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Unveiling Key Aspects Related to Citrus Fruit Consumption and the Physiology of Blood Oranges

PhD Thesis

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No seré yo, ciertamente, quien niegue que los más ilustres iniciadores científicos pertenecen a la aristocracia del espíritu, y han sido capacidades mentales muy elevadas, a las cuales no llegaremos nunca, por mucho que nos esforcemos, los que figuramos en el montón de los trabajadores modestos. Pero después de hacer esta concesión, que es de pura justicia, sigo creyendo que a toda persona de regular entendimiento y ansiosa de nombradía, le queda todavía mucho campo donde ejercitar su actividad y de tener la fortuna que, a semejanza de la lotería, no sonríe siempre a los ricos, sino que se complace, de vez en cuando, en alegrar el hogar de los humildes, además, que todo ser humano puede ser, si se lo propone, escultor de su propio cerebro, y que aun en el peor dotado es susceptible, al modo de las tierras pobres, pero bien cultivadas y abonadas, de rendir copiosa mies.

Santiago Ramón y Cajal

Pública flor i publicat enigma,

pública flor del taronger, de pètals
d'algun antic calendari de pedra,
principi dens, remotíssim, perdut,
oh flor del sí, oh flor sagramental!
Arriba març: com un vedell arribes
atarantant els matins del meu poble.
Molt has torbat la meua adolescència
i encara més la meua maduresa.
Olor dels horts, preponderant, total,
que convocaves guitarres molt dolces,
aigua del rec, fertilitats incògnites.

El meu cant ol a flor de taronger,
com el meu cos, tota la meua vida.

Segell, anell, lenta olor que penetres
cambra i cervell i te'n duus els amants
a fer l'amor i fer les seues coses,
olor profunda i rica en savieses.

Per ella sols, tan sols per ella, dic
el compromís de tornar altre jorn:
que creuarà els carrers del meu cant
com ha creuat tota la meua vida.

Un pam més alt que el més alt, arribava
la seua olor de vida i descendència.

Vicent Andrés Estellés
La flor del taronger

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ABSTRACT

In the current context of growing competitiveness in the markets, the Spanish citrus fruit sector, which is the world's leading exporter, is facing the challenge of extending its offer with superior quality varieties. To this end, in the case of mandarins, the main approach has been the introduction of triploid, seedless hybrids, while for oranges, the focus in recent years has been on the reintroduction of blood orange varieties, mainly varieties from other countries.

This thesis consists of two parts. In the first part, consumer studies are presented with the aim of gaining in-depth knowledge of the different profiles of citrus fruit consumers, both Spanish and from other countries, in order to be able to adapt the supply to the preferences and needs of consumers. The second part focuses on the physiology of blood oranges, mainly on the processes related to the synthesis of aromas and anthocyanins, the latter being responsible for the characteristic red colour of these varieties.

Chapter I presents a cross-cultural study aimed at identifying the organoleptic attributes of mandarins that are key to the satisfaction of Spanish and Italian consumers. The results showed that consumers in both countries can be divided into two groups: “consumers focused on mouth perceptions” and “multisensory consumers”. Attributes such as ‘sweet’, ‘sweet-acid balance’, ‘intense flavour’, ‘juicy’ and ‘non-fibrous’ were key organoleptic attributes for both groups. In addition, flesh colour and attributes related to ease of consumption such as ‘easy to peel’ and ‘seedless’ were also important for “multisensory consumers”. The proportion of “multisensory consumers” was higher in Spain (59%) than in Italy (48%).

Chapter II evaluated the use of the CATA (Check-All-That-Apply) technique in combination with the description of the Ideal Product to select new mandarin varieties obtained in the IVIA breeding programme. For this purpose, 134 citrus fruit consumers evaluated four new varieties (‘Pri-88’, ‘Pri-89’, ‘Pri-90’ and ‘Tri-707’) in comparison with two of their parents, ‘Clemenules’ and ‘Oronules’, which are important commercial

varieties with the same harvest period. The results revealed that there are two reference profiles among consumers. However, both groups used the same attributes to describe their ideal mandarin (‘refreshing taste’, ‘very aromatic’, ‘sweet’, ‘juicy’, ‘very intense taste’, ‘sour’ and ‘not very fibrous’), suggesting that the use of attributes is conditioned by preferences. Among the new varieties evaluated, ‘Pri-89’ and ‘Tri-707’ were preferred by one of the consumer profiles, being liked more than their parent variety ‘Clemenules’ and, therefore, representing an improvement in organoleptic quality compared to it. The other group of consumers showed a preference for ‘Pri-90’, a variety that is particularly suitable for consumers sensitive to bitterness and mandarins with a certain level of acidity.

One of the objectives of the IVIA's mandarin breeding programme is to obtain new varieties of pigmented mandarins. These new varieties are hybrids obtained by crosses between blood oranges and traditional mandarins and can have a wide range of flesh colouring. In **Chapter III** of this thesis, the influence of the intensity of reddish flesh pigmentation of blood mandarin varieties (light, medium and intense) on their acceptance by Spanish and Italian consumers was evaluated. The study was based on an online questionnaire with images of mandarins of different pigmentation intensities. Half of the participants in each country received information about the health benefits of these pigmented varieties due to their high anthocyanin content. The other half did not receive this information. In general, Italian consumers were more receptive to the new pigmented mandarins, probably due to their greater familiarity with blood oranges, and showed a good purchase intention towards light and medium pigmented mandarins. Spanish consumers, less familiar with blood oranges, showed a preference for lightly pigmented mandarins. Very intense flesh pigmentation did not appeal to consumers in either country. Information related to the health benefits of these mandarins had a positive effect on the acceptance of Spanish consumers, while it had no significant impact on the attitude of Italian consumers.

In order to deepen the knowledge of orange consumers and to understand the possible effect of cultural context on their behaviour

towards traditional (blond) and blood oranges, **Chapter IV** assessed the barriers and motivations for consumption, as well as the main consumption contexts and key organoleptic attributes for consumers in four countries (China, Spain, Italy and Mexico). The data obtained showed that Italy leads in consumption and availability of blood oranges. The main motivations for consuming both types of oranges were the "liking" and the "health properties", regardless of the country. However, important differences in consumption contexts and barriers to consumption were identified according to nationality. While both types of orange can coexist in markets with high levels of consumption, as is the case in Italy, achieving this balance requires country-specific strategies.

All the information generated by the consumer studies included in the first part of this thesis can be of great use both to citrus breeders in setting their quality objectives and to the citrus sector in adapting supply to consumer preferences.

The second part of this thesis, focused on the physiology of blood oranges, comprises chapters V, VI and VII. **Chapter V** evaluated the volatile compound profile of three blood orange varieties ('Tarocco Rosso', 'Moro' and 'Sanguinelli') compared to three non-blood varieties ('Cadenera', 'Salustiana' and 'Hamlin'). In the blood varieties, samples were obtained from oranges with lightly and intensely pigmented flesh to determine whether sugar concentration and volatile compound profile depend on the degree of pigmentation. The results show that blood oranges do not have a unique aromatic profile that distinguishes them from non-blood oranges. In addition, samples of intensely pigmented blood oranges were found to have a higher concentration of sugars, mainly sucrose, as well as of certain volatile compounds (ethanol, ethyl acetate, ethyl-2-methyl butanoate and ethyl-2-butenoate) compared to less pigmented ones. These results suggest that not only is there a metabolic relationship between sugars and anthocyanins, but that there may also be an interaction between the biosynthetic pathways of anthocyanins and aromatic compounds.

In **Chapter VI**, which deepened the study of the volatile compounds of blood oranges, the evolution of the aromatic profile during the last stages

of ripening of the 'Tarocco Ippolito' variety was evaluated. This variety was chosen for the study because of its growing commercial interest. Among the volatile compounds identified, three patterns of evolution were observed during the ripening of the fruit: 1) compounds whose concentration decreases, mainly aldehydes; 2) compounds whose concentration increases, mainly esters and monoterpenes; and 3) compounds whose concentration remains stable, including compounds relevant to citrus aroma such as valencene and D-limonene.

Finally, in **Chapter VII**, the involvement of different environmental factors (thermal amplitude, chilling and solar radiation) in the accumulation of anthocyanins in ‘Tarocco Ippolito’ blood orange was evaluated. For this purpose, meteorological data were collected during three consecutive seasons and the fruit was monitored to determine the moment when anthocyanin synthesis began in both the peel and the pulp. The first thing that was observed is that anthocyanin synthesis in the flesh and peel of this variety does not occur simultaneously, as it started 8 to 15 days earlier in the flesh than in the peel. Of the three environmental factors evaluated, chilling was identified as the key factor for the initiation of anthocyanin synthesis. Four cold requirement calculation models were evaluated: Chilling Hours, Utah, Utah Positive and the Dynamic model. The Dynamic and Utah Positive models revealed that there are specific chilling requirements for anthocyanin synthesis to initiate. Anthocyanin accumulation in ‘Tarocco Ippolito’ pulp started after an accumulation of 4-5 chilling portions (Dynamic model) and approximately 128 chilling units (Utah Positive model), while the initiation of the process in the peel was associated with ≈ 10 chilling portions and ≈ 240 chilling units.

The three studies presented in the second part of this thesis are of great scientific value, as they represent an important advance in the knowledge of the physiology of blood oranges. In addition, knowing the cold requirements for the initiation of anthocyanin synthesis can be of great help when determining the ideal regions for the cultivation of the ‘Tarocco Ippolito’ variety. The identification of the most suitable models for this purpose opens the door to calculating the specific cold requirements of different varieties.

