casi un 90% el contenido de licopeno en el zumo en comparación con la extracción manual. De forma similar, otros estudios indican que la extracción industrial, al ser mecánicamente más agresiva que la manual, incrementa la liberación de carotenoides de la pulpa al zumo (Gil-Izquierdo et al., 2002; Bai et al., 2013; Li et al., 2021). Sin embargo, la metodología de extracción no afecto de forma relevante al contenido de carotenoides de los zumos de Kirkwood.

La extracción industrial aumentó ligeramente el contenido de tocoferoles en el zumo de Cara Cara y Kirkwood, y el de vitamina C tan solo en el de Cara Cara, en comparación con el zumo manual (Fig. 3A-B, Capítulo 5). En otros trabajos se ha observado hasta un 25% de aumento en la extracción de vitamina C mediante procedimientos industriales respecto a exprimidores domésticos (Gil-Izquierdo et al., 2002). Por el contrario, el contenido de flavonoides es mayor en el zumo manual de Navel y Cara Cara respecto al zumo industrial. Gil-Izquierdo et al. (2003) describe que, aunque la extracción industrial extrae más flavanonas totales que una extracción manual, disminuye su contenido en la fase acuosa y se aumenta en la nube (‘cloud fraction’, en inglés) del zumo.

Por otro lado, la metodología de extracción apenas tuvo influencia en la concentración de azúcares y ácidos orgánicos de los diferentes zumos. En la práctica, los zumos comerciales se elaboran a partir de mezclas ('blending', en inglés) de zumos de distintas variedades, estados de madurez y orígenes, de tal forma que homogenice e iguale el contenido de azúcares y ácidos orgánicos y poder garantizar su calidad organoléptica constante durante todo el año (Chanson-Rolle et al., 2016).

Tabla 4. Resumen de los principales efectos de la metodología de extracción (manual o industrial), homogeneización a altas presiones (HPH) (150MPa/1 min) y pasteurización (P) (85 °C/30 s) en el zumo industrial de la variedad de naranja común Navel y los zumos de las naranjas de pulpa roja Cara Cara y Kirkwood.

Compuestos	Extracción	Tratamiento
Carotenoides	Industrial + 18% Navel Industrial + 32% Cara Cara + 90% licopeno	P y HPH – 40% Violaxantina Navel P y HPH Kirkwood + 9% Fitoeno + 19% Fitoflueno + 40 % Licopeno
Vitamina C	Industrial + 6% Cara Cara	HPH + 15% Navel
Tocoferoles	Industrial + 43% Cara Cara Industrial + 20% Kirkwood	P – 10% Cara Cara – 10% Kirkwood
Fenoles totales	=	=
Flavonoides	Manual + 17% Navel Manual + 25% Cara Cara Manual + 28% Kirkwood	=
Azúcares	=	=
Ácidos orgánicos	Industrial Navel + 25% Quínico + 25% Succínico	=
CAH	=	P – 20% Cara Cara ABTS-H – 25% Kirkwood ABTS-H
CAL	Manual + 25% Navel	HPH – 30 % Todas P – 20 % Todas

Efecto de la homogeneización a altas presiones y pasteurización

En la Tabla 4 se resume el efecto de los dos tratamientos aplicados en los distintos compuestos analizados en este trabajo para los zumos de las tres variedades.

A pesar de que los tratamientos térmicos convencionales como la pasteurización siguen siendo las metodologías más empleadas para la inocuidad y estabilidad de los zumos, existe un creciente interés por parte de la industria en desarrollar técnicas alternativas de procesado de los zumos que impliquen cambios mínimos en su calidad organoléptica y nutricional. Por

ello, en este trabajo se han comparado los efectos de la pasteurización con la homogenización a alta presión (HPH) en el zumo industrial de las tes variedades de naranja, cuyos resultados para los diferentes parámetros analizados en los zumos se indican de forma resumida en la Tabla 4.

Clásicamente, se ha descrito que los tratamientos térmicos pueden degradar, oxidar o isomerizar a los carotenoides y, por ende, afectar negativamente a su concentración y biodisponibilidad en el zumo (Lee & Coates, 2003; Sentandreu et al., 2020; Stinco et al., 2020). La variedad común Navel, presentó una reducción significativa del contenido total de carotenoides debido a la pérdida del 40% de violaxantina por efecto de la pasteurización y homogeneización (Tabla 3, Capítulo 5). Estos resultados coinciden con los observados por Etzbach et al. (2020) en los que sugiere que los di-epoxi carotenoides son muy susceptibles a la degradación térmica. Por su parte, la pasteurización y HPH tuvieron menores consecuencias en los zumos de Cara Cara, debido principalmente a que los carotenos lineales no se vieron alterados por ambos tratamientos. Sin embargo, en los zumos de la variedad Kirkwood el tratamiento por HPH y pasteurización aumentó entre un 9-19% los niveles de fitoeno y fitoflueno, y casi un 50% el de licopeno (Tabla 3, Capítulo 5). Estos resultados sugieren que ambos tratamientos pueden disgregar de forma importante las estructuras subcelulares donde se almacenan los carotenoides y favorecer su liberación en el medio acuoso o zumo (Mapelli-Brahm et al., 2018).

El tratamiento por HPH tuvo un efecto positivo en el contenido de vitamina C en el zumo de la variedad Navel, mientras que redujo levemente los niveles en Cara Cara y Kirkwood (Fig. 3A, Capítulo 5). Diferentes estudios coinciden en que solo la aplicación de altas presiones que supongan un elevado aumento de la temperatura puede degradar u oxidar la vitamina C (Sánchez-Moreno et al., 2005). Por otro lado, la pasteurización (85 °C/30 s) que requiere temperaturas más elevadas que la HPH (150 MPa/55 °C/min) no tuvo efectos negativos en los niveles de vitamina C. Por tanto, estos resultados y los obtenidos en diferentes estudios indican que la estabilidad de la vitamina C depende de una combinación de factores, como la extracción, las condiciones del tratamiento (temperatura, presión, tiempo, etc) y las características particulares de las variedades empleadas (Martí et al., 2009).

Por otro lado, mientras los tocoferoles se mantuvieron estables en los zumos de Navel tras la aplicación de HPH y pasteurización, en el zumo pasteurizado de las variedades rojas se observó una ligera reducción (Fig. 3B, Capítulo 5). Los tocoferoles se consideran compuestos sensibles al calor y determinados trabajos también han observado una reducción de su contenido en diferentes bebidas tras la aplicación de tratamientos térmicos (Romeu-Nadal et al., 2008; Cilla et al., 2012).

El contenido de flavonoides no se vio afectado por la pasteurización ni por el tratamiento HPH en el zumo de ninguna de las variedades estudiadas respecto al zumo industrial de referencia (Tabla 4, Capítulo 5), lo que coincide con múltiples observaciones en otros trabajos con zumos de naranja (Bai et al., 2013; Velázquez-Estrada et al., 2013; Sentandreu et al., 2020).

La pasteurización y homogeneización disminuyó de modo similar la CAL de las tres variedades (Fig. 4D, Capítulo 5). Este resultado coincide con la reducción en el contenido de carotenoides en los zumos de Navel, pero no con los niveles de carotenoides y tocoferoles en los zumos de Cara Cara y Kirkwood. Por tanto, los datos sugieren que la reducción de CAL en los zumos de las naranjas rojas puede estar relacionada con la degradación de otro tipo de compuestos liposolubles no evaluados en este trabajo. Existe una gran disparidad de resultados en la literatura respecto a la influencia de los tratamientos térmicos y no térmicos en la capacidad antioxidante de los zumos, sin embargo, esta variabilidad puede explicarse en cierto modo en base a las diferencias en la composición de los zumos, las condiciones de procesamiento, el tipo de extracto (hidro- o liposoluble) y el ensayo antioxidante utilizado (Galaverna & Dall'Asta, 2014).

El contenido individual y total de azúcares y ácidos orgánicos permaneció prácticamente inalterado, indicativo de que la aplicación de altas presiones o los tratamientos térmicos no compromete la estabilidad de estos compuestos en los zumos de las tres variedades analizadas (Verboort et al., 2011; Brasili et al., 2018).

Análisis de la composición y revalorización del subproducto de naranja de pulpa roja

Además de los estudios anteriores relacionados con los zumos de dos variedades de pulpa roja y una tradicional, también se llevó a cabo una caracterización de la composición en compuestos bioactivos y del potencial antioxidante del subproducto generado tras la extracción industrial del zumo de cada una de las variedades. Es importante destacar que el

subproducto de naranja se compone de restos de los diferentes tejidos del fruto, principalmente la piel (flavedo y albedo), y restos de las vesículas de la pulpa y, por tanto, la composición del mismo es una mezcla en proporciones variables de los diferentes tejidos, pero comparable entre las diferentes variedades. La composición de carotenoides del subproducto de la variedad Navel y de las naranjas de pulpa roja Cara Cara y Kirkwood se asemejan a la descrita en otras secciones de este trabajo (Tabla 1 y 2; Tabla Suplementaria 5 y 6, Capítulo 1) y en estudios anteriores (Alquézar et al., 2008a,b; Rodrigo et al., 2015; De Ancos et al., 2020). Destaca que el subproducto de Cara Cara y Kirkwood contiene alrededor de 10 veces más carotenoides totales que el correspondiente de Navel, con niveles extremadamente elevados de fitoeno, e importantes cantidades de licopeno, y una capacidad pro-vitamina A 40% mayor (Tabla 5, Capítulo 5).

Además, el análisis de otros compuestos de interés reveló algunas diferencias interesantes entre variedades (Tabla 6, Capítulo 5). Por ejemplo, el subproducto de Kirkwood contiene menores niveles de vitamina C y flavonoides que las otras dos variedades, y el contenido de tocoferoles de Cara Cara es superior al de Navel. De forma similar a lo descrito para los zumos, la capacidad antioxidante del extracto hidrosoluble del subproducto de Navel es superior al de las naranjas rojas, mientras la capacidad antioxidante de los extractos liposolubles determinados por el método ABTS es mayor en Cara Cara y Kirkwood. Sin embargo, a diferencia de los ensayos DPPH, FRAP y ABTS que emplean radicales sintéticos, la capacidad antioxidante frente al oxígeno singlete (ROS que se genera en sistemas biológicos) es notablemente superior en el subproducto procedente de las naranjas rojas.

El residuo o subproducto de los frutos de naranja resultante de la extracción y elaboración de zumos comerciales es uno de los principales inconvenientes que afronta la industria del sector y, en particular, la del zumo de naranja por su alta producción y consumo. Este subproducto es un material con gran potencial industrial debido al contenido de fibra soluble (pectina), aceites esenciales, y otros compuestos bioactivos con actividad antioxidante u otras propiedades funcionales. Sin embargo, la revalorización de este material para posteriores procesos o aplicaciones es escasa y costosa y, en general, se emplea para la alimentación animal, obtención de aceites esenciales, fertilizantes, biorefinería, etc. (Cyprinao et al., 2018; Zema et al., 2018). Los resultados de este trabajo ponen de manifiesto el alto contenido en determinados compuestos bioactivos, especialmente carotenoides y capacidad antioxidante

del subproducto de las naranjas de pulpa roja, en cantidades muy superiores respecto al residuo de las naranjas tradicionales, y, además, es una fuente muy rica en fitoeno y fitoflueno, carotenoides poco abundantes en la mayoría de materias primas. Por lo tanto, este subproducto puede tener un alto potencial y valor añadido para su revalorización en determinados sectores industriales como el alimentario, nutraceútico, cosmética y afines.

1. La principal característica fenotípica de las variedades de naranja dulce Kirkwood Navel y Ruby Valencia es el color rojo intenso de la pulpa, que es distinguible desde estados tempranos del desarrollo del futo. Otras características del fruto, como la tasa de crecimiento, el color externo y maduración interna fueron similares a las de las variedades comunes de referencia, Foios Navel y Midknight Valencia, respectivamente.

2. La pulpa de ambas variedades contiene altas concentraciones de carotenoides totales, con una composición totalmente alterada respecto a las variedades comunes. Destacan las concentraciones inusualmente elevadas de los carotenos incoloros fitoeno y fitoflueno, concentraciones medias de licopeno, presencia de β-caroteno y niveles inferiores de violaxantina. También se detectaron niveles reducidos de la hormona ácido abscísico (ABA) y su conjugado ABA-glucosil éster. Además, los cromoplastos de las vesículas de la pulpa presentaron una ultraestructura alterada, con menos plastoglóbulos, presencia de grandes vesículas y cristales de licopeno.

3. No se detectaron diferencias relevantes en la expresión de un amplio número de genes del metabolismo de carotenoides en la pulpa durante el desarrollo y maduración del fruto. Por tanto, el inusual contenido y composición de carotenoides en las las variedades de pulpa roja no está asociada a alteraciones transcripcionales ni a cambios en la secuencia de los genes de las enzimas licopeno ciclasas respecto a las variedades de referencia.

4. La parte interna de la corteza (floema/cambium) de los tallos de los dos mutantes presentó coloración roja, una composición de carotenoides totalmente alterada y menores niveles de ABA. Las hojas de las variedades Kirkwood y Ruby contenían mayor concentración de clorofilas, fitoeno y carotenoides totales.

5. La alteración en las variedades de pulpa roja no está específicamente relacionada con la acumulación de carotenoides durante la maduración del fruto y parece deberse a un bloqueo parcial en la actividad licopeno β-ciclasa (capacidad de conversión de licopeno en β-caroteno) o en factores asociados a la misma, que reduciría el flujo de metabolitos posteriores de la ruta e incrementaría los de etapas anteriores. La mutación no afecta exclusivamente al fruto, sino que también se manifiesta en otros tejidos de la planta con una carotenogénesis activa.

6. El contenido en vitamina C, tocoferoles, fenoles totales, flavonoides, azúcares y capacidad antioxidante hidrosoluble en la pulpa de las variedades Kirkwood y Ruby y sus cambios durante la maduración fueron esencialmente similares a los de las variedades de naranja común. El contenido en ácidos orgánicos fue más variable entre las variedades y, en general, las naranjas de pulpa roja presentaron concentraciones ligeramente superiores de ácido cítrico. En conjunto, no se observaron efectos negativos en parámetros o atributos relacionados con la calidad del fruto en las dos variedades de pulpa roja.

7. Los extractos de pulpa de las naranjas Kirkwood y Ruby presentaron mayor capacidad antioxidante liposoluble por ABTS y frente al oxígeno singlete (SOAC) respecto a las variedades de naranja común. Además, el ensayo antioxidante SOAC se correlacionó

significativamente con el elevado contenido en carotenoides en ambas variedades de pulpa roja.

8. El contenido de vitamina C (mayor en los frutos de naranja Ruby y Valencia que en las mandarinas Clemenules y Nadorcott, y pomelos Star Ruby y Marsh) se correlacionó positivamente con la capacidad antioxidante hidrosoluble confirmando una contribución significativa a la misma. Los carotenoides tuvieron una contribución variable a la capacidad antioxidante liposoluble frente al radical $ABTS^{\bullet+}$, siendo superior la de la naranja Ruby, asociada principalmente a la alta concentración de fitoeno y fitoflueno.

9. Los frutos de las variedades Kirkwood y Ruby presentaron valores comparables de pérdida de peso y firmeza, y un menor índice de daños por frío que las naranjas Navel y Valencia comunes. Además, la mayoría de compuestos bioactivos y relacionados con calidad organoléptica, así como la mayor capacidad antioxidante liposoluble y SOAC se mantuvieron o incluso aumentaron en los frutos de Kirkwood y Ruby durante la conservación refrigerada (4 semanas a 2 °C) y posterior vida comercial (5 días a 20 °C). Estos resultados indican una buena aptitud para el manejo y conservación en frío y que los frutos de las naranjas rojas pueden llegar a los consumidores con sus características de calidad y valor añadido intactos.

10. El perfil de compuestos volátiles fue notablemente diferente en la pulpa de las naranjas Kirkwood y Ruby respecto a las tradicionales, y con cambios diferentes durante la conservación refrigerada. Así, ambas mutaciones presentaron niveles más altos de norisoprenoides, monoterpenos, aldehídos de cadena larga y diferencias significativas en ésteres alifáticos. El mayor contenido en los norisoprenoides: MHO, geranilacetona, neral y geranial en las naranjas de pulpa roja, estuvo asociado a los altos niveles de sus carotenos precursores fitoeno, fitoflueno y licopeno. Por tanto, el inusual contenido y composición de carotenoides en la pulpa de Kirkwood y Ruby no solo afectó al color, sino también al perfil de compuestos volátiles y posiblemente al aroma.

11. El mayor contenido en tocoferoles y monoterpenos en la pulpa de las naranjas rojas durante la postcosecha, así como la menor concentración de sesquiterpenos, sugiere que la mutación responsable de la inusual composición de carotenoides también parece alterar de forma directa o indirecta el metabolismo de otros compuestos isoprenoides.

12. Los zumos frescos (manual e industrial) de las naranjas de pulpa roja Kirkwood y Cara Cara presentaron entre 3 y 6 veces mayor contenido total de carotenoides que los zumos de Navel, además, con una composición similar a la de la pulpa: elevadas concentraciones de carotenos incoloros, bajas concentraciones de licopeno, mayor capacidad provitamina A y menor contenido en violaxantina.

13. Los tratamientos de pasteurización y homogeneización a altas presiones (HPH) afectaron de forma variable a la concentración de carotenoides, debido principalmente a las diferencias en la composición de los zumos en cada variedad, pero, en general, fueron

efectivos manteniendo la composición en carotenoides respecto a los correspondientes zumos frescos.

14. La mayor capacidad antioxidante hidrosoluble de los zumos de Navel se correlacionó con el mayor contenido en vitamina C, mientras que la mayor capacidad antioxidante liposoluble de Cara Cara y Kirkwood se asoció con el mayor contenido en carotenoides y tocoferoles.

15. Los subproductos de la extracción industrial del zumo de las naranjas de pulpa roja Cara Cara y Kirkwood representaron un mayor valor añadido en comparación con el de la naranja tradicional, debido principalmente a una alta concentración de carotenoides y tocoferoles, y mayor capacidad antioxidante frente al oxígeno singlete. Así, el subproducto de la naranja de pulpa roja es una fuente importante de compuestos bioactivos con alto potencial para su revalorización industrial.

Abeysinghe, D., Li, X., Sun, C., Zhang, W., Zhou, C., & Chen, K. (2007). Bioactive compounds and antioxidant capacities in different edible tissues of citrus fruit of four species. *Food Chemistry*, 104, 1338-1344.

Al-Babili, S., & Bouwmeester, H.J. (2015). Strigolactones, a novel carotenoid-derived plant hormone. *Annual Review of Plant Biology*, 66, 161–186.

Albertini, M.V., Carcouet, E., Pailly, O., Gambotti, C., Luro, F., & Berti, L. (2006). Changes in organic acids and sugars during early stages of development of acidic and acidless citrus fruit. *Journal of Agricultural and Food Chemistry*, 54(21), 8335-8339.

Alhabeeba, H., Sohoulib, M.H., Larib, A., Fatahic, S., Shidfarc, F., Alomar, O., et al. (2022). Impact of orange juice consumption on cardiovascular disease risk factors: asystematic review and meta-analysis of randomized-controlled trials. *Critical Review of Food Science and Nutrition,* 62(12), 3389-3402.

Alòs, E., Rodrigo, M.J., & Zacarías, L. (2014). Differential transcriptional regulation of L-ascorbic acid content in peel and pulp of citrus fruits during development and maturation. *Planta*, 239(5), 1113–1128.

Alquézar, B., Rodrigo, M. J., & Zacarías, L. (2008b). Regulation of carotenoid biosynthesis during fruit maturation in the red-fleshed orange mutant Cara Cara. *Phytochemistry,* 69, 1997-2007.

Alquézar, B., Rodrigo, M. J., Lado, J., & Zacarías, L. (2013). A comparative physiological and transcriptional study of carotenoid biosynthesis in white and red grapefruit (*Citrus paradisi* Macf.). *Tree Genetics and Genomes,* 9, 1257-1269.

Alquézar, B., Rodrigo, M.J., & Zacarías, L. (2008b). Carotenoid biosynthesis and their regulation in citrus fruits. *Tree and Forestry Science and Biotechnology*, 2(1), 23-37.

Alquézar, B., Zacarías, L., & Rodrigo, M.J. (2009). Molecular and functional characterization of a novel chromoplast-specific lycopene β-cyclase from *Citrus* and its relation to lycopene accumulation. *Journal of Experimental Bontany*, 60, 1783-1797.

Amengual, J. (2019). Bioactive Properties of Carotenoids in Human Health. *Nutrients,* 11:2388.

Apak, R., Gorinstein, S., Böhm, V., Schaich, K.M., Özyürek, M. & Güçlü, K. (2013) Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report). *Pure and Applied Chemistry*, 85(5), 957–998.

Arballo, J., Amengual, J., Erdman, J.W. (2021). Lycopene: A critical review of digestion, absorption, metabolism, and excretion. *Antioxidants*, 10(3), 342.

Arena, E., Fallico, B., & Maccarone, E. (2000). Influence of carotenoids and pulps on the color modification of blood orange juice. *Journal of Food Science*, 65, 458-460.

Arnao, M.B., Cano, A., & Acosta, M. (2001). The hydrophilic and lipophilic contribution to total antioxidant activity. *Food Chemistry*, 73, 239-244.

Arunkumar, R., Calvo, C.M., Conrady, C.D., & Bernstein, P.S. (2018) What do we know about the macular pigment in AMD: the past, the present, and the future. *Eye (Lond),* 32, 992–1004.

Astley, S., Elliott, R., Archer, D., & Southon, S. (2004). Evidence that dietary supplementation with carotenoids and carotenoid-rich foods modulates the DNA damage: repair balance in human lymphocytes. *British Journal of Nutrition*, 91(1), 63-72.

Aust, O., Stahl, W., Sies, H., Tronnier, H., & Heinrich, U. (2005) Supplementation with tomato-based products increases lycopene, phytofluene, and phytoene levels in human serum and protects against UV-light-induced erythema. *International Journal for Vitamin and Nutrition Research*, 75, 54–60.