RESUMEN

En el contexto actual de creciente competitividad en los mercados, el sector citrícola español, que es el primer exportador a nivel mundial, se enfrenta al reto de ampliar su oferta con variedades de calidad superior. Para ello, en el caso de las mandarinas, se está apostando principalmente por la introducción de híbridos triploides, sin semillas, y, en el caso de las naranjas, se ha puesto el foco en la reintroducción de variedades sanguinas, principalmente procedentes de otros países.

Esta tesis consta de dos partes. En la primera, se llevaron a cabo estudios con consumidores con el objetivo de conocer en profundidad los diferentes perfiles de consumidores de cítricos, tanto españoles como de otros países, para poder adaptar así la oferta a las preferencias y necesidades de los consumidores. La segunda parte se centra en la fisiología de las naranjas sanguinas, principalmente en los procesos relacionados con la síntesis de aromas y de antocianinas, estas últimas responsables del color rojo característico de estas variedades.

En el **Capítulo I** se presenta un estudio *cross-cultural* orientado a identificar los atributos organolépticos de las mandarinas que son clave para la satisfacción de los consumidores españoles e italianos. Los resultados mostraron que los consumidores de ambos países pueden dividirse en dos grupos: "consumidores centrados en las percepciones en boca" y "consumidores multisensoriales". Atributos como 'dulce', 'equilibrio dulce-ácido', 'sabor intenso', 'jugoso' y 'no fibroso' fueron atributos organolépticos clave para la satisfacción de ambos grupos. Para los "consumidores multisensoriales" fueron importantes también el color de la pulpa y atributos relacionados con la facilidad de consumo, como 'fácil de pelar' y 'sin semillas'. La proporción de "consumidores multisensoriales" fue mayor en España (59 %) que en Italia (48 %).

En el **Capítulo II** se evaluó la utilización de la técnica CATA (*Check-All-That-Apply*) en combinación con la descripción del Producto Ideal para seleccionar nuevas variedades de mandarina obtenidas en el programa de mejora del IVIA. Para ello, 134 consumidores de cítricos evaluaron cuatro nuevas variedades ('Pri-88', 'Pri-89', 'Pri-90' y 'Tri-707') en

comparación con dos de sus parentales, las variedades ‘Clemenules’ y ‘Oronules’, ambas de importancia comercial y con las que coinciden en periodo de recolección. Los resultados revelaron que existen dos perfiles de preferencia entre los consumidores. Sin embargo, ambos grupos usaron los mismos atributos para describir su mandarina ideal (‘sabor refrescante’, ‘muy aromática’, ‘dulce’, ‘jugosa’, ‘sabor muy intenso’, ‘ácida’ y ‘poco fibrosa’), lo que sugiere que el uso de los atributos está condicionado por las preferencias. Entre las nuevas variedades evaluadas, la ‘Pri-89’ y la ‘Tri-707’ fueron las preferidas de uno de los perfiles de consumidor, gustando más que su parental ‘Clemenules’ y, por lo tanto, suponiendo una mejora de la calidad organoléptica respecto a esta. El otro grupo de consumidores mostró preferencia por la ‘Pri-90’, variedad que resulta especialmente adecuada para los consumidores sensibles al amargor y a las mandarinas con cierto nivel de acidez.

Uno de los objetivos del programa de mejora de mandarinas del IVIA es obtener nuevas variedades de mandarinas pigmentadas. Estas nuevas variedades son híbridos que se obtienen mediante cruces entre naranjas sanguinas y mandarinas tradicionales, y pueden presentar una amplia gama de coloración de pulpa. En el **Capítulo III** de esta tesis se evaluó la influencia de la intensidad de pigmentación rojiza de la pulpa de estas variedades (ligera, media e intensa) en su aceptación por parte de consumidores españoles e italianos. El estudio se basó en un cuestionario en línea con imágenes de mandarinas de diferentes intensidades de pigmentación. La mitad de los participantes de cada país recibieron información sobre las propiedades beneficiosas para la salud que estas variedades pigmentadas presentan debido a su alto contenido en antocianinas. La otra mitad no recibió esta información. En general, los consumidores italianos se mostraron más receptivos a las nuevas mandarinas pigmentadas, probablemente debido a su familiaridad con las naranjas sanguinas, y mostraron una buena intención de compra hacia las mandarinas de pigmentación ligera y media. Los consumidores españoles, menos familiarizados con las naranjas sanguinas, mostraron preferencia por las mandarinas ligeramente pigmentadas. Una pigmentación muy intensa de la pulpa no resultó atractiva para los consumidores de ninguno de los dos países. La información relacionada con los beneficios para la salud de estas mandarinas tuvo un efecto positivo en la aceptación de los consumidores españoles, mientras que no tuvo un impacto significativo sobre la actitud de los consumidores italianos.

Con el objetivo de profundizar en el conocimiento del consumidor de naranjas y entender el posible efecto del contexto cultural en su comportamiento respecto a la naranja tradicional (blanca) y la naranja sanguina, en el **Capítulo IV** se evaluaron las barreras y motivaciones al consumo, los contextos de consumo y los atributos organolépticos clave para los consumidores de cuatro países (China, España, Italia y México). Los datos obtenidos mostraron que Italia lidera en consumo y disponibilidad de naranjas sanguinas. Las principales motivaciones para consumir ambos tipos de naranja fueron el "*liking*" y las "propiedades saludables", independientemente del país. Sin embargo, se identificaron diferencias importantes en los contextos y barreras al consumo en función de la nacionalidad. Si bien ambos tipos de naranja pueden coexistir en los mercados con amplios niveles de consumo, como ocurre en Italia, para alcanzar este equilibrio se requieren estrategias diseñadas específicamente para cada país.

La información generada en los estudios con consumidores incluidos en la primera parte de esta tesis puede ser de gran utilidad, tanto para los mejoradores de cítricos a la hora de establecer sus objetivos de calidad, como para el sector citrícola a la hora de adaptar la oferta a las preferencias de los consumidores.

La segunda parte de esta tesis, centrada en la fisiología de las naranjas sanguinas, engloba los capítulos V, VI y VII. En el **Capítulo V** se evaluó el perfil de compuestos volátiles de tres variedades de naranja sanguina ('Tarocco Rosso', 'Moro' y 'Sanguinelli') en comparación con tres variedades no sanguinas ('Cadenera', 'Salustiana' y 'Hamlin'). Además, en las variedades sanguinas se compararon muestras de naranjas con pulpa ligera e intensamente pigmentada para determinar si la concentración de azúcares y el perfil de compuestos volátiles dependen del grado de pigmentación. Los resultados mostraron que las naranjas sanguinas no tienen un perfil aromático exclusivo que las distinga de las naranjas no sanguinas. Además, se observó que las muestras de sanguinas intensamente pigmentadas tienen una mayor concentración de azúcares, principalmente sacarosa, así como de ciertos compuestos volátiles (etanol, acetato de etilo, etil-2-metil butanoato y etil-2-butenoato) en comparación con las menos pigmentadas. Estos resultados sugieren que no solo existe una relación metabólica entre los azúcares y las antocianinas, sino que también puede haber una interacción entre las vías biosintéticas de las antocianinas y los compuestos aromáticos.

En el **Capítulo VI**, profundizando en el estudio de los compuestos volátiles de las naranjas sanguinas, se evaluó la evolución del perfil aromático durante las últimas etapas del proceso de maduración de la variedad ‘Tarocco Ippolito’. Esta variedad se seleccionó para su estudio debido al creciente interés comercial que está despertando. Entre los compuestos volátiles identificados se observaron tres patrones de evolución durante la etapa de maduración del fruto: 1) compuestos cuya concentración disminuye, entre los que se encuentran mayoritariamente aldehídos; 2) compuestos cuya concentración aumenta, mayoritariamente ésteres y monoterpenos, y 3) compuestos cuya concentración permanece estable, entre los que se encuentran compuestos relevantes para el aroma de los cítricos como el valenceno y el D-limoneno.

Por último, en el **Capítulo VII** se evaluó la implicación de distintos factores medioambientales (amplitud térmica, exposición al frío y radiación solar) en la acumulación de antocianinas en la naranja sanguina ‘Tarocco Ippolito’. Para ello, durante tres campañas consecutivas se recogieron los datos meteorológicos y se hizo un seguimiento del fruto para determinar el momento en que se inició la síntesis de antocianinas tanto en la piel como en la pulpa. Lo primero que se observó es que la síntesis de antocianinas en la pulpa y la piel de esta variedad no se produce simultáneamente, ya que en la pulpa se inició entre 8 y 15 días antes que en la piel. De los tres factores ambientales evaluados, la exposición al frío se identificó como el factor clave para que la síntesis de antocianinas se inicie. Se evaluaron cuatro modelos de cálculo de requerimientos de frío: Horas Frío, Utah, Utah Positivo y el modelo Dinámico. Los modelos Dinámico y Utah Positivo revelaron que existen unos requerimientos específicos de frío para que se inicie la síntesis de antocianinas. La acumulación de antocianinas en la pulpa de 'Tarocco Ippolito' comenzó tras una acumulación de 4-5 porciones de frío (modelo Dinámico) y aproximadamente 128 unidades de frío (modelo Utah Positivo), mientras que el inicio del proceso en la piel se asoció con ≈ 10 porciones de frío y ≈ 240 unidades de frío.