Bai, J., Manthey, J.A., Ford, B.L., Luzio, G., Cameron, R.G., Narciso, J., Baldwin, E.A. (2013). Effect of extraction, pasteurization and cold storage on flavonoids and other secondary metabolites in fresh orange juice. *Journal of the Science of Food and Agriculture,* 93(11), 2771–2781.

Bailey, J.R. (2015) Lycopene: Food sources potential role in human health and antioxidant effects. New York, USA: Nova Publishers.

Bain, J.M. (1958). Morphological anatomical and physiological changes in the developing fruit of the Valencia orange, *Citrus sinensis* L. Osbeck. Aust. *Journal of Botany*, 6, 1-24.

Baldwin, E.A., Bai, J., Plotto, A., & Ritenour, M. (2014) Citrus fruit quality assessment, producer and consumer perspectives. *Stewart Postharvest Rev*iew, 10, 1–7.

Baltazari, A., Mtui, H.D., Mwatawala, M.W., Chove, L.M., Msogoya, T., Samwel, J., & Subramanian, J. (2020). Effects of storage conditions, storage duration and post-harvest treatments on nutritional and sensory quality of orange (*Citrus sinensis* (L) Osbeck) Fruit. *International Journal of Fruit Science*, 20(4), 737-749.

Barros, M.P., Rodrigo, M.J., & Zacarias, L. (2018). *Journal of Agriculture and Food Chemistry,* 66(23), 5733-5740.

Barry, G. H., Caruso, M., & Gmitter, F. (2020). Commercial scion varieties. In M. Talon, M. Caruso, & F. G. Gmitter (Eds.), The Genus *Citrus* (pp. 83-104). Cambridge, UK: Woodhead Publishing.

Basu, A., & Imrhan, V. (2007). Tomatoes versus lycopene in oxidative stress and carcinogenesis: conclusions from clinical trials. *European Journal of Clinical Nutrition*, 61: 295–303.

Berman, J., Zorrilla-López, U., Farré, G., Zhu, C., Sandmann, G., Twyman, R.M., Capell, T., & Christou, P. (2014). Nutritionally important carotenoids as consumer products. *Phytochemistry Reviews,* 14, 727–743.

Bermejo, A., & Cano, A. (2012). Analysis of nutritional constituents in twenty citrus cultivars from the mediterranean area at different stages of ripening. *Food Science and Nutrition*, 03(05), 639–650.

Blas, A. L., Ming, R., Liu, Z., Veatch, O.J., Paull, R.E., Moore, P. H., et al. (2010). Cloning of the papaya chromoplast-specific lycopene beta-cyclase, CpCYC-b, controlling fruit flesh color reveals conserved microsynteny and a recombination hot spot. *Plant Physiology*, 152(4), 2013-22.

Böhm, F., Edge, R., & Truscott, G. (2012). Interactions of dietary carotenoids with activated (singlet) oxygen and free radicals: potential effects for human health. *Molecular Nutrition and Food Research,* 56, 205–216.

Böhm, V., & Bitsch, R. (1999) Intestinal absorption of lycopene from different matrices and interactions to other carotenoids, the lipid status, and the antioxidant capacity of human plasma. *European Journal of Nutrition*, 38, 118–125 (1999).

Bohn, T., Bonet, M.L., Borel, P., Keijer, J., Landrier, J.F., Milisav, I., Ribot, J., et al. (2021). Mechanistic aspects of carotenoid health benefits - where are we now? *Nutrition Research Reviews,* 34(2), 276-302.

Bramley, P. (2000). Is lycopene beneficial to human health? *Phytochemistry*, 54(3), 233-236.

Brasili, E., Hassimotto, N.M.A., Del Chierico, F., Marini, F., Quagliariello, A., Sciubba, F., Miccheli, A., et al. (2019). Daily Consumption of Orange Juice from *Citrus* sinensis L. Osbeck cv. Cara Cara and cv. Bahia Differently Affects Gut Microbiota Profiling as Unveiled by an Integrated Meta-Omics Approach. *Journal of Agricultural and Food Chemistry*, 67(5), 1381-1391.

Brasili, E., Seixas Chaves, D.F., Xavier, A.A.O., Mercadante, A.Z., Hassimotto, N.M.A., & Lajolo, F.M. (2017). Effect of pasteurization on flavonoids and carotenoids in *Citrus sinensis* (L.) Osbeck cv. 'Cara Cara' and 'Bahia' juices. *Journal of Agricultural and Food Chemistry*, 65, 1371–1377.

Britton, G. (1995). Structure and properties of carotenoids in relation to function. *The Faseb Journal*, 9, 1551-8.

Britton, G. (2008). Functions of intact carotenoids. In G. Britton, S. Liaaen-Jensen, H. Pfander (Eds.), Carotenoids, vol 4: natural functions (pp. 189–212). Basel, Switzerland: Birkhäuser Verlag.

Caballero, P., & Fernández-Zamudio, M.A. (2007). Factores que inciden en los costes de producción y en los precios percibidos por el citricultor. Estrategias de actuación. VII Congrés citrícola de L'Horta Sud. pp: 20-28. Fundación Ruralcaja Valencia. [Disponible en: http://www.fundacioncajamarvalencia.es/es/comun/actividades/fondo-editorial/vii-congres-citricola/vii-congres-citricola.pdf].

Caballero, P., Carmona, B., & Fernández-Zamudio M.A. (2010). Opciones en la reducción de los costes de producción y sus efectos en la competitividad y en la rentabilidad de los agrios. *Levante Agrícola*, 403, 376-386.

Cano, A., & Arnao, M.B. (2004) Lipophilic and hydrophilic antioxidant activity and vitamin C content of commercial orange juices: correlation with organoleptic parameters. *Ciencia y Tecnolología Alimentaria* 4(3), 185-189.

Cano, A., Medina, A., & Bermejo A (2008) Bioactive compounds in different citrus varieties. Discrimination among cultivars. *Journal of Food Composition and Analysis*, 21, 377-381.

Carmona, L., Zacarías, L., & Rodrigo, M.J. (2012). Stimulation of coloration and carotenoid biosynthesis during postharvest storage of 'Navelina' orange fruit at 12 °C. *Postharvest Biology and Technology*, 74, 108–117.

Cazzonelli, C.I. (2011). Carotenoids in nature: Insights from plants and beyond. *Functional Plant Biology*, 38(11), 833–847.

CGCT. 2016. La citricultura en España presente y futuro. Plan Star Cítricos. Comité de Gestión de Cítricos. [Disponible en http://www.agronegocios.es/digital/files/planstar/Sanfeliu_pstar_citricos_valencia.pdf]

Chanson-Rolle, A., Braesco, V., Chupin, J., & Bouillot, L. (2016). Nutritional Composition of Orange Juice: A Comparative Study between French Commercial and Home-Made Juices. *Food and Nutrition Sciences*, 7(4), 65813.

Chaudhary, P.R., Yu, X., Jayaprakasha, G.K., & Patil, B.S. (2016). Influence of storage temperature and low-temperature conditioning on the levels of health-promoting compounds in Rio Red grapefruit. *Food Science and Nutrition*, 5, 545–553.

Chen, J., Song, Y., & Zhang, L. (2013). Effect of lycopene supplementation on oxidative stress: an exploratory systematic review and meta-analysis of randomized controlled trials. *Journal of Medicinal Food*, 16(5), 361–374.

Chen, J., Zhang, H., Pang, Y., Cheng, Y., Deng, X., & Xu, J. (2015). Comparative study of flavonoid production in lycopene-accumulated and blonde-flesh sweet oranges (*Citrus sinensis*) during fruit development. *Food Chemistry*, 184, 238-246.

Chen, J., Zhang, H., Pang, Y., Cheng, Y., Deng, X., & Xu, J. (2015). Comparative study of flavonoid production in lycopene-accumulated and blonde-flesh sweet oranges (*Citrus sinensis*) during fruit development. *Food Chemistry*, 184, 238–246.

Cilla, A., Alegría, A., de Ancos, B., Sánchez-Moreno, C., Cano, M.P., Plaza, L.; Clemente, G., et al. (2012). Bioaccessibility of tocopherols, carotenoids, and ascorbic acid from milk- and soy-based fruit beverages: influence of food matrix and processing. *Journal of Agricultural and Food Chemistry*, 60, 7282– 7290.

Cilla, A., Rodrigo, M.J., Zacarías, L., de Ancos, B., Sánchez-Moreno, C., Barberá, R., & Alegría A. (2018). Protective effect of bioaccessible fractions of citrus fruit pulps against H2O2-induced oxidative stress in Caco-2 cells. *Food Research International*, 103, 335–344.

Clinton, S.K. (1998). Lycopene: Chemistry, biology and implications for human health and disease. *Nutrition Reviews*, 56, 35-51.

Cohen, E., Shapiro, E., Shalom, Y., & Klein, J.D (1994). Water Loss: A Nondestructive Indicator of Enhanced Cell Membrane Permeability of Chilling-injured Citrus Fruit. *American Society of Horticultural Science*, 19(5), 983-986.

Cunningham F.X. (2002). Regulation of carotenoid synthesis and accumulation in plants. *Pure and Applied Chemistry*, 74(8), 1409–1417.

Curk, F., Ollitrault, F., Garcia-Lor, A., Luro, F., Navarro, L., & Ollitrault, P. (2016). Phylogenetic origin of limes and lemons revealed by cytoplasmic and nuclear markers. *Annals Botany*, 117(4), 565-83.

Cypriano, D.Z., da Silva, L.L., & Tasic, L. (2018). High value-added products from the orange juice industry waste. *Waste Manage*, 79, 71–78.

Da Graça, J., Louzada, E., & Sauls, J.W. (2004). The origins of red pigmented grapefruits and the development of new varieties. *Proceedings of the International Society of Citriculture*, 1, 369-374.

De Ancos, B., Cilla, A., Barberá, R., Sánchez-Moreno, C., & Cano, M.P. Influence of orange cultivar and mandarin postharvest storage on polyphenols, ascorbic acid and antioxidant activity during gastrointestinal digestion. *Food Chemistry*, 225, 114–124.

De Ancos, B., Rodrigo, M.J, Sánchez-Moreno, C., Cano, M.P, & Zacarías, L. (2020). Effect of high-pressure processing applied as pretreatment on carotenoids, flavonoids and vitamin C in juice of the sweet oranges 'Navel' and the red-fleshed 'Cara Cara'. *Food Research International*, 132, 109105.

de Oliveira, R., Orlando, C., Cadavid, M., Carmona, L., Peña, L., De, R., Oliveira, P., & Scotti, L. (2019). Pasteurized Orange Juice Rich in Carotenoids Protects *Caenorhabditis elegans against* Oxidative Stress and β-Amyloid Toxicity through Direct and Indirect Mechanisms. *Oxidative Medicine and Cellular Longevity.*

Deng, Y., Tu, Y., Lao, S., Wu, M., Yin, H., Wang, L., & Liao W. (2022). The role and mechanism of citrus flavonoids in cardiovascular diseases prevention and treatment. *Critical Review of Food Science and Nutrition*, 62(27), 7591-7614.

Devitt, L.C., Fanning, K., Dietzgen, R.G., & Holton, T.A. (2010). Isolation and functional characterization of a lycopene beta-cyclase gene that controls fruit colour of papaya (*Carica papaya* L.). *Journal of Experimental Botany*, 61(1), 33-9.

Dhalaria, R., Verma, R., Kumar, D., Puri, S., Tapwal, A., Kumar, V., Nepovimova, E., & Kuca, K. (2020). Bioactive compounds of edible fruits with their anti-aging properties: A comprehensive review to prolong human life. *Antioxidants*, 9(11), 1123.

Dhuique-Mayer, C., Fanciullino, A.L., Dubois, C., & Ollitrault, P. (2009). Effect of genotype and environment on citrus juice carotenoid content *Journal of Agricultural and Food Chemistry*, 57, 9160–9168.

Di Vaio, C., Graziani, G., Marra, L., Cascone, A., & Ritiene, A. (2008). Antioxidant capacities, carotenoids and polyphenols evaluation of fresh and refrigerated peach and nectarine cultivars from Italy. *European Food Research and Technology*, 227, 1225–1231.

Echeverría, E., & M. Ismail. (1987). Changes in sugars and acids of citrus fruits during storage. *Proceedings of the Florida State Horticultural Society*, 100, 50- 52.

Eggersdorfer, M., & Wyss, A. (2018). Carotenoids in human nutrition and health. *Archives of Biochemistry and Biophysics*, 652, 18–26.

Engelmann, N.J., Clinton, S.K. & Erdman, J.W. (2011). Nutritional aspects of phytoene and phytofluene, carotenoid precursors to lycopene. *American Society for Nutrition*, 2, 51–61.

Fanciullino, A.L., Cercós, M., Dhuique-Mayer, C., Froelicher, Y., Talón, M., Ollitrault, P., & Morillon, R. (2008) Changes in carotenoid content and biosynthetic gene expression in juice sacs of four orange varieties (*Citrus sinensis*) differing in flesh fruit color. *Journal of Agricultural and Food Chemistry* 56, 3628-3638

Fanciullino, A.L., Dhuique-Mayer, C., Luro, .F, Casanova, J., Morillon, A., & Ollitrault, P. (2006). Carotenoid diversity in cultivated citrus is highly influenced by genetic factors. *Journal of Agricultural and Food Chemistry*, 54, 4397- 4406.

FAOSTAT (2020). FAOSTAT statistical database. Food and Agriculture Organization of the United Nations (FAO). Roma, Italia. [Disponible en: https://www.fao.org/3/cb6492en/cb6492en.pdf]

Felemban, A., Braguy, J., Zurbriggen, M.D., & Al-Babili, S. (2019) Apocarotenoids involved in plant development and stress response. *Frontiers in Plant Science*, 10:1168.

Fernández-Zamudio, M.A. (2021). La citricultura valenciana, la evolución de sus costes de producción e insumos que los determinan. *Levante Agrícola*, 455, 57-65.

Fernández-Zamudio, M.A., & Lliso, J.J. (2017). Aspectos económicos y agroculturales que caracterizan a la citricultura convencional y ecológica. *Levante Agrícola*, 436, 113-118.

Fiedor, J., & Burda, K. (2014). Potential role of carotenoids as antioxidants in human health and disease. *Nutrients*, 6(2), 466-88.

Forman, H.J., Davies, K.J.A., & Ursini, F. (2014). How do nutritional antioxidants really work: Nucleophilic tone and para-hormesis versus free radical scavenging *in vivo*. *Free Radical Biology and Medicine*, 66, 24–35.

Galaverna, G., & Dall'Asta, C. (2014). Production processes of orange juice and effects on antioxidant components. In V.R Preedy (Eds.), Processing and Impact on Antioxidants in Beverages (pp. 2013-214). Amsterdam, Netherlands: Elservier.

Gardner, P.T., White. T.A.C., McPhail, D.B., & Duthie, G.G. (2000). The relative contributions of vitamin C, carotenoids and phenolics to the antioxidant potential of fruit juices. *Food Chemistry*, 68, 471–474.

Gil-Izquierdo, A., Gil, M.I., & Ferreres, F. Effect of processing techniques at industrial scale on orange juice antioxidant and beneficial health compounds. *Journal of Agricultural and Food Chemistry,* 50(18), 5107–5114.

Gil-Izquierdo, A., Gil, M.I., Tomas-Barberan, F.A., Ferreres, F. (2003). Influence of industrial processing on orange juice flavanone solubility and transformation to chalcones under gastrointestinal conditions. *Journal of Agricultural and Food Chemistry,* 51(10), 3024-3028.

Giuffrida, D., Cacciola, F., Mapelli-Brahm, P., Stinco, C.M., Dugo, P., Oteri, M., Mondello, L., & Meléndez-Martínez, A.J. (2019). Free carotenoids and carotenoids esters composition in Spanish orange and mandarin juices from diverse varieties. *Food Chemistry*, 300:125139.

Giuliano, G., Bartley, G.E.P., & Scolnik, A. (1993). Regulation of carotenoid biosynthesis during tomato development. *The Plant Cell*, 5(4), 379–387.

Gmitter, F.G. (1995). Origin, evolution, and breeding of the grapefruit. *Plant Breeding Review*, 13, 345–363.

González-Mas, M.C., Rambla, J.L., Alamar, M.C., Gutiérrez, A., & Granell, A. (2011). Comparative analysis of the volatile fraction of fruit juice from different citrus species. *PLoS One*, 6, e22016.

Gross, J. (1987). Carotenoids: Pigments in Fruits. London, UK: London Academic Press.

Guo, L.-X., Shi, C.-Y., Liu, X., Ning, D.-Y., Jing, L.-F., Yang, H., & Liu, Y.Z. (2016). Citrate accumulation-related gene expression and/or enzyme activity analysis combined with metabolomics provide a novel insight for an orange mutant. *Scientific Reports*, 6, 29343.

Habibi, F., Ramezanian, A., Guillén, F., Castillo, S., Serrano, M., & Valero, D. (2020a). Changes in bioactive compounds, antioxidant activity, and nutritional quality of blood orange cultivars at different storage temperatures. *Antioxidants*, 9, 1016.

Habibi, F., Ramezanian, A., Guillén, F., Serrano, M., & Valero D. (2020b). Effect of various postharvest treatment on aroma volatile compounds of blood orange fruit exposed to chilling temperature after long-term storage. *Food and Bioprocess Technology*, 13, 2054-2064.

Hemmerlin, A. (2013). Post-translational events and modifications regulating plant enzymes involved in isoprenoid precursor biosynthesis. *Plant Science*, 203–204, 41–54.

Huang, R., Xia, R., Hu, L., Lu, H., & Wang, M. (2007). Antioxidant activity and oxygen-scavenging system in orange pulp during fruit ripening and maturation. *Scientia Horticulturae*, 113(2), 166-172.

Hussain, S.B., Shi, C.-Y., Guo, L.-X., Kamran, H.M., Sadka, A., & Liu, Y.-Z. (2017). Recent advances in the regulation of citric acid metabolism in citrus fruit. *Critical Review in Plant Science*, 36, 241–256.

Ikoma, Y., Matsumoto, H., & Kato, M. (2016). Diversity in the carotenoid profiles and the expression of genes related to carotenoid accumulation among citrus genotypes. *Breeding Science*, 66(1), 139–147.

Kafkas, E., Polatöz, S., Koç, N.K. (2011). Quantification and comparison of sugars, carboxylic acids and vitamin C components of various citrus species using HPLC techniques. *Journal of Agriculture, Science and Technology*, 5, 175-180.

Kato, M. (2012). Mechanism of carotenoids accumulation in Citrus fruit. *Journal of the Japanese Society of Horticultural Science*, 81(3), 219-233.

Kato, M., Ikoma, Y., Matsumoto, H., Sugiura, M., Hyodo, H., & Yano, M. (2004). Accumulation of carotenoids and expression of carotenoid biosynthetic genes during maturation in citrus fruit. *Plant Physiology*, 134(2), 824-37.

Kato, M., Matsumoto, H., Ikoma, Y., Okuda, H., & Yano, M. (2006). The role of carotenoid cleavage dioxygenases in the regulation of carotenoid profiles during maturation in citrus fruit. *Journal of Experimental Botany*, 57(10), 2153-2164.

Khachik, F., Carvalho, L., Bernstein, P.S., Muir, G.J., Zhao, D.Y., & Katz, N.B. (2002). Chemistry, distribution, and metabolism of tomato carotenoids and their impact on human health. *Experimental Biology and Medicine*, 227(10), 845-851.

Khan, M.U., Mackinney, G. (1953). Carotenoids in Grapefruit, *Citrus Paradisi*. *Plant Physiology*, 28(3), 550-2.

Krinsky, N.I., & Johnson, E.J. (2005). Carotenoid actions and their relation to health and disease. *Molecular Aspects of Medicine*, 26(6):459-516.

Kuromori, T., Sugimoto, E., & Shinozaki, K. (2014). Intertissue signal transfer of abscisic acid from vascular cells to guard cells. *Plant Physiology*, 164(4), 1587-92.

Ladaniya, M.S. Citrus fruit: Biology, Technology and Evaluation. Elsevier Inc: Atlanta, USA, 2008.

Lado, J., Cronje, P. J., Rodrigo, M. J., & Zacarías, L. (2019). *Citrus*. In T. de F. Sergio & P. Sunil (Eds.), Postharvest Physiological Disorders in Fruits and Vegetables (pp. 377–398). https://doi.org/10.1201/b22001-17

Lado, J., Cronje, P., Alquézar, B., Page, A., Manzi, M., Gómez-Cadenas, A., et al. (2015a). Fruit shading enhances peel color, carotenes accumulation and chromoplast differentiation in red grapefruit. Physiologia Plantarum, 154(4), 469–484. https://doi.org/10.1111/ppl.12332

Lado, J., Rodrigo, M. J., Cronje, P., & Zacarías, L. (2015b). Involvement of lycopene in the induction of tolerance to chilling injury in grapefruit. *Postharvest Biology and Technology*, 100, 176–186.

Lado, J., Rodrigo, M.J., & Zacarías, L. (2014). Maturity indicators and citrus fruit quality. *Stewart Postharvest Review*, 10, 1–6.

Lafuente, M.T, Ballester, A.R., Callejero, J., & González-Candelas, L. (2011). Effect of high-temperature-conditioning treatments on quality, flavonoid composition and vitamin C of cold stored 'Fortune' mandarins. *Food Chemistry*, 128, 1080-1086.

Lana, G., Zacarias-Garcia, J., Distefano, G., Gentile, A., Rodrigo, M.J., & Zacarias, L. (2020). Transcriptional analysis of carotenoids accumulation and metabolism in a pink-fleshed lemon mutant. *Genes,* 11(11), 1294.

Lattanzio, V., Cardinali, A., & Palmieri, S. (1994). The role of phenolics in the postharvest physiology of fruit and vegetables: Browning reaction and fungal diseases. *Italian Journal of Food Science*, 1, 3-22.

Lee, H.S, & Coates, G.A. (2003).Effect of thermal pasteurization on Valencia orange juice color and pigments. *LWT*, 36, 153-156.