Los tres estudios presentados en la segunda parte de esta tesis tienen un gran valor científico, ya que suponen un avance importante en el conocimiento de la fisiología de las naranjas sanguinas. Además, conocer los requerimientos de frío para el inicio de la síntesis de antocianinas puede ser de gran ayuda a la hora de decidir las regiones idóneas para el cultivo de la variedad 'Tarocco Ippolito'. La identificación de los modelos más adecuados para ello, abren la puerta a calcular las necesidades específicas de frío de diferentes variedades.

RESUM

En el context actual de creixent competitivitat en els mercats, el sector citrícola espanyol, que és el primer exportador a escala mundial, s'enfronta al repte d'ampliar la seua oferta amb varietats de qualitat superior. Amb tal fi, en el cas de les mandarines, s'està apostant principalment per la introducció d'híbrids triploides, sense llavors, i, en el cas de les taronges, s'ha posat el focus en la reintroducció de varietats sanguines, principalment procedents d'altres països.

Aquesta tesi consta de dos parts. En la primera, es van dur a terme estudis amb consumidors amb l'objectiu de conéixer en profunditat els diferents perfils de consumidors de cítrics, tant espanyols com d'altres països, per poder adaptar així l'oferta a les preferències i necessitats d'aquests. La segona part es centra en la fisiologia de les taronges sanguines, principalment en els processos relacionats amb la síntesi d'aromes i d'antocianines, aquestes últimes responsables del color roig característic d'aquestes varietats.

En el **Capítol I** es presenta un estudi *cross-cultural* orientat a identificar els atributs organolèptics de les mandarines que són clau per a la satisfacció dels consumidors espanyols i italians. Els resultats van mostrar que els consumidors de tots dos països poden dividir-se en dos grups: "consumidors centrats en les percepcions en boca" i "consumidors multisensorials". Atributs com 'dolç', 'equilibri dolç-àcid', 'sabor intens', 'sucós' i 'no fibrós' van ser atributs organolèptics clau per a la satisfacció dels dos grups. A més, per als "consumidors multisensorials" també van ser importants el color de la polpa i atributs relacionats amb la facilitat de consum, com 'fàcil de pelar' i 'sense llavors'. La proporció de "consumidors multisensorials" va ser major a Espanya (59 %) que a Itàlia (48 %).

En el **Capítol II** es va avaluar la utilització de la tècnica CATA (*Check-All-That-Apply*) en combinació amb la descripció del Producte Ideal per seleccionar noves varietats de mandarina obtingudes en el programa de millora de l'IVIA. Per aquest motiu, 134 consumidors de cítrics van avaluar quatre noves varietats ('Pri-88', 'Pri-89', 'Pri-90' i 'Tri-707') en comparació

amb dos dels seus parentals, les varietats ‘Clemenules’ i ‘Oronules’, totes dues d’importància comercial i amb les quals coincideixen en període de recol·lecció. Els resultats van revelar que existeixen dos perfils de preferència entre els consumidors. No obstant això, els dos grups van utilitzar els mateixos atributs per descriure la seua mandarina ideal (‘sabor refrescant’, ‘molt aromàtica’, ‘dolça’, ‘sucosa’, ‘sabor molt intens’, ‘àcida’ i ‘poc fibrosa’), la qual cosa suggereix que l'ús dels atributs està condicionat per les preferències. Entre les noves varietats avaluades, la ‘Pri-89’ i la ‘Tri-707’ van ser les preferides d'un dels perfils de consumidor, ja que van agradar més que la varietat ‘Clemenules’ i, per tant, van suposar una millora de la qualitat organolèptica respecte a aquesta. L'altre grup de consumidors va mostrar preferència per la ‘Pri-90’, varietat que resulta especialment adequada per als consumidors sensibles a l'amargor i a les mandarines amb un cert nivell d'acidesa.

Un dels objectius del programa de millora de mandarines de l'IVIA és obtenir noves varietats de mandarines pigmentades. Aquestes noves varietats són híbrids que s'obtenen mitjançant encreuaments entre taronges sanguines i mandarines tradicionals, i poden presentar una àmplia gamma de coloració de polpa. En el **Capítol III** d'aquesta tesi es va avaluar la influència de la intensitat de pigmentació vermellosa de la polpa d'aquestes varietats (lleugera, mitjana i intensa) en la seua acceptació per part de consumidors espanyols i italians. L'estudi es va basar en un qüestionari en línia amb imatges de mandarines de diferents intensitats de pigmentació. La mitat dels participants de cada país van rebre informació sobre les propietats beneficioses per a la salut que aquestes varietats pigmentades presenten a causa del seu alt contingut en antocianines. L'altra mitat no va rebre aquesta informació. En general, els consumidors italians es van mostrar més receptius a les noves mandarines pigmentades, probablement a causa de la seua familiaritat amb les taronges sanguines, i van mostrar una bona intenció de compra cap a les mandarines de pigmentació lleugera i mitjana. Els consumidors espanyols, menys familiaritzats amb les taronges sanguines, van mostrar preferència per les mandarines lleugerament pigmentades. Una pigmentació molt intensa de la polpa no va resultar atractiva per als consumidors de cap dels dos països. La informació relacionada amb els beneficis per a la salut d'aquestes mandarines va tindre un efecte positiu en l'acceptació dels consumidors espanyols, mentre que no va tindre un impacte significatiu sobre l'actitud dels consumidors italians.

Amb l'objectiu d'aprofundir en el coneixement del consumidor de taronges i entendre el possible efecte del context cultural en el seu comportament cap a la taronja tradicional (blanca) i la taronja sanguina, en el **Capítol IV** es van avaluar les barreres i motivacions al consum, els contextos de consum i els atributs organolèptics clau per als consumidors de quatre països (la Xina, Espanya, Itàlia i Mèxic). Les dades obtingudes van mostrar que Itàlia lidera en consum i disponibilitat de taronges sanguines. Les principals motivacions per consumir els dos tipus de taronja eren el "*liking*" i les "propietats saludables", independentment del país. No obstant això, es van identificar diferències importants en els contextos i barreres al consum en funció de la nacionalitat. Si bé els dos tipus de taronja poden coexistir en els mercats amb amplis nivells de consum, com ocorre a Itàlia, per aconseguir aquest equilibri es requereixen estratègies dissenyades específicament per a cada país.

Tota la informació generada en els estudis amb consumidors inclosos en la primera part d'aquesta tesi pot ser de gran utilitat, tant per als milloradors de cítrics a l'hora d'establir els seus objectius de qualitat, com per al sector citrícola a l'hora d'adaptar l'oferta a les preferències dels consumidors.

La segona part d'aquesta tesi, centrada en la fisiologia de les taronges sanguines, engloba els capítols V, VI i VII. En el **Capítol V** es va avaluar el perfil de compostos volàtils de tres varietats de taronja sanguina ('Tarocco Rosso', 'Moro' i 'Sanguinelli') en comparació amb tres varietats no sanguines ('Cadenera', 'Salustiana' i 'Hamlin'). A més, en les varietats sanguines es van comparar mostres de taronges amb polpa lleugerament i intensament pigmentada per determinar si la concentració de sucres i el perfil de compostos volàtils depenen del grau de pigmentació. Els resultats mostren que les taronges sanguines no tenen un perfil aromàtic exclusiu que les distingisca de les taronges no sanguines. A més, es va observar que les mostres de sanguines intensament pigmentades tenen una major concentració de sucres, principalment sacarosa, així com de certs compostos volàtils (etanol, acetat d'etil, etil-2-metil butanoat i etil-2-butenoat) en comparació amb les menys pigmentades. Aquests resultats suggereixen que no sols existeix una relació metabòlica entre els sucres i les antocianines, sinó que també pot haver-hi una interacció entre les vies biosintètiques de les antocianines i els compostos aromàtics.

En el **Capítol VI**, aprofundint en l'estudi dels compostos volàtils de les taronges sanguines, es va avaluar l'evolució del perfil aromàtic durant les últimes etapes del procés de maduració de la varietat 'Tarocco Ippolito'. Aquesta varietat es va seleccionar per al seu estudi degut al creixent interés comercial que està despertant. Entre els compostos volàtils identificats es van observar tres patrons d'evolució durant l'etapa de maduració del fruit: 1) compostos la concentració dels quals disminueix, entre els quals es troben majoritàriament aldehids; 2) compostos la concentració dels quals augmenta, majoritàriament èsters i monoterpens, i 3) compostos la concentració dels quals roman estable, entre els quals es troben compostos rellevants per a l'aroma dels cítrics com el valencé i el D-limoné.

Finalment, en el **Capítol VII** es va avaluar la implicació de diferents factors mediambientals (amplitud tèrmica, exposició al fred i radiació solar) en l'acumulació d'antocianines en la taronja sanguina 'Tarocco Ippolito'. Per a això, durant tres campanyes consecutives es van arreplegar les dades meteorològiques i es va fer un seguiment del fruit per determinar el moment en què es va iniciar la síntesi d'antocianines tant en la pell com en la polpa. El primer que es va observar és que la síntesi d'antocianines en la polpa i la pell d'aquesta varietat no es produeix simultàniament, ja que en la polpa es va iniciar entre 8 i 15 dies abans que en la pell. Dels tres factors ambientals avaluats, l'exposició al fred es va identificar com el factor clau perquè la síntesi d'antocianines s'inicie. Es van avaluar quatre models de càlcul de requeriments de fred: Hores Fred, Utah, Utah Positiu i el model Dinàmic. Els models Dinàmic i Utah Positiu van revelar que existeixen uns requeriments específics de fred perquè s'inicie la síntesi d'antocianines. L'acumulació d'antocianines en la polpa de 'Tarocco Ippolito' va començar després d'una acumulació de 4-5 porcions de fred (model Dinàmic) i aproximadament 128 unitats de fred (model Utah

Positiu), mentre que l'inici del procés en la pell es va associar amb ≈ 10 porcions de fred i ≈ 240 unitats de fred.