Lee, H.S. (2001). Characterization of carotenoids in juice of red navel orange (Cara Cara). . *Journal of Agricultural and Food Chemistry*, 49(5), 2563-2568.

Leonardi, C., Ambrosino, P., Esposito, F., & Fogliano, V. (2000). Antioxidative activity and carotenoid and tomatine contents in different typologies of fresh consumption tomatoes. *Journal of Agricultural and Food Chemistry*, 48(10), 4723-4727.

Lewinsohn, E., Sitrit, S., Bar, E., Azulay Y., Meir, A., Zamir, D., & Tadmor, Y. (2005b). Carotenoid pigmentation affects the volatile composition of tomato and watermelon fruit, as revealed by comparative genetic analyses. *Journal of Agricultural and Food Chemistry*, 53, 3142-3148.

Lewinsohn, E., Sitrit, Y., Bar, E., Azulay, Y., Ibdah, M., Meir, A. Yosef, E., Zamir, E., & Tadmor, Y. (2005a). Not just colors-carotenoid degradation as a link between pigmentation and aroma in tomato and watermelon fruit. *Trends of Food Science and Technology*, 16, 407–415.

Li, Q., Li, T., Baldwin, E.A., Manthey; J.A., Plotto, A., Zhang, Q., Gao, W., et al. (2021). Extraction Method Affects Contents of Flavonoids and Carotenoids in Huanglongbing-Affected "Valencia" Orange Juice. *Foods*, 10, 783.

Lime, B. J., Griffiths, F. P., O'connor, R. T., Heinzelman, D. C., & McCall, E. R. (1957). Grapefruit pigment determination, spectrophotometric methods for determining pigmentation-beta-carotene and lycopene-in ruby red grapefruit. *Journal of Agricultural and Food Chemistry*, 5(12), 941-944.

Liu, C., He, M., & Xu, J. (2022) The constitutions of terpenoids and hormones of leaves in red-flesh citrus mutants and their wild types. *Horticultural Plant Journal*, 8(3), 297–310.

Liu, C., He, M., Wang, Z., & Xu, J. (2019). Integrative analysis of terpenoid profiles and hormones from fruit of red-flesh citrus mutants and their wild types. *Molecules*, 24, 3456.

Liu, C., Yan, F., Gao, H., He, M., Wang, Z., Cheng, Y., Deng, X., et al. (2015). Features of citrus terpenoid production as revealed by carotenoid, limonoid and aroma profiles of two pummelos (*Citrus maxima*) with different flesh color. *Journal of the Science of Food and Agriculture*, 95, 111–119.

Liu, C., Zhang, H., He, M., Liu, X., Chen, S., He, Z., et al. (2021). Lycopene accumulation in Cara Cara red-flesh Navel orange is correlated with weak abscisic acid catabolism. *Journal of Agricultural and Food Chemistry*, 69(29), 8236-8246.

Liu, Q., Xu, J., Liu, Y., Zhao, X., Deng, X., Guo, L., et al. (2007). A novel bud mutation that confers abnormal patterns of lycopene accumulation in sweet orange fruit (*Citrus sinensis* L. Osbeck). *Journal of Experimental Bontany*, 58, 4161-4171.

Liu, W., Ye, Q., Jin, X., Han, F., Huang, X., Cai, S., et al. (2016). A spontaneous bud mutant that causes lycopene and β-carotene accumulation in the juice sacs of the parental Guanxi pummelo fruits (*Citrus grandis* (L.) Osbeck). *Scientia Horticulturae*, 198, 379-384.

Liu, W., Ye, Q., Jin, X., Han, F., Huang, X., Cai, S., et al. (2016). A spontaneous bud mutant that causes lycopene and β-carotene accumulation in the juice sacs of the parental Guanxi pummelo fruits (*Citrus grandis* (L.) Osbeck). *Scientia Horticulturae*, 198, 379-384.

Liu, Y., Heying, E., & Tanumihardjo, S.A. (2012). History, global distribution, and nutritional importance of citrus fruits. *Comprehensive Reviews in Food Science and Food Safety*, 11, 530–545.

Lu, P.J., Wang, C.Y., Yin, T.T., Zhong, S.L., Grierson, D., Chen, K.S., et al. (2017). Cytological and molecular characterization of carotenoid accumulation in normal and high-lycopene mutant oranges. *Scientific Reports*, 7, 1-15.

Lu, S., Zhang, Y., Zheng, X., Zhu, K., Xu, Q., & Deng, X. (2016). Molecular characterization, critical amino acid identification, and promoter analysis of a lycopene β-cyclase gene from citrus. *Tree Genetics and Genomes* 12, 106.

Lu, X., Zhao, C., Shi, H., Liao, Y., Xu, F., Du, H., Xiao, H., et al. (2021). Nutrients and bioactives in citrus fruits: Different citrus varieties, fruit parts, and growth stages. *Critical Review of Food Science and Nutrition*, 5, 1–24.

Lux, P. E., Carle, R., Zacarías, L., Rodrigo, M.-J., Schweiggert, R. M., & Steingass, C. B. (2019). Genuine carotenoid profiles in sweet orange [*Citrus sinensis* (L.) Osbeck cv. Navel] peel and pulp at different maturity stages. *Journal of Agricultural and Food Chemistry*, 67(47), 13164-13175.

Lv, X., Zhao, S., Ning, Z., Zeng, H., Shu, Y., Tao, O., Xiao, C., Lu, C., & Liu, Y. (2015) Citrus fruits as a treasure trove of active natural metabolites that potentially provide benefits for human health. *Chemistry Central Journal*, 9, 68.

Lykkesfeldt, J. (2020). On the effect of vitamin C intake on human health: How to (mis)interprete the clinical evidence. *Redox Biology*, 34:101532.

Ma, G., Zhang, L. & Kato, M. (2023) Molecular regulation of carotenoid biosynthesis in citrus juice sacs: new advances. *Scientia Horticulturae*, 309, 111629.

Ma, G., Zhang, L., Iida, K., Madono, Y., Yungyuen, W., Yahata, M., et al. (2017). Identification and quantitative analysis of β-cryptoxanthin and β-citraurin esters in Satsuma mandarin fruit during the ripening process. *Food Chemistry*, 234, 356-364.

Mahattanatawee, K., Rouseff, R., Valim, M.F., & Naim, M. (2005). Identification and aroma impact of norisoprenoids in orange juice. *Journal of Agricultural and Food Chemistry*, 53, 393-397.

Mapelli-Brahm, P., & Meléndez-Martínez, A.J. (2019). The colourless carotenoids phytoene and phytofluene: Sources, consumption, bioavailability and health effects. *Current Opinion in Food Science*, 41, 201–209.

Mapelli-Brahm, P., Corte-Real, J., Meléndez-Martínez, A.J., & Bohn, T. (2017). Bioaccessibility of phytoene and phytofluene is superior to other carotenoids from selected fruit and vegetable juices. *Food Chemistry*, 229, 304-311.

Mapelli-Brahm, P., Desmarchelier, C., Margier, M., Reboul, E., Meléndez Martínez, A.J., & Borel, P. (2018). Phytoene and phytofluene isolated from a tomato extract are readily incorporated in mixed micelles and absorbed by caco-2 cells, as compared to lycopene, and SR-BI is involved in their cellular uptake. *Molecular Nutrition and Food Research*, 62(22), e1800703.

Marcilla, A., Martínez, M., Carot, J.M., Palou, L., & del Río, M.A (2009). Relationship between sensory and physico-chemical quality parameters of cold-stored" Clemenules" mandarins coated with two commercial waxes. *Spanish Journal of Agricultural Research,* 7(1), 181-189.

Mariutti, L.R.B., & Mercadante, A.Z. (2018). Carotenoid esters analysis and occurrence: What do we know so far? *Archives Biochemistry and Biophysics*, 648, 36-43.

Martí, N., Mena, P., Cánovas, J.A., Micol, V., & Saura, D. (2009). Vitamin C and the role of citrus juices as functional food. *Natural Products Communication*, 4, 677–700.

Martínez, A., Stinco, C.M., & Meléndez-Martínez, A.J. (2014). Free radical scavenging properties of phytofluene and phytoene isomers as compared to lycopene: A combined experimental and theoretical study. *The Journal of Physical Chemistry*, 118, 9819–9825.

Matsumoto, H., Ikoma, Y., Kato, M., Kuniga, T., Nakajima, N., & Yoshida, T. (2007). Quantification of Carotenoids in Citrus Fruit by LC-MS and Comparison of Patterns of Seasonal Changes for Carotenoids among Citrus Varieties. *Journal of Agricultural and Food Chemistry*, 55(6), 2356–2368.

Matsumoto, H., Ikoma, Y., Kato, M., Nakajima, N., & Hasegawa, Y. (2009). Effect of postharvest temperature and ethylene on carotenoid accumulation in the flavedo and juice sacs of Satsuma mandarin (*Citrus unshiu* Marc.) fruit. *Journal of Agricultural and Food Chemistry*, 57, 4724–4732.

Maugeri, A.A., Cirmi, S.A., Minciullo, P.L.B., Gangemi, S.B., Calapai, G.C., Mollace, V.D., & Navarra M.A. (2019). Citrus fruits and inflammaging: A systematic review. *Phytochemistry Reviews,* 18(4).

Mayne, S.T., Cartmel, B., Silva, F., Kim, C.S., Fallon, B.G., Briskin, K., Zheng, T., Baum, M., Shor-Posner, G., Goodwin, W.J. (1999). Plasma lycopene concentrations in humans are determined by lycopene intake, plasma cholesterol concentrations and selected demographic factors. *Journal of Nutrition,* 129(4), 849-854.

Mditshwaa, A., Magwazab, L.S., Tesfaya, S.Z., & Oparac, U.L. (2017). Postharvest factors affecting vitamin C content of citrus fruit: A review. *Scientia Horticulturae*, 128, 95-104.

Mein, J.R., Lian. F., & Wang, X.D. (2008). Biological activity of lycopene metabolites: implications for cancer prevention. *Nutrition Reviews,* 66(12): 667–683.

Meléndez-Martínez, A.J. (2019). An overview of carotenoids, apocarotenoids, and vitamin a in agro-food, nutrition, health, and disease. *Molecular Nutrition and Food Research*, 63, 1801045C.

Meléndez-Martínez, A.J., Böhm, V., Borge, G.I., Cano, M.P., Fikselová, M., Gruskiene, R., Lavelli, V., Loizzo, M.R., Mandic, A., Mapelli-Brahm, P. et al. (2021). Carotenoids: considerations for their use in functional foods, nutraceuticals, nutricosmetics, supplements, botanicals and novel foods in the context of sustainability, circular economy and climate change. *Annual Review of Food Science and Technology,* 12, 433-460.

Meléndez-Martínez, A.J., Mapelli-Brahm, P. & Stinco C.M. (2018). The colourless carotenoids phytoene and phytofluene: from dietary sources to their usefulness for the functional foods and nutricosmetics industries. *Journal of Food Composition and Analysis*, 68, 91–103.

Meléndez-Martínez, A.J., Mapelli-Brahm, P., Benítez-González, A. & Stinco, C.M. (2015). A comprehensive review on the colorless carotenoids phytoene and phytofluene. *Archives of Biochemistry and Biophysics,* 572, 188–200.

Mendes, A.F.S., Chen, C., Gmitter, F.G., Moore, G.A., & Costa, M.G.C. (2011). Expression and phylogenetic analysis of two new lycopene β-cyclases from *Citrus paradisi*. *Physiology Plantarum*, 141, 1-10.

Meredith, F.I., & Young, R.H. (1969). Effect of temperature on pigment development in Red Blush grapefruit and Ruby Blood oranges. *Procedures First International Citrus Symposium,* 1, 271-276.

Miles, E.A., & Calder, P.C. (2021). Effects of Citrus Fruit Juices and Their Bioactive Components on Inflammation and Immunity: A Narrative Review. *Front in Immunology*, 12:712608.

Ministerio de Agricultura, Pesca y Alimentación de España (2021). Avance de datos de Frutales Cítricos año 2020. [Disponible en https://www.mapa.gob.es/es/estadistica/temas/estadisticas-agrarias/agricultura/superficies-producciones-anuales-cultivos/].

Moise, A.R., Al-Babili, S., & Wurtzel, E.T. (2014). Mechanistic aspects of carotenoid biosynthesis. *Chemical Reviews*, 114, 164–193.

Monselise, S.P., & Halvey, A.H. (1961). Detection of lycopene in pink orange fruit. *Science*, 133, 1478.

Müller, L., Fröhlich, K., & Böhm, V. (2011). Comparative antioxidant activities of carotenoids measured by ferric reducing antioxidant power (FRAP) ABTS bleaching assay (αTEAC) DPPH assay and peroxyl radical scavenging assay. *Food Chemistry*, 129, 139-148.

Multari, S., Licciardello, C., Caruso, M., & Martens, S. (2020). Monitoring the changes in phenolic compounds and carotenoids occurring during fruit development in the tissues of four citrus fruits. *Food Research International*, 134:109228.

Nadi, R., Golein, B., Gómez-Cadenas, A., & Arbona, V. (2019). Developmental Stage- and Genotype-Dependent Regulation of Specialized Metabolite Accumulation in Fruit Tissues of Different Citrus Varieties. *International Journal of Molecular Sciences*, 20(5), 1245.

O'Neil, C.E., Nicklas, T.A., Rampersaud, G.C., & Fulgoni III, V.L. (2012). 100% Orange juice consumption is associated with better diet quality, improved nutrient adequacy, decreased risk for obesity, and improved biomarkers of health in adults: National Health and Nutrition Examination Survey, 2003-2006. *Nutrition Journal*, 11, 107.

Obenladn, D., Colli, S., Mackey, B., Sievert, J., & Arpaia, M.L. (2011). Storage temperature and time influences sensory quality of mandarins by altering soluble solids, acidity and aroma volatile composition. *Postharvest Biology and Technology*, 59(2), 187-193.

Obenland, D., Collin, S., Sievert, J., & Arpaia, M.L. (2013). Mandarin flavor and aroma volatile composition are strongly influenced by holding temperature. *Postharvest Biology and Technology*, 82, 6–14.

Pan, Z., Zeng, Y., An, J., Ye, J., Xu, Q., & Deng, X. (2012). An integrative analysis of transcriptome and proteome provides new insights into carotenoid biosynthesis and regulation in sweet orange fruits. *Journal of Protemoics*, 75(9), 2670-84.

Patil, B.S., Jayaprakasha, G.K., & Murthy, K.N.C. (2017). Beyond vitamin C: the diverse, complex health-promoting properties of citrus fruits. *Citrus Research and Technology* 38(1), 107-121.

Perera, C.O., & Yen, G.M. (2007) Functional Properties of Carotenoids in Human Health, *International Journal of Food Properties*, 10:2, 201-230.

Pérez-Cacho, P.R., & Rouseff, R.L. (2008a). Fresh squeezed orange juice odor: a review. *Critical Review of Food Science and Nutrition*, 48, 681-695.

Pérez-Cacho, P.R., & Rouseff, R.L. (2008b). Processing and storage effects on orange juice aroma: a review. *Journal of Agricultural and Food Chemistry*, 56, 9785-9796.

Petry, F.C., de Nadai, F.B., Cristofani-Yaly, M., Latado, R.R., & Mercadante, A.Z. (2019). Carotenoid biosynthesis and quality characteristics of new hybrids between tangor (*Citrus reticulata* x *C. sinensis*) cv. 'Murcott' and sweet orange (*C. sinensis*) cv. 'Pêra'. *Food Research International*, 122, 461-470.

Philip, T. (1973). Nature of carotenoid esterification in citrus fruits. *Journal of Agricultural and Food Chemistry*, 21(6), 964–966.

Pogson, B.J., Rissler, H.M., & Frank, H.A. (2005). The Role of Carotenoids in Energy Quenching. In Wydrzynski, T.J., Satoh, K., Freeman, J.A. (Eds.), Photosystem II. Advances in Photosynthesis and Respiration (pp. 515-537). Berlin, Germany: Springer.

Promkaew, P., Pongprasert, N., Wongs-Aree, C., Kaewsuksaeng, S., Opio, P., Kondo, S., et al. (2020). Carotenoids accumulation and carotenoids biosynthesis gene expression during fruit development in pulp of Tubtim-Siam pummelo fruit. *Scientia Horticulturae*, 260, 108870.

Rajendran, P., Nandakumar, N., Rengarajan, T., Palaniswami, R., Gnanadhas, E.N., & Lakshminarasaiah, U. (2014). Antioxidants and human diseases. *Clinica Chimica Acta*, 436, 332–347.

Rampersaud, G.C & Valim, M.F. (2015). 100% Citrus Juice: Nutritional Contribution, Dietary Benefits, and Association with Anthropometric Measures. *Critical Reviews in Food Science and Nutrition,* 57(1).

Rao, A.V., & Agarwal, S. (1999). Role of lycopene as antioxidant carotenoid in the prevention of chronic diseases: a review. *Nutrition Research*, 19, 305-323.

Rapisarda, P., Bianco, M.L., Pannuzzo, P., & Timpanaro, N. (2008). Effect of cold storage on vitamin C, phenolics and antioxidant activity of five orange genotypes (*Citrus sinensis* (L.) Osbeck). *Postharvest Biology and Technology*, 49, 348–354.

Rey, F., Rodrigo, M.J., & Zacarias, L. (2021). Regulation of tocopherol biosynthesis during fruit maturation of different Citrus species. *Frontiers in Plant Science*, 12, 743993.

Rey, F., Zacarías, L., & Rodrigo, M.J. (2020). Carotenoids, vitamin c, and antioxidant capacity in the peel of mandarin fruit in relation to the susceptibility to chilling injury during postharvest cold storage. *Antioxidants*, 9(12), 1–21.

Rodrigo, M. J., Alquézar, B., Alós, E., Lado, J., & Zacarias, L. (2013a). Biochemical bases and molecular regulation of pigmentation in the peel of Citrus fruit. *Scientia Horticulturae*, 163, 46-62.

Rodrigo, M. J., Cilla, A., Barberá, R., & Zacarías, L. (2015). Carotenoid bioaccessibility in pulp and fresh juice from carotenoid-rich sweet oranges and mandarins. *Food Function*, 6, 1950-1959.

Rodrigo, M. J., Lado, J., Alós, E., Alquézar, B., Dery, O., Hirschberg, J., et al. (2019). A mutant allele of ζ-carotene isomerase (*Z-ISO*) is associated with the yellow pigmentation of the "Pinalate" sweet orange mutant and reveals new insights into its role in fruit carotenogenesis. *BMC Plant Biology*, 19(1), 465.

Rodrigo, M.J., Alquezar, B., & Zacarías, L. (2006). Cloning and characterization of two 9-cis-epoxycarotenoid dioxygenase genes, differentially regulated during fruit maturation and under stress conditions, from orange (*Citrus sinensis* L. Osbeck). *Journal of Experimental Botany*, 57(3), 633-43.

Rodrigo, M.J., Alquézar, B., Alós, E., Lado, J., & Zacarias, L. (2013). Biochemical bases and molecular regulation of pigmentation in the peel of Citrus fruit. *Scientia Horticulturae*, 163, 46-62.

Rodrigo, M.J., Alquézar, B., Alós, E., Medina, V., Carmona, L., Bruno, M., et al. (2013b). A novel carotenoid cleavage activity involved in the biosynthesis of Citrus fruit-specific apocarotenoid pigments. *Journal of Experimental Botany*, 64, 4461-4478.

Rodrigo, M.J., Marcos, J.F., & Zacarías, L. (2004). Biochemical and molecular analysis of carotenoid biosynthesis in flavedo of orange (*Citrus sinensis* L.) during fruit development and maturation. *Journal of Agricultural and Food Chemistry*, 52, 6724-6731.

Rodríguez-Amaya, D.B. (2010). Quantitative analysis in vitro assessment of bioavailability and antioxidant activity of food carotenoids—A review. *Journal of Food Composition and Analysis*, 23, 726–740.

Rodríguez-Concepción M. (2010). Supply of precursors for carotenoid biosynthesis in plants. *Archives of Biochemistry and Biophysics,* 504(1), 118-22.

Rodríguez-Concepcion, M.J., Avalos, M.L., Bonet, A., Boronat, L., Gómez-Gómez, D., Hornero-Mendez, M.C., Limon, A.J., Meléndez-Martíınez, B., Olmedilla-Alonso, A., Palou, A., et al. (2018). A global perspective on carotenoids: Metabolism, biotechnology, and benefits for nutrition and health. *Progress in Lipid Research*, 70, 62–93.

Romeu-Nadal, M., Castellote, A.I., Gayà, A., López-Sabater, M.C. (2008). Effect of pasteurisation on ascorbic acid, dehydroascorbic acid, tocopherols and fatty acids in pooled mature human milk. *Food Chemistry*, 107(1), 434–438.

Rouseff, R.L., Sadler, G.D., Putnam, T.J., & Davis, J.E. (1992). Determination of β-Carotene and Other Hydrocarbon Carotenoids in Red Grapefruit Cultivars. *Journal of Agricultural and Food Chemistry*, 40, 47-51.

Ruiz-Sola, M.A., & Rodríguez-Concepción M. (2012). Carotenoid biosynthesis in *Arabidopsis*: a colorful pathway. *Arabidopsis Book*, 10:e0158.

Sadali, N.M., Sowden, R.G., Ling, Q., & Jarvis, R.P. (2019). Differentiation of chromoplasts and other plastids in plants. *Plant Cell Reports*, 38(7), 803-818.

Saini, R.K., Ranjit, A., Sharma, K., Prasad, P., Shang, X., Gowda, K.G.M., & Keum, Y.S. (2022). Bioactive compounds of Citrus fruits: A review of composition and health benefits of carotenoids, flavonoids, limonoids, and terpenes. *Antioxidants*, 11, 239.

Sánchez-Moreno, C. (2002) Review: Methods used to evaluate the free radicals scavenging activity in foods and biological systems. *Food Science and Technology International*, 8, 121–137.