Els tres estudis presentats en la segona part d'aquesta tesi tenen un gran valor científic, ja que suposen un avanç important en el coneixement de la fisiologia de les taronges sanguines. A més, conéixer els requeriments de fred per a l'inici de la síntesi d'antocianines pot ser de gran ajuda a l'hora de decidir les regions idònies per al cultiu de la varietat 'Tarocco Ippolito'. La identificació dels models més adequats per a això, obrin la porta a calcular les necessitats específiques de fred de diferents varietats.

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I. INTRODUCTION

I. INTRODUCTION

I.1. Citrus Fruit Production

Citrus fruits, grown in many tropical and subtropical regions of both hemispheres, are among the world's most consumed and economically important crops. World citrus production is estimated at more than 160 million tonnes (FAO, 2022). The main citrus-producing countries are China, Brazil, India, Mexico, Spain and the United States (Table 1). Oranges account for 45.9% of total citrus production, followed by mandarins with 26.6%. Lemons and limes contribute 12.9%, while grapefruit accounts for 5.9% of world citrus production (FAO, 2022).

Table 1. Main citrus-producing countries in the world (Source: FAO, 2022).

	Citrus production (tonnes)
China	48,321,259.6
Brazil	19,732,041.9
India	14,756,000
Mexico	9,317,400.52
Spain	5,558,090
United States	5,112,415.58
Egypt	4,750,806.7
Türkiye	4,710,808
Iran	4,378,959.34
Nigeria	4,192,296.09
Argentina	3,387,833.88
Italy	3,094,430

Spain ranked 5th in world citrus production in 2022 (Table 1). It is the main exporter on the world citrus market. In the 2023-2024 season, it exported 2,342,298 tonnes, with the main destinations within the European Union being Germany, France, the Netherlands and Poland. The main export markets outside the EU included countries such as the United Kingdom, Norway, Switzerland and Canada (MAPA, 2024).

The total area planted with the different citrus species in Spain is of around 300,000 ha (FAO, 2022). Of this area, 48.8% is devoted to oranges, 32.7% to mandarins, 17.6% to lemons and limes, 1.1% to grapefruit, and 0.25% to other citrus species (FAO, 2022).

Citrus production in Spain is concentrated in the Mediterranean region. The Valencian Community has the largest share of the national area under citrus fruit production, with 52.9%, followed by Andalusia with 29.6% and the Region of Murcia with 13.3% (MAPA, 2024). As a result, the Valencian Community is the most important citrus-producing region in Spain, accounting for 48.8% of the country's total production (MAPA, 2024).

Of the citrus fruits produced in the Valencian Community, mandarins constitute approximately 42% of production while sweet oranges account for around 45% (Conselleria d'Agricultura, Ramaderia i Pesca, 2024).

I.2. Mandarins

I.2.1. Varietal Range and Its Expansion

In Spain, mandarins, which are harvested from late August to mid-May, represent the citrus group with the widest range of varieties (Figure 1). The main mandarin variety groups traditionally grown in Spain are satsumas (*Citrus unshui*) and clementines (*Citrus x clementina*). Satsumas include ‘Hashimoto’, ‘Owari’, ‘Clausellina’, ‘Okitsu Wase’ and ‘Iwasaki’. The main clementine varieties include ‘Fina’, ‘Clemenules’, ‘Arrufatina’, ‘Oroval’, ‘Marisol’, ‘Oronules’, ‘Clemenrubí’, ‘Clemensoon’ and 'Hernandina'.

In recent years, increasing market competitiveness due to the entry of third countries such as Morocco, South Africa, and Egypt, among others, has made the marketing of high-quality mandarin varieties a priority for the citrus sector. This challenge has been addressed by cultivating new superior-quality varieties developed in other countries, such as ‘Orri’ from Israel, as well as through the development of new hybrids and mutations via national breeding programs.

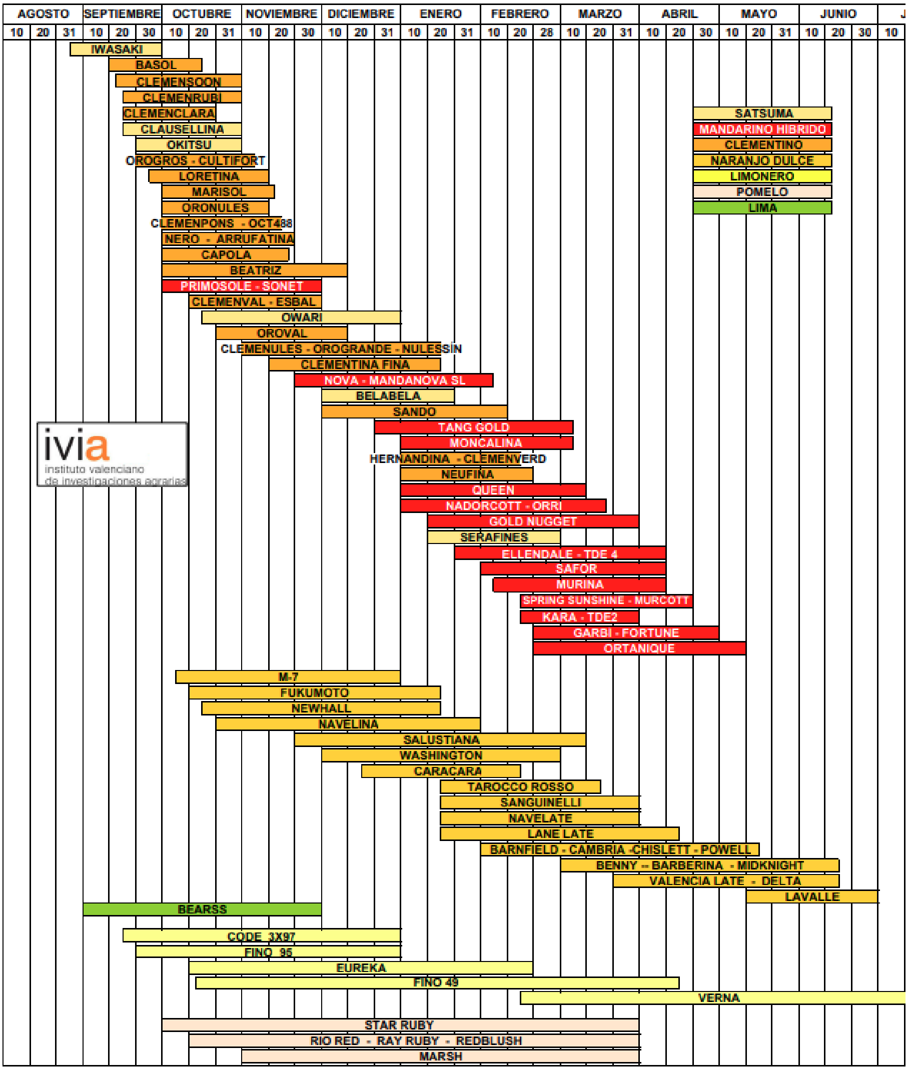

Figure 1. Harvesting periods of citrus varieties grown in Spain (Source: IVIA, 2012).

In this line, a breeding programme has been established at the Valencian Institute for Agricultural Research (IVIA, Spain) with the main objectives of obtaining varieties that are seedless, have late or early ripening, are resistant to pathogens and are of superior organoleptic quality. Varieties such as ‘Garbí’ and ‘Safor’ have been obtained through this programme (Aleza *et al.*, 2010; Cuenca *et al.*, 2010). The programme is still ongoing and new pre-selected varieties are currently in the final stages of evaluation.

In addition to having a good agronomic performance, any newly released cultivar intended to replace or compete with the cultivars already available on the market must be of comparable or superior quality for consumers (Hampson *et al.*, 2000). Consumer studies are key to determine to what extent new varieties meet this requirement. Thus, sensory studies performed with consumers investigate the extent to which the new pre-selected varieties have their own characteristics that differentiate them from those already on the market, and whether consumers will like them less, the same, or more. These studies have begun to be carried out at IVIA and are providing valuable information for the breeding programme (Tarancón *et al.*, 2020).

In addition to the objectives mentioned above, the IVIA's breeding programme has for some years been aimed at obtaining pigmented mandarins by crossing blood oranges with traditional mandarins. To date, a large number of this type of mandarins have been obtained and are being evaluated for their agronomic performance.

This objective had also been pursued by other breeding programmes and some varieties had already been developed. The main one is the ‘Mandared’ variety developed by the *Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria* (CREA, Italy). This variety was obtained by crossing the clementine ‘Clemenules’ and the blood orange ‘Tarocco’ (Barry *et al.*, 2020) and has been marketed under the names of ‘Red Nules’ and ‘MandaRosa’. Other examples are the varieties ‘Tacle’ and ‘Alkantara’, obtained from the same breeding programme, although their presence in the market is less prominent (Recupero *et al.*, 2005; Russo *et al.*, 2016). None of these varieties are currently being marketed in the Spanish market.

I.2.2. Organoleptic Characteristics of Mandarins

Mandarins are highly valued in the marketplace for their diverse sensory attributes. Notably, flavour is a critical factor, characterised by an optimal balance of sugars and acids. In addition, studies indicate that they have characteristic organoleptic properties that differentiate them from oranges (Goldenberg *et al.*, 2017).

Mandarins are generally considered more convenient to eat than oranges. Their natural segmentation into easily separable sections allows consumers to enjoy them without the need for utensils or cutting (Tarancón *et al.*, 2021a). In addition, the easy-to-peel rind of most varieties allows quick access to the fruit, making them an ideal choice for on-the-go snacking (Tarancón *et al.*, 2020).

Another desirable feature of this fruit is the absence of seeds, this is why the breeding of seedless varieties through techniques such as triploidy and gamma irradiation has been one of the main objectives of breeding programmes (Ali *et al.*, 2013). The breeding of numerous seedless varieties in recent years has been an important achievement as seedlessness eliminates the need to remove seeds, making mandarins a perfect snack for both children and adults.

Therefore, not only flavour but also convenience attributes like easy-peeling and seedlessness have become increasingly important in determining consumer satisfaction and preference, as the demand for fruit that is not only tasty but also easy to consume has grown with busy lifestyles. Moreover, recent studies demonstrate that fruit appearance also plays a key role in consumer choice (Morales *et al.*, 2021; Tarancón *et al.*, 2021b). These studies show that not only rind colour but also flesh colour have a significant impact on purchase intention.

Knowing that the pulp colouration influences consumer response and given that the new pigmented mandarin varieties have a flesh colouration very different from what consumers are accustomed to, it is necessary to conduct studies to understand how the colouration of these new mandarins affects their acceptance by consumers.

I.3. Sweet Oranges

I.3.1. Varietal Range and Its Expansion

Sweet oranges (*Citrus sinensis* [L.] Osbeck), which are harvested from late September to mid-to-late June, are the citrus group with the second largest varietal range in Spain (Figure 1). The main varieties grown in Spain can be divided into three groups: blond, navel and blood oranges.

Blond varieties, which are of great commercial importance, include 'Salustiana', 'Valencia Late', 'Valencia Delta Seedless' and 'Valencia Midknight'. Navel varieties include early varieties ('Navelina', 'Newhall' and 'Washington Navel') and late varieties (mainly 'Navelate' and 'Lane Late'). The 'Cara Cara' variety, whose flesh has a pinkish colour due to the presence of lycopene, also stands out in this group (Simons *et al.*, 2019). Blood oranges, also known as pigmented oranges, are characterised by their anthocyanin content, which gives them a distinctive reddish colouration (Barry *et al.*, 2020). Although blood oranges were once quite popular in Spain, their cultivation declined, probably due to difficulty of achieving consistent colouring in some seasons (Hodgson, 1967). Recently, however, there has been a resurgence of interest in introducing these varieties to the market to broaden the range of varieties available with these characteristically coloured varieties.

Historically, the most well-known blood orange variety in Spain was 'Sanguinelli', which originated in Castellón (Spain) as a mutation of the 'Doble Fina' variety (Hodgson, 1967), alongside other appreciated varieties like 'Tarocco Rosso' introduced from Italy (Cebadera-Miranda *et al.*, 2019; Morales *et al.*, 2021; Melgarejo *et al.*, 2023). In 2021, blood oranges represented only 0.3% of the total area planted with oranges in Spain (MAPA, 2021), but the increasing interest has led to the introduction of newer varieties from Italy, such as 'Moro' and 'Tarocco Ippolito', which are also gaining in popularity (Cebadera-Miranda *et al.*, 2019; Legua *et al.*, 2021; Morales *et al.*, 2021).

For the successful introduction of blood orange varieties, it is necessary to know their behaviour, particularly with regard to anthocyanin accumulation, as the lack of colour has made their cultivation difficult in some countries (Butelli *et al.*, 2012). Currently, the IVIA maintains a germplasm bank of blood orange cultivars, which serves as a valuable resource for evaluating the performance of different cultivars and improving our understanding of their physiology.

As a significant part of the research in this thesis is dedicated to blood oranges, an overview of the current state of knowledge on this topic is provided below.

I.3.2. Blood Oranges

I.3.2.1. Origin and Global Spread of Blood Oranges

The origin of blood oranges has been the subject of much debate. The first written references mentioning a red-fleshed orange fruit date from the 17th century and are attributed to an Italian botanist (Caruso *et al.*, 2016). These varieties were originally thought to have evolved from a bud mutation in blond Mediterranean oranges. However, more recent studies suggest that they may have originated in southeast Asia or China and were later imported to Europe via trade routes (Seminara *et al.*, 2023).

Once introduced to Europe, blood oranges were initially cultivated for their ornamental appeal. Over time, their use shifted to production for fresh consumption. Commercial cultivation was first established in Italy, where it has been documented since the early 19th century, when growers began to make clonal selections to improve characteristics such as fruit size, firmness, and especially flesh pigmentation (Catara *et al.*, 2015).

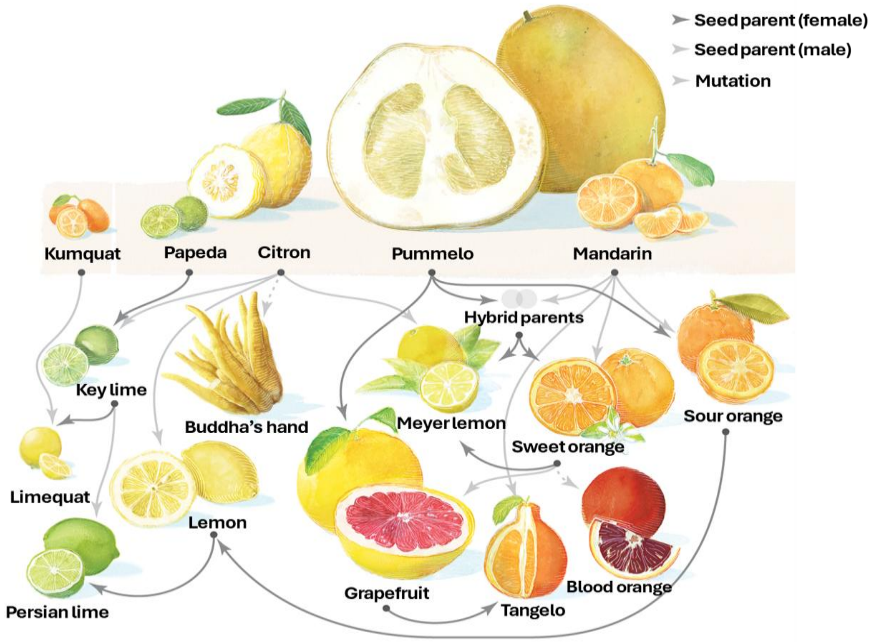

Figure 2. The Citrus Family Tree (Source: National Geographic).

Since their appearance in the Mediterranean area, blood oranges have been diversified into three main groups. The first group includes three varieties from Sicily (Italy) ('Sanguinello', 'Moro', and 'Tarocco') and the 'Maltese Sanguigno', which is of uncertain origin but was probably developed in Malta before being introduced into various regions of North Africa (Barry *et al.*, 2020). The second group is derived from the Spanish 'Doble Fina' type, from which several selections have been made. Notably, the 'Sanguinelli' variety, which is not the same as the Sicilian 'Sanguinello', was identified in 1929 in Castellón (Spain) as a bud mutation of the 'Doble Fina' and became popular because of its significantly enhanced pigmentation in both flesh and rind compared to the original. Lastly, the third group consists of 'Shamouti' or 'Palestine Jaffa' blood oranges, which includes a few varieties like 'Shamouti Maouardi' and 'Maouardi Beladi', characterized by traits similar to the slight coloured 'Shamouti', but differing in fruit pigmentation (Hodgson, 1967; Butelli *et* *al.*, 2012; Barry *et al.*, 2020).

The variety 'Tarocco', which originated as a bud mutation of 'Sanguinello' in Sicily, is probably the most widespread of the blood varieties, mainly due to the large number of vegetative mutations and clones derived from it. As a result, nowadays we can find diverse 'Tarocco' cultivars with different pigmentation degrees and ripening times such as 'Dalmuso', 'Gallo', 'Ippolito', 'Lempso', 'Meli', 'Messina', 'Rosso', 'Sant' Alfio', 'Sciara', 'Scirè', 'TDV' and 'Vigo' (Cebadera-Miranda *et al.*, 2019; Fabroni *et al.*, 2020; Strano *et al.*, 2021; Forner-Giner *et al.*, 2023; Seminara *et al.*, 2023).

Today, blood oranges are cultivated in various countries outside of Europe, including Brazil, China, and the United States (Magalhães *et al.*, 2019; Simons *et al.*, 2019; Chen *et al.*, 2023). All varieties currently grown worldwide trace their origins directly or indirectly to the Mediterranean. However, in China, the 'Jingxian' variety is noteworthy as the only blood orange of non-Mediterranean origin, although it has not gained popularity due to its limited quality and seed content (Butelli *et al.*, 2012; Rapisarda *et al.*, 2022).

The current worldwide expansion of blood varieties is largely due to the fact that over the last two decades, several studies have linked anthocyanin content to health benefits (Grosso *et al.*, 2013; Li *et al.*, 2017; Vendrame and Klimis-Zacas, 2019; Solverson, 2020), attracting renewed attention from the citrus industry and scientists alike.