Sánchez-Moreno, C., Plaza, L., de Ancos, B., & Cano, M.P. Quantitative bioactive compounds assessment and their relative contribution to the antioxidant capacity of commercial orange juices. *Journal of the Science of Food and Agriculture*, 83(5), 430–439.

Sánchez-Moreno, C., Plaza, L., De Ancos, B., & Cano, P. (2003) Quantitative bioactive compounds assessment and their relative contribution to the antioxidant capacity of commercial orange juices. *Journal of the Science of Food and Agriculture*, 83, 430–439.

Sentandreu, E., Stinco, C.M., Vicario, I.M., Mapelli-Brahm, P., Navarro, J.L., & Meléndez-Martínez A.J. (2020). High-pressure homogenization as compared to pasteurization as a sustainable approach to obtain mandarin juices with improved bioaccessibility of carotenoids and flavonoids. *Journal of Cleaner Production*, 262: 121325.

Shamel, A.D. (1932). A pink-fruited lemon. In Citrus-Fruit Improvement: A Study of Bud Variation in the Eureka Lemon, US Department of Agriculture: Washington, DC, USA, 1932, Volume 23, pp. 23–27.

Shin, Y. (2012). Correlation between antioxidant concentrations and activities of Yuja (*Citrus junos* Sieb ex Tanaka) and other citrus fruit. *Food Science and Biotechnology*, 21(5), 1477-1482.

Sicari, V., Pellicano, T.M., Giuffrè, A.M., Zappia, C. & Capocasale, M. (2016) Bioactive compounds and antioxidant activity of citrus juices produce from varieties cultivated in Calabria. *Food Measure,* 10, 773-780.

Sies, H., & Stahl, W. (1998). Lycopene: antioxidant and biological effects and its bioavailability in the human. *Proceedings of the Society for Experimental Biology*, 218(2), 121-4.

Sies, H., Berndt, C., & Jones, D.P. (2017). Oxidative stress. *Annual Review of Biochemistry*, 86, 715−748.

Silveira, J.Q., Dourado, G.K.Z.S. & Cesar, T.B. (2015). Red-fleshed sweet orange juice improves the risk factors for metabolic syndrome. *International Journal of Food Science and Nutrition*, 66(7), 830–836.

Stahl, W., & Sies, H. (1996). Lycopene: a biologically important carotenoid for humans? *Archives of Biochemistry and Biophysics*, 336, 1-9.

Stahl, W., & Sies, H. (2003). Antioxidant activity of carotenoids. *Molecular Aspects of Medicine*, 24, 345–351.

Statista, 2022. "Orange Juice - Worldwide." [Disponible en: https://www.statista.com/outlook/cmo/non-alcoholic-drinks/juices/orange-juice/worldwide.]

Stinco, C.M., Fernández-Vázquez, R., Escudero-Gilete, M.L., Heredia, F.J., Meléndez-& Martínez, A.J., & Vicario, I.M. (2012). Effect of orange juice's processing on the color, particle size, and bioaccessibility of carotenoids. *Journal of Agricultural and Food Chemistry*, 60(6), 1447−1455.

Stinco, C.M., Sentandreu, E., Mapelli-Brahm, P., Navarro, J.L., Vicario, I.M., Meléndez-& Martínez, A.J. (2020). Influence of high-pressure homogenization and pasteurization on the in vitro bioaccessibility of carotenoids and flavonoids in orange juice. *Food Chemistry*, 331:127259.

Strano, M.C., Altieri, G., Allegra, M., Di Renzo, G.C., Paterna, G., Matera, A., & Genovese, F. (2022). Postharvest technologies of fresh citrus fruit: advances and recent developments for the loss reduction during handling and storage. *Horticulturae*, 8, 612.

Sun, T., Yuan, H., Cao, H., Yazdani, M., Tadmor, Y., & Li, L. (2018). Carotenoid metabolism in plants: the role of plastids. *Molecular Plant,* 11, 58-74.

Sun, X.H., Xiong, J.J., Zhu, A.D., Zhang, L., Ma, Q.L., Xu, J., Cheng, Y.J., et al. (2012). Sugars and organic acids changes in pericarp and endocarp tissues of pumelo fruit during postharvest storage. *Scientia Horticulturae*, 142, 112–117.

Tadeo, F. R., Terol, J., Rodrigo, M. J., Licciardelo, C., & Sadka, A. (2020). Fruit growth and development. In M. Talon, M. Caruso, & F. G. Gmitter (Eds.), The Genus *Citrus* (pp. 245–289). Cambridge, UK: Woodhead Publishing.

Tan, B.L., & Norhaizan, M.E. (2019). Carotenoids: How effective are they to prevent age-related diseases. *Molecules,* 24: 1801.

Tao, N., Hu, Z., Liu, Q., Xu, J., Cheng, Y., Guo, L., Guo, W. et al. (2007). Expression of phytoene synthase gene (Psy) is enhanced during fruit ripening of Cara Cara navel orange (*Citrus sinensis* Osbeck). *Plant Cell Reports*, 26, 837–843.

Tao, N., Wang, C., Xu, J., & Chen, Y. (2012). Carotenoid accumulation in postharvest Cara Cara navel orange (*Citrus sinensis* Osbeck) fruit stored at different temperatures was transcriptionally regulated in a tissue-dependent manner. *Plant Cell Reports*, 31, 1667–1676.

Tatmala, N., Ma, G., Zhang, L., Kato, M., & Kaewsuksaeng, S. (2020). Characterization of carotenoid accumulation and carotenogenic gene expression during fruit ripening in red colored pulp of 'siam red ruby' pumelo (*Citrus grandis*) cultivated in Thailand. *The Horticulture Journal*, 89, 237-243.

Turner, T & Burri, B.T. (2013). Potential Nutritional Benefits of Current Citrus Consumption. Agriculture, 30(1), 170-187.

Velázquez-Estrada, R.M., Hernández-Herrero, M.M., Rüfer, C.E., Guamis-López, B., & Roig-Sagués, A.X. (2013). Influence of ultra high pressure homogenization processing on bioactive compounds and antioxidant activity of orange juice. *Innovative Food Science and Emerging Technologies*, 18, 89–94.

Vervoort, L., Van der Plancken, I., Grauwet, T., Timmermans, R.A.H., Mastwijk, H.C., Matser, A.M.; Hendrickx, M.E., et al. (2011). Comparing equivalent thermal, high pressure and pulsed electric field processes for mild pasteurization of orange juice Part II: impact on specific chemical and biochemical quality parameters. *Innovative Food Science and Emerging Technologies*, 12(4), 466–477.

Vogel, J.T., Tan, B.C., McCarty, D.R., & Klee, H.J. (2008). The carotenoid cleavage dioxygenase 1 enzyme has broad substrate specificity, cleaving multiple carotenoids at two different bond positions. *Journal of Biological Chemistry*, 283(17), 11364-11373.

Walter, M.H., & Strack, D. (2011). Carotenoids and their cleavage products: biosynthesis and functions. *Natural Products Reports*, 28(4), 663-92.

Wang, X.D. (2012). Lycopene metabolism and its biological significance. *American Journal of Clinical Nutrition*, 96, 1214S–1122S.

Wright, L.P., Rohwer, J.M., Ghirardo, A., Hammerbacher, A., Ortiz-Alcaide, M., Raguschke, B., Schnitzler, J.P., Gershenzon, J., & Phillips, M.A. (2014). Deoxyxylulose 5-phosphate synthase controls flux through the methylerythritol 4-phosphate pathway in *Arabidopsis. Plant Physiology*, 165, 1488–1504.

Wu, G.A., Prochnik, S., Jenkins, J., Salse, J., Hellsten, U., Murat, F., Perrier, X., et al. (2014). Sequencing of diverse mandarin, pummelo and orange genomes reveals complex history of admixture during citrus domestication. *Nature Biotechnology*, 32, 656–662.

Wu, G.A., Terol, J., Ibanez, V., López-García, A., Pérez-Román, E., Borredá, C., Domingo, C., et al. (2018). Genomics of the origin and evolution of *Citrus*. *Nature* 554, 311–316.

Xu, C.J., Fraser, P.D., Wang, W.J., & Bramley, P.M. (2006). Differences in the carotenoid content of standard citrus and lycopene-accumulating mutants *Journal of Agricultural and Food Chemistry*, 54, 5474-5481.

Xu, Q., Chen, L.L., Ruan, X., Chen, D., Zhu, A., Chen, C., & Bertrand, D. (2913). The draft genome of sweet orange (*Citrus sinensis*). *Nature Genetics*, 45(1), 59-66.

Xu, Q., Yu, K., Zhu, A., Te, J., Liu, Q., Zhang, J., et al. (2009). Comparative transcripts profiling reveals new insight into molecular regulating lycopene in a sweet orange (*Citrus sinensis)* red-flesh mutant. *BMC Genomics,* 10:540.

Yan, F., Shi, M., He, Z., Wu, L., Xu, X., He, M., et al. (2018). Largely different carotenogenesis in two pummelo fruits with different flesh colors. *PLoS ONE,* 13, e0200320.

Yoo, K.M., & Moon, B. (2016) Comparative carotenoid compositions during maturation and their antioxidative capacities of three citrus varieties. *Food Chemistry*, 196: 544-549.

Yoo, K.M., Lee, K.W., Park, J.B., Lee, H.J., & Hwang, I.K. (2004). Variation in major antioxidants and total antioxidant activity of Yuzu (*Citrus Junos* Sieb ex Tanaka) during maturation and between cultivars. *Journal of Agricultural and Food Chemistry*, 52, 5907–5913.

Yu, K., Xu, Q., Da, X., Guo, F., Ding, Y., & Deng, X. (2012) Transcriptome changes during fruit development and ripening of sweet orange (*Citrus sinensis*). *BMC Genomics*, 13:10.

Zacarias, L., Cronje, P. J. R., & Palou, L. (2020). Postharvest technology of citrus fruits. In M. Talon, M. Caruso, & F. G. Gmitter (Eds.), The Genus *Citrus* (pp. 421–446). Cambridge, UK: Woodhead Publishing.

Zacarías-García. J., Lux, P.E., Carle, R., Schweiggert, R.M., Steingass, C.B., Zacarías, L., et al. (2021). Characterization of the Pale Yellow Petal/Xanthophyll Esterase gene family in citrus as candidates for carotenoid esterification in fruits. *Food Chemistry*, 342:128322.

Zema, D.A., Calabrò, P.S., Folino, A., Tamburino, V., Zappia, G., & Zimbone S.M. (2018). Valorisation of citrus processing waste: A review. *Waste Manage*, 80, 252–273.

Zhang, H., Chen, J., Peng, Z., Shi, M., Liu, X., Wen, H., Jiang, Y., et al. (2021), Integrated Transcriptomic and Metabolomic analysis reveals a transcriptional regulation network for the biosynthesis of carotenoids and flavonoids in 'Cara Cara' navel Orange. *BMC Plant Biology*, 21(1), 29.

Zhang, J., Liang, Y., He, L., Kaliaperumal, K., Tan, H., Jiang, Y., Zhong, B., & Zhang, J. (2022). Effects of storage time and temperature on the chemical composition and organoleptic quality of Gannan navel orange (*Citrus sinensis* Osbeck cv. Newhall). J. Food Meas. Charact. 16, 935–944.