I.3.2.2. Anthocyanin Accumulation in Blood Oranges

The intriguing reddish colour of blood oranges is due to their accumulation of anthocyanins (Lo Piero, 2015; Fallico et al., 2017). The production of these compounds in this group of oranges is attributed to the Ruby transcription factor, which not only regulates the expression of structural genes essential for anthocyanin production, but also interacts with other regulatory factors. This interaction forms a regulatory system that coordinates the activation of genes involved in anthocyanin synthesis (Chen *et al.*, 2015; Catalano *et al.*, 2020). Recent studies suggest that these varieties have inherited this transcription factor from the various hybridisations that have been conducted on citrus ancestors (Figure 2), with some studies identifying the pummelo as the primary contributor to this trait (Butelli *et al*., 2012, 2017; Lu *et al*., 2022).

Recent studies have shown that all sweet oranges have the structural and regulatory genes necessary to produce anthocyanins. However, the regulatory genes are inactive in non-pigmented oranges. In blood oranges, on the other hand, both types of genes, structural and regulatory, are active allowing anthocyanin production (Cotroneo *et al.*, 2006; Butelli *et al.*, 2012; Huang *et al.*, 2018; Catalano *et al.*, 2020).

The process of anthocyanin accumulation in blood oranges occurs in both the flesh and the rind, but not always in a balanced way (Figure 3). There are varieties with intense internal colouring but slight rind colouring, and vice versa. There are also varieties with intense or slight pigmentation in both the flesh and the rind (Cebadera-Miranda *et al.*, 2019; Forner-Giner *et al.*, 2023). So, anthocyanin accumulation depends on the genotype, being different among varieties (Bernardi *et al.*, 2010; Legua *et al.*, 2021; Modica *et al.*, 2024).

Cultivar	Fruit Rind	Fruit flesh
'Violeta de los Valles'		
'Tarocco Messina'		
'Sanguinelli'		

Figure 3. Blood orange varieties with different rind-flesh pigmentation ratios grown in the IVIA germplasm bank under the agro-climatic conditions of Valencia (Spain).

Anthocyanins in blood oranges are synthesised through the flavonoid pathway, which involves a series of enzymatic reactions (Figure 4). Synthesis begins with the conversion of phenylalanine to cinnamic acid, catalysed by phenylalanine ammonia-lyase (PAL). Cinnamic acid is then converted to naringenin by several enzymes, including chalcone synthase (CHS) and chalcone isomerase (CHI). Anthocyanidins are formed from leucoanthocyanidins through the action of anthocyanidin synthase (ANS). Finally, anthocyanins are modified and stabilised by glycosylation and acylation, which contributes to the structural diversity of these pigments in blood oranges (Cotroneo *et al.*, 2006; Chen *et al.*, 2015; Lo Piero, 2015; Carmona *et al.*, 2017).

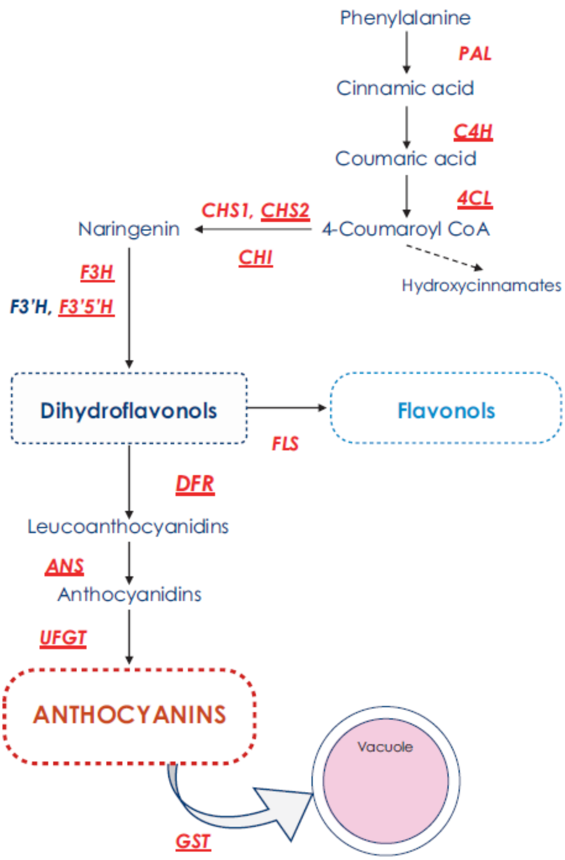

Figure 4. Anthocyanin biosynthetic pathway (Source: Carmona *et al.*, 2017).

The major anthocyanins that play an important role in the colour profile and health benefits of blood oranges include cyanidin-3-O-glucoside, delphinidin-3-O-glucoside, cyanidin-3-(6”malonyl)-O-glucoside and cyanidin-3-(6”dioxalyl)-O-glucoside. Other important anthocyanins are cyanidin 3,5-diglucoside, cyanidin 3-(feruloyl) glucoside, cyanidin 3-(p-coumaroyl) glucoside and delphinidin 3-(diferuloyl) (Cebadera-Miranda *et al.*, 2019; Rapisarda *et al.*, 2022; Forner-Giner *et al.*, 2023; Habibi *et al.*, 2023). Pigmented mandarins have an anthocyanin profile very similar to that of blood oranges, as they are hybrids obtained by crossing blood oranges with common mandarins (Rapisarda *et al.*, 2003, 2008, 2009).

In addition to the intrinsic characteristics of each variety, the environment in which the trees grow is known to play a key role in the accumulation of anthocyanins (Li and Ahammed, 2023). It is believed that the temperature difference between day and night, or thermal amplitude, play a significant function in the accumulation of these pigments in blood oranges, and various authors have suggested this in the introductions of some of their scientific papers (Zarbà and Pulvirenti, 2006; Lo Piero, 2015; Caruso *et al.*, 2016). However, there is no empirical evidence for this, since no scientific studies have experimentally monitored how this day-night temperature range affects the anthocyanin production of the fruit while on the tree.

What has been well demonstrated through post-harvest studies is that exposure to cold plays a key role in enhancing the expression of the Ruby transcription factor, which subsequently increases the synthesis of anthocyanins. So, it has been observed that storing the fruit at low temperatures promotes the accumulation of anthocyanins (Butelli *et al.*, 2012; Carmona *et al.*, 2017, 2020, 2022; Chen *et al.*, 2023). This cold dependency of anthocyanins, first observed in Mediterranean varieties, was also found to be a trait of the Chinese origin variety ‘Jingxian’ (Butelli *et al.*, 2012).

Other factors such as light have also been shown to be important determinants of anthocyanin production. Intense light exposure can promote the upregulation of structural and regulatory genes involved in anthocyanin synthesis (Zhang *et al.*, 2018). In this regard, Huang *et al.* (2019) showed that the use of bags on blood oranges during their development on the tree inhibited anthocyanin biosynthesis in the fruit rind, while the accumulation process in the flesh was not affected.

While the above-mentioned studies provide enough information to be sure that cold and sun exposure are involved in the accumulation of anthocyanins, at a practical level it is not known what environmental conditions must be present for the fruit to develop the expected colouration. This means that in some areas where blood oranges have recently been introduced, they cannot be consolidated because the fruit does not develop enough colour in certain seasons (Butelli *et al.*, 2012). It is therefore necessary to further investigate the role of environmental factors in anthocyanin development.

I.3.2.3. Organoleptic Characteristics of Blood Oranges

The organoleptic characteristics of citrus fruit, like those of other fruits, can be divided into those that can be perceived by sight at the time of purchase and those that can be perceived when the fruit is eaten.

Among the external characteristics, the red colour of blood oranges is an organoleptic feature that makes them recognisable and can play a key role in their purchase. Internally, what we perceive as 'flavour' is given by the taste (sweet, sour and bitter in the case of fruit) and the aroma. It should be also noted that the texture of the fruit contributes to the overall perception when eating it, and in the case of citrus fruits, the amount of juice is key to the perception of juiciness (Goldenberg *et al.*, 2015, 2017; Tarancón *et al.*, 2020).

Blood orange varieties are not only highly variable in terms of colour, but also in other external parameters such as size and physicochemical characteristics related to flavour. The variability among the different blood orange varieties has been observed in several studies, as in the case of Forner-Giner *et al.* (2023), who after characterising eight blood orange varieties, 'Sanguinelli', 'Moro' and six 'Tarocco' cultivars grown in an experimental orchard in Orihuela (Spain), observed that, at the same maturity stage, fruit diameter ranged from 70.1 mm in 'Tarocco Scirè' to 86.4 mm in 'Tarocco Dalmuso'. In the same line, Caruso *et al.* (2016) evaluated eighty-eight clones from the CREA (Italy) germplasm bank, with genotypes belonging to the 'Moro', 'Sanguigno', 'Sanguinello' and 'Tarocco' groups, and found that at the same time of harvest, the TSS ranged from 10.59 °Brix to 13.19 °Brix and the acidity ranged from 0.70% to 1.70% in the 'Tarocco' group.

It is worthy to comment that in Spain, the quality requirements for exportation of blood oranges to the UE are regulated by the UNECE standards, in which a minimum juice yield of 30% and maturity index of 6.5 are required. A minimum size of 53 mm is also required for oranges (UNECE, 2023).

In terms of aroma, the aroma of sweet oranges is mainly formed by monoterpenes, sesquiterpenes, hydrocarbons, alcohols, aldehydes, esters and ketones (Pan *et al.*, 2023). Among these, D-limonene has been identified as the most abundant aromatic compound in oranges (Rodríguez *et al.*, 2017; Kvittingen *et al.,* 2021). The aroma of oranges compared to other citrus fruits seems to be characterized by its high content of esters and certain aldehydes. Thus, Feng *et al.* (2017), when comparing the aromatic profile of oranges and mandarins, observed that oranges are richer in esters that contribute to their fruity aroma, as well as some aldehydes such as octanal and decanal. Similarly, González-Mas *et al.* (2011) found that oranges have a higher diversity and concentration of volatile compounds compared to other citrus fruits. This includes a large number of esters and aldehydes, which contribute to a more intense and complex fruit aroma.

With regard to blood oranges' aroma, research has suggested that they have a unique volatile composition that sets them apart from blond oranges (Näf *et al.*, 1996; Moufida and Marzouk, 2003). The findings from these studies primarily involved comparisons between a single variety of blood orange versus a blond variety (Näf *et al.*, 1996), or with other types of citrus fruits (Moufida and Marzouk, 2003). Similarly, Arena *et al.* (2006) reported that when analysing the volatile profiles of two blood orange varieties alongside two blond varieties, specific volatile compounds were found in odour-active concentrations exclusively in the blood oranges. As it has been shown that volatile profiles can vary significantly between different varieties of the same species and group, studies with a larger number of varieties are needed to reach a conclusion on this point (González-Mas *et al.*, 2011; Ren *et al.*, 2015; Sdiri *et al.*, 2017).

On the other hand, in a recent study on blood orange 'Sanguinelli', Tarancón *et al.* (2022) found that there is a certain relation between pigmentation intensity of rind and flesh. Thus, the authors reported that at harvest time the more intense the rind pigmentation was, the more intense the internal pigmentation and the higher the total soluble solids. Moreover, through a blind taste test, the researchers demonstrated that consumers were able to differentiate between fruit with intense pigmentation and those with slight pigmentation. This observation prompts the question of whether the perceived differences are solely linked to variations in total soluble solids content, or if aroma compounds might also play a role in shaping distinct flavour perceptions.

These results highlight the need to further investigate the relationship between anthocyanin accumulation and other physico-chemical parameters responsible for sensory characteristics of blood oranges.

I.4. Consumer Studies as a Key Tool to Guarantee Successful Expansion of Varietal Range in Markets

I.4.1. Sensory Studies for Selecting New Varieties

Traditionally, the quality assessment of varieties introduced from other countries or new varieties obtained in breeding programmes has focused on the main physico-chemical parameters. However, the organoleptic characteristics perceived by consumers, which are critical for market acceptance, depend on a multitude of interacting factors that are difficult to quantify through analytical methods alone. Because of that, in the last 20 years sensory studies have become increasingly important for evaluation of new varieties.

Initially, the organoleptic characterisation of new varieties was carried out using sensory trained panels (Hampson, 2000; Vaysse *et al.*, 2005; Wismer *et al.*, 2005; Oraguzie *et al.*, 2009). Although this methodology can generate reliable data, the information may not always resonate with consumers or address their specific needs. Moreover, maintaining a trained panel is expensive and requires a lot of time to train the panelists. Therefore, in recent years, sensory science has made significant research efforts to develop methods for evaluating sensory properties without the need to train panelists. These “rapid” methods have the advantage of saving time and money, but also that they can be used to assess consumer perception. Most of these techniques are based on questions or tasks that are simple and easy-to-understand for the evaluator and the use of specific statistical tests to deal with the variability on consumers assessment. Many techniques and versions of techniques have been proposed, such as Check-All-That-Apply (CATA) questions, Flash Profile (FP), Free Choice Profile (FCP) or Projective Mapping (Tárrega, 2022). These new methodologies have already been successfully used for the characterisation of new citrus varieties (Castro *et al.*, 2013; Tarancón *et al.*, 2020) as well as other types of fruits and vegetables (Lado *et al.*, 2010; Laurie *et al.*, 2012; Rocha *et al.*,2013; Swegarden *et al.*, 2019). In addition, these studies often address not only the characterisation of properties, but also the degree of acceptance of the new varieties, allowing us to know whether they like them less, the same or more than the sensory-reference varieties, i.e., those already available on the market.

Among these sensory methods involving consumers, the CATA question has gained significant relevance in recent years. In this technique, the consumer is asked to describe the sensory properties of the product by selecting all the terms that apply to the sample from a list of terms. Using CATA questions in sensory evaluations with consumers has proven to be an effective way of profiling new fruit varieties, yielding insights of considerable commercial value (Lado *et al.*, 2010, 2021; Tarancón *et al.*, 2020). Additionally, several studies have highlighted the effectiveness of using CATA questions alongside Ideal Product (IP) descriptions to refine the formulations of developed products and improve their sensory profiles, aligning them more closely with consumer preferences (Ares *et al.*, 2014). In this method, consumers first respond to the CATA questions for each sample. They are then asked to use the same list of attributes to describe their ideal product. This practice aims to identify differences between product characteristics and consumers' ideal product, thus facilitating reformulation.

I.4.2. The Role of External and Cultural Factors in Consumer Response

Several studies have highlighted that certain factors not directly related to the sensory properties of a product play a significant role in influencing consumer acceptance and purchase intention. These factors, often referred to as credence attributes, represent pieces of information provided by sellers that consumers cannot verify at the time of purchase nor once consumed. Examples of credence attributes include claims of environmental friendliness, certifications for organic products, water-saving efforts, sustainability practices, health benefits and guarantees regarding food safety (Darby and Karni, 1973; Wessells, 2002; Carlsson *et al.*, 2007; Wilcock and Ball, 2014; Holland, 2016; Lee and Hwang, 2016).

The degree of influence of certain credence attributes may be closely related to the cultural background of the consumer (Bonne and Verbeke, 2008; Heiman *et al.*, 2019), since it plays a significant role in shaping individuals' perceptions of food, which in turn influences their willingness to try and accept certain products (Jeong and Lee, 2021). For example, consumers from cultures that prioritise healthy eating may be more inclined to purchase products that align with their values, while those from other backgrounds may not prioritise these attributes as highly (Goto *et al.*, 2014; Levine *et al.*, 2016).

Another important cultural factor that can influence consumer response is familiarity with the product. Familiarity can significantly reduce uncertainty and help align consumer expectations with product attributes. When consumers are familiar with a type of food, they are more likely to trust its properties, which makes them more willing to try it. This is particularly relevant when considering new food products that come with novel attributes or claims that may be unfamiliar to the consumer base (Onwezen and Bartels, 2011; Jeong and Lee, 2021).

In the current context of globalisation, where Spanish citrus fruits are exported to different regions of the world, understanding the influence of cultural background becomes particularly important when assessing export opportunities. Different markets may have unique food preferences and perceptions, influenced by local traditions, eating habits and values (Ajetunmobi and Laobangdisa, 2024). For example, a market that places a high value on organic products may be more receptive to Spanish citrus bearing such certification, while another market may prioritize price or aesthetic appeal.

It is therefore essential to conduct research that takes these cultural factors into account. Understanding the cultural nuances that drive consumer behaviour allow to design more informed and effective marketing strategies, fostering greater consumer engagement and loyalty. By tailoring marketing strategies that resonate with the specific values and preferences of different consumer groups, companies can increase the likelihood of acceptance of their products in new markets.

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II. OBJECTIVES

II. OBJECTIVES

The general objective of this thesis is to obtain new knowledge that helps to improve the quality of citrus fruits in market, by identifying key factors for consumer satisfaction and by advancing the understanding of physiological processes linked to organoleptic quality.

To this end, the following specific objectives were pursued:

To understand the preferences of citrus fruit consumers:

- To identify the aspects and attributes that act as drivers of liking of mandarin and orange consumers.
- To evaluate the impact of flesh colour of new pigmented mandarin varieties on consumer acceptance.
- To determine the extent to which credence attributes and cultural background influence consumers' response.

To deepen the knowledge of the physiology of blood oranges:

- To determine if there is a specific volatile profile of blood oranges and explore a potential relationship between anthocyanin and volatile accumulation.
- To determine the changes in the profile of volatile compounds associated with the ripening of blood oranges.
- To determine the role of environmental factors (chilling, solar radiation and thermal amplitude) in the synthesis of anthocyanins in blood oranges.

III. THESIS OUTLINE

III. THESIS OUTLINE

The research carried out in this thesis has resulted in different scientific publications in line with the stated objectives, and the results are divided into two distinct sections:

The first section includes consumer studies that provide valuable information to help mandarin breeding programmes define their objectives and to help the citrus sector adapt commercial strategies that contribute to the successful marketing of pigmented citrus fruits. The following scientific publications are collected in this section:

- Giménez-Sanchis, A., Farina, V., Tarancón, P. and Besada, C. (2023). How to Satisfy Consumer Expectations for Mandarin? A Cross-Cultural Study in Spain and Italy. *Acta Horticulturae*, *1364*, 395-402. https://doi.org/10.17660/ActaHortic.2023.1364.50 **[Chapter I]**

- Giménez-Sanchis, A., Tárrega, A., Tarancón, P., Aleza, P., & Besada, C. (2021). Check-All-That-Apply Questions Including the Ideal Product as a Tool for Selecting Varieties in Breeding Programs. A Case Study with Mandarins. *Agronomy*, *11*(11), 2243. https://doi.org/10.3390/agronomy11112243 **[Chapter II]**

- Giménez-Sanchis, A., Pons-Gómez, A., Farina, V., & Besada, C. (2022). Effect of Pulp Pigmentation Intensity on Consumer Acceptance of New Blood Mandarins: A Cross-Cultural Study in Spain and Italy. *Agronomy*, *12*(12), 3058. https://doi.org/10.3390/agronomy12123058 **[Chapter III]**