Zhang, J., Sun, H., Guo, S., Ren, Y., Li, M., Wang, J., Zhang, H., et al. (2020). Decreased protein abundance of lycopene β-cyclase contributes to red flesh in domesticated watermelon. *Plant Physiology*, 183(3), 1171-1183.

Zhang, L., Ma, G., Shirai, Y., Kato, M., Yamawaki, K., & Ikoma, Y. et al. (2012). Expression and functional analysis of two lycopene β-cyclases from citrus fruits. *Planta,* 236(4), 1315-25.

Zheng X., Zhu K., Sun Q., Zhang W., Wang X., Cao H., Tan M., et al. (2019). Natural variation in CCD4 promoter underpins species-specific evolution of red coloration in citrus peel. *Molecular Plant*, 12(9). 1294-1307.

Zou, Z., Xi, W., Hu, Y., Nie, C., & Zhiquin, Z. (2016). Antioxidant activity of Citrus fruits. *Food Chemistry*, 196, 885–896.

7.1. Anexo 1: publicación correspondiente al capítulo 1

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A comprehensive analysis of carotenoids metabolism in two red-fleshed mutants of Navel and Valencia sweet oranges (*Citrus sinensis*)

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Kirkwood Navel and Ruby Valencia are two spontaneous bud mutations of the respective parental lines of sweet orange (*Citrus sinensis*) Palmer Navel and Olinda Valencia, showing an atypical red pigmentation of the pulp. These red-fleshed varieties are commercially available and highly attractive for consumers but their carotenoid metabolism and the basis of the mutation have not been investigated. The red colour of Kirkwood and Ruby pulp was observed from the very early stages of fruit development until full maturity and associated with an altered carotenoid profiling. The red-fleshed varieties accumulated from 6- up to 1000-times more total carotenoids compared to the standard oranges. Specifically, the pulp of Kirkwood and Ruby accumulated large amounts of phytoene and phytofluene, and moderate contents of lycopene. Moreover, the red-fleshed oranges contained other unusual carotenes as δ-carotene, and lower concentrations of downstream products such as β,β-xanthophylls, abscisic acid (ABA) and ABA-glucosyl ester. This peculiar profile was associated with chromoplasts with lycopene crystalloid structures and round vesicles likely containing colourless carotenes. The flavedo and leaves of Kirkwood and Ruby showed minor changes in carotenoids, mainly limited to higher levels of phytoene. The carotenoid composition in Kirkwood and Ruby fruits was not explained by differences in the transcriptional profile of 26 genes related to carotenoid metabolism, covering the main steps of biosynthesis, catabolism and other processes related to carotenoid accumulation. Moreover, sequence analysis of the lycopene cyclase genes revealed no alterations in those of the red-fleshed oranges compared to the genes of the standard varieties. A striking event observed in Kirkwood and Ruby trees was the reddish coloration of the inner side of the bark tissue, with larger amounts of phytoene, accumulation of lycopene and lower ABA content. These observation lead to the conclusion that the mutation is not only manifested in fruit, affecting other carotenogenic tissues of the mutant plants, but with different consequences in the carotenoid profile. Overall, the carotenoid composition in the red-fleshed

7.2. Anexo 2: publicación correspondiente al capítulo 2

antioxidants

MDPI

Article

Bioactive Compounds, Nutritional Quality and Antioxidant Capacity of the Red-Fleshed Kirkwood Navel and Ruby Valencia Oranges

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Abstract: Kirkwood Navel and Ruby Valencia are two spontaneous bud-mutations of the ordinary Washington Navel and Valencia late oranges characterized by the red coloration of their flesh. The purpose of this study was to analyze the physiological features, internal fruit quality, contents of relevant bioactive compounds and antioxidant capacity in the pulps of the red-fleshed fruits compared with the ordinary oranges during late development and maturation. In general, the content of sugars, organic acids, vitamin C, tocopherols, total phenolics and flavonoids, the hydrophilic antioxidant capacity and their changes during maturation were similar in the red-fleshed oranges and in the corresponding blond oranges. However, the mature Ruby fruits contained lower concentrations of sugars, malic and succinic acid and higher levels of citric acid than the ordinary Valencia. The major difference between the pulps of the Kirkwood and Ruby oranges and those of the ordinary oranges was the higher lipophilic antioxidant capacity and SOAC (singlet oxygen absorption capacity) of the former. Together, the high and unique content and composition of carotenoids in Kirkwood and Ruby may contribute to an enhanced antioxidant capacity without any detrimental effects on other fruit-quality attributes, making these varieties good sources of phytochemicals for the fresh-fruit and juice-processing citrus industries.

Keywords: red-fleshed oranges; maturation; nutritional quality; bioactive compounds; antioxidant

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1. Introduction

Citrus is one of the most important commercial fruit crops worldwide in terms of production, market trade and consumption. The demand for and acceptance of citrus fruits for fresh consumption or juice production is highly variable across different national and international markets, depending on the consumer choices and preferences but, in general, it is mainly based on the external appearance, organoleptic properties, nutritional quality and health-related benefits [1].

The consumption of citrus fruits is associated with anti-inflammatory effects, obesity control, a decrease in the incidence of cardiovascular diseases and a reduction in the risk of certain cancers [2–4]. Therefore, the current concern over the prevention of health problems through nutrition is leading to intensive studies of the nutritional composition and antioxidant properties of citrus fruits. In this sense, oxidative stress plays a pivotal role in the development of human diseases and evidence indicates that the several beneficial effects of citrus metabolites in human health are associated with their antioxidant activity [5,6].

The content and composition of nutrients and bioactive and antioxidant metabolites in citrus fruits vary widely among the different species and varieties, tissues and maturation stages, influencing their quality, nutritional value and health-promoting effects [7]. Orange fruits (*Citrus sinensis*) are relevant sources of various nutrients and phytochemicals,

7.3. Anexo 3: publicación correspondiente al capítulo 3

Research Article

FOOD SCIENCE AND TECHNOLOGY INTERNATIONAL

Antioxidant capacity in fruit of Citrus cultivars with marked differences in pulp coloration: Contribution of carotenoids and vitamin C

Jaime Zacarías-García[1], Florencia Rey[1], José-Vicente Gil[1,2], María J Rodrigo[1] and Lorenzo Zacarías[1]

Abstract

The purpose of this study was to evaluate the specific contribution of carotenoids and vitamin C to the lipophilic and hydrophilic antioxidant capacity, respectively, of the pulp of citrus fruits using the genetic diversity in pigmentation and in the carotenoid complement. To this end, six citrus varieties were selected: two mandarins, Clemenules (*Citrus clementina*) and Nadorcott (*C. reticulata*); two grapefruits (*C. paradisi*), Marsh and Star Ruby; and two sweet oranges (*C. sinensis*), Valencia late and Valencia Ruby. Total carotenoid content and composition in the pulp of fruits were very different, in relation to their color singularities. Valencia Ruby and Nadorcott had the highest carotenoid content, accumulating the former large amounts of linear carotenes (phytoene, phytofluene, and lycopene) and Nadorcott of β-cryptoxanthin. Orange fruits contained the highest amount of vitamin C while in Nadorcott mandarin it was substantially lower. Analysis of antioxidant capacity, evaluated by 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays, in the pulp of the different fruit varieties indicated a high and positive correlation between vitamin C content and hydrophilic antioxidant capacity. Nevertheless, a weak correlation was observed between carotenoids content and lipophilic antioxidant capacity in the pulp extracts assayed by ABTS. Overall, vitamin C in the pulp of citrus fruit had an important contribution to the hydrophilic antioxidant capacity, whereas that of carotenoids to lipophilic antioxidant capacity was very variable, being the highest that of Valencia Ruby orange, with large concentrations of lycopene and phytoene, followed by Nadorcott mandarin, with high β-cryptoxanthin content.

Keywords

Antioxidant capacity, carotenoids, citrus fruit, vitamin C

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INTRODUCTION

Citrus fruits are one of the primary fruit crops in international trade in terms of value and are highly demanded worldwide for fresh consumption, juice processing, freeze concentrates, and also as food additives for dishes and beverages (Talón et al., 2020). Color is one of the most remarkable features among the different Citrus species and cultivars and a key factor of external and internal quality and consumer's acceptance. The genus *Citrus* shows a high diversity in external and internal fruit coloration: from the yellow color of lemons (*Citrus limon*), pummelos (*C. maxima*), many

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7.4. Anexo 4: publicación correspondiente al capítulo 5

foods

MDPI

Article

Juices and By-Products of Red-Fleshed Sweet Oranges: Assessment of Bioactive and Nutritional Compounds

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Abstract: The content of nutrients and bioactive compounds, and antioxidant capacity were assessed in the juices from two red-fleshed oranges, Cara Cara and Kirkwood, and compared with that of a standard Navel orange. Two juice extraction procedures, hand-squeezing and industrial, and two treatments, pasteurization (85 °C/30 s) and high-pressure homogenization (HPH, 150 MPa/55 °C/1 min), were evaluated. For most of the nutrients and bioactive compounds, the hand and industrial juice squeezing rendered similar extraction efficiency. Individual composition of carotenoids in the juices were differentially affected by the extraction procedure and the treatments, but the red-fleshed orange juices contained between 3- to 6-times higher total carotenoids than the standard Navel juices, being phytoene and phytofluene the main carotenoids. The industrial and treated juices of both red-fleshed oranges contained 20–30% higher amounts of tocopherols but about 20% lower levels of vitamin C than Navel juices. Navel juices exhibited higher hydrophilic antioxidant capacity, while the red-fleshed orange juices showed an improved lipophilic antioxidant capacity. The main distinctive characteristic of the industrial juice by-product of the red-fleshed oranges was a higher content of carotenoids (×10) and singlet oxygen antioxidant capacity (×1.5–2) than the Navel by-product.

Keywords: red-fleshed orange juice; pasteurization; high-pressure homogenization; juice quality; bioactive compounds; antioxidant capacity

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1. Introduction

Citrus is one of the main horticultural crops worldwide in terms of production and economic value. Spain produces over 50% of total citrus fruits in the EU and is the main exporter for fresh consumption. Other producer countries, such as Brazil, assign 90% of their production to the juice industry, while in Spain, about 80% of the total production is marketed as fresh, and nearly 20% is processed [1,2].

Citrus fruits and juices are widely consumed worldwide and are a natural source of micro- and macronutrients such as minerals, sugars, organic acids, vitamins C, E, and folates, and other bioactive phytochemicals such as carotenoids and phenolic compounds (including flavonoids and coumarins), among others [3–5]. Citrus fruit and juice consumption have been related to numerous beneficial effects on health, mainly attributed to the balanced content of nutrients and bioactive compounds [3,6–9].

Among the bioactive compounds present in citrus fruits and juices, carotenoids also determine their characteristic pigmentation [10,11]. Carotenoids are very efficient quenchers of singlet oxygen and scavengers of other ROS [12–14]. The carotenoid profile in the pulp and juice of standard blond sweet oranges can vary among varieties [15–17], but, in general, the β,β-xanthophylls content represents over 90% of total carotenoids, and linear carotenes are at low concentrations [18–22]. Within β,β-xanthophylls, violaxanthin is the