- Giménez-Sanchis, A., Zhong, K., Pintor, A., Farina, V., & Besada, C. (2022). Understanding Blood versus Blond Orange Consumption: A Cross-Cultural Study in Four Countries. *Foods*, *11*(17), 2686. https://doi.org/10.3390/foods11172686 **[Chapter IV]**

The second section focuses on the physiology of blood oranges, trying to understand the relationship between anthocyanin accumulation and volatile compounds and the influence of different environmental factors in anthocyanin synthesis. This second part includes the following scientific publications:

- Giménez-Sanchis, A., Bermejo, A., & Besada, C. (2024). Changes in the Sugars and Volatile Compounds Profiles Associated with Anthocyanin Accumulation in Oranges: Blood vs. Blond varieties, and Slightly Pigmented vs. Intensely Pigmented Blood Fruit. *Food Research International*, *197*, 115199. https://doi.org/10.1016/j.foodres.2024.115199 **[Chapter V]**
- Giménez-Sanchis, A., & Besada, C. Volatile Organic Compounds Profile of 'Tarocco Ippolito' Blood Orange: Changes Associated with the Final Phases of Ripening. Submitted to *International Journal of Fruit Science*. **[Chapter VI]**
- Giménez-Sanchis, A., Bonet, L., Bermejo, A., Pérez-Pérez, J. G., & Besada, C. Determination of Chilling Requirements for Anthocyanin Accumulation in Blood Orange 'Tarocco Ippolito'. Submitted to *Journal of Agriculture and Food Research*. **[Chapter VII]**

IV. RESULTS

IV.1. UNDERSTANDING THE PREFERENCES OF CITRUS FRUIT CONSUMERS

CHAPTER I

How to Satisfy Consumer Expectations for Mandarin? A Cross-Cultural Study in Spain and Italy

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Abstract

Determining consumer quality requirements is needed to provide markets with high quality fruit, which helps to reduce fruit waste both at home and at points of sale. This cross-cultural study investigated how important are different mandarin attributes in the repurchase intention of Spanish and Italian consumers. 450 consumers from each country participated in an online survey where they scored mandarin attributes according to their importance. Cluster analysis revealed that in both countries consumers can be divided into two groups: “Consumers focused on mouth perceptions” and “Multisensory consumers”. Among Spanish participants, 41% belonged to the first group and 59% to the second one, while in Italy 52% of consumers belonged to the first group and 48% to the second. “Consumers focused on mouth perceptions” reported that the most important attributes that mandarins should have to buy them again are: ‘sweet’, ‘balance sweet-acid’, ‘intense flavour’, ‘juicy’ and ‘not fibrous’. For this group of consumers, other attributes that are mainly perceived with the hands and sight (easy peeling, not messy when eating, pulp colour) and ‘seedless’ were not important aspects. “Multisensory consumers” stated that all the attributes mentioned above that are related to taste and texture perceived in mouth are very important. Moreover, they reported that characteristics like easy peeling, seedless or pulp colour are also relevant. The less important attribute for both groups of consumers was ‘not messy when eating’. In addition to the percentage of participants in each cluster, the main differences among Italian and Spanish consumers were that in general Italian consumers gave more importance to ‘aromatic’ and ‘pulp colour’ attributes and less importance to ‘not fibrous’ than the Spanish ones.

Keywords: consumer; mandarin; cluster; attributes; cross-cultural; purchase

1. Introduction

Among fruit species, citrus fruit represents the major fruit tree crop in the world, with a global estimated production close to 158 million tonnes. In terms of quantity, oranges are the most widely produced and traded citrus fruit. However, production of mandarins has increased by around 60% in the last 10 years, while orange production has remained relative stable. Thus, mandarin consumption has experienced the most significant increase among citrus cultivars in the last decade (FAO, 2022).

Due to the growing consumer interest in mandarins, several breeding programmes have been established worldwide in citrus fruit-producing countries. In many cases, these programs have focused on extending the commercial season by obtaining early or late season cultivars (Smith, 2014; Navarro *et al.*, 2015). In parallel to these agronomic objectives, obtaining new cultivars that satisfy consumer convenience and sensory quality requirements has always been on the minds of breeders. Clear examples of convenience-based breeding are obtaining seedless (Ali *et al.*, 2013) or easy-peeling cultivars (Yu *et al.*, 2021).

The success of the new cultivars will depend on the extent to which they satisfy and meet consumer expectations. If fruit from new cultivars satisfies consumers, the likelihood of repurchase will increase resulting in higher consumption. Therefore, determining consumer quality requirements is needed so that breeding programs can establish their quality objectives on a sound basis. Moreover, providing markets with high quality fruit is essential to reduce fruit waste both at the point of sale and at home.

Since cultural background has an effect on food acceptability (Laaksonen *et al.*, 2020; Jeong and Lee, 2021), performing cross-cultural studies that allow to compare response of consumers from different countries is necessary to provide both fruit breeders and marketers with essential information (Bonany *et al.*, 2014).

On another note, determining consumer requirements from a sensory and hedonic point of view may also be important to increase fruit consumption for health reasons (Pettigrew, 2016; Lado *et al.*, 2021).

Efforts must be made to increase fruit consumption in Mediterranean countries as a decreasing trend in consumption has been detected in the last decade (Leone *et al.*, 2017).

In this context, this study aims to investigate how important different sensory attributes of mandarins are for consumers' satisfaction and therefore for their intention to repurchase the product. By means of a cross-cultural questionnaire, this question was explored in two different Mediterranean countries, Spain and Italy.

2. Material and Methods

2.1. Consumer Sample

Four hundred and fifty consumers of each nationality, Spain and Italy, participated in this study. Sampling was carried out by members of the two institutions involved in this study (the Valencian Institute for Agricultural Research and the *Università degli Studi di Palermo*). Participants were recruited using three methods: street surveys using a tablet device or a QR that link to the survey, student mailing list of both institutions, and snowball recruitment (word-of-mouth). Consent indicating voluntary participation was obtained from each participant at the beginning of the questionnaire. Only people who reported consuming mandarin fruit at least once a month during the season were invited to participate.

Table 1 shows the demographic information of each nationality. The recruitment of participants was carried out to obtain a similar percentage of responses from young adults (18-30 years old), middle-aged adults (31-50 years old) and late-aged adults (more than 50 years old). Besides, the proportion of men and women was similar in both countries, with a percentage of women of around 60%.

Table 1. Demographic characteristics of Spanish and Italian respondents. Data is expressed in percentages of participants.

		Country	
		Spain	**Italy**
Gender	**Female**	62.1	60.5
	Male	37.9	39.5
Age	**18-30**	31.7	20.7
	31-50	37.5	39.3
	>50	30.7	40

2.2. Survey Design

This study was based on an online questionnaire via the platform Google Forms (www.googleforms.com).

In order to contextualize participants, a picture of mandarins was shown to them before proceeding with the questions. Participants were requested to answer the following question: "Imagine that you are eating a mandarin that you just bought. How important are the following characteristics to buy them again?". They had to rate the importance of certain sensory attributes using a 7-point Likert scale where 1 = not important at all, 7 = very important (Galati *et al.*, 2019). The sensory attributes to evaluate were: 'being seedless', 'being easy to peel', 'not being messy when eating', 'being sweet', 'having balance between sweetness and acidity', 'being aromatic', 'having an intense flavour', 'being juicy', 'not being fibrous (be easy to chew)' and 'having an intense pulp colour'.

The list of attributes presented to participants was preliminarily created by the Spanish research group based on their previous experience and literature about citrus fruit (Goldenberg *et al.*, 2015, 2018; Tarancón *et al.*, 2020). A preliminary list was designed after performing a test run with 10 consumers in order to add any missing option relevant for them. Based on the list generated in Spain, a pre-test was carried out with Italian consumers, and the final list was then created. The presentation order of list terms was balanced among consumers in order to avoid bias.

Lastly, in the demographic data section, gender and age data were recorded.

The questionnaire was originally generated in Spanish language by the Spanish research group and underwent pilot trial with consumers (n=10) to check for errors or ambiguity. Afterwards, members of the *Università degli Studi di Palermo* translated the questionnaire into Italian. The questionnaires in each language were then carefully assessed in order to ensure the meaning of the original content was maintained and that the two versions were consistent with each another.

2.3. Statistical Analysis

To assess the dispersion of the importance scores data, descriptive statistics were calculated and expressed as box-and-whisker plots. A hierarchical cluster analysis (HCA) was then performed to identify groups of consumers giving similar importance scores. Euclidean distances (dissimilarity), Ward's techniques (agglomeration method) and automatic truncation were used.

Analysis of variance (ANOVA) was used to evaluate the difference in the importance scores among the identified consumers' clusters and attributes. Significant difference between them were determined by calculating the Least Significant Difference test ($p < 0.05$).

3. Results and Discussion

Figure 1 shows the box and whisker plots for each attribute and country. The mean importance scores ranged between 3.7 and 6.3 for data collected in Spain (Figure 1A), and between 4 and 6.3 for data collected among Italian consumers (Figure 1B). In both cases, consumers gave the lower importance scores to 'not messy' attribute. In Spain, the highest scores were recorded for 'juicy', while 'juicy' and 'flavour intensity' shared the first position in importance in Italy. These attributes, 'juicy' in both counties and 'flavour intensity' in Italy, were the ones with more consensus among consumers. For the rest of the attributes, the box and whisker plots revealed that the importance scores were spread over the whole scale range. Moreover, the difference between the first quartile (Q1, lower edge of the box) and third quartile (Q3, upper edge of the box) was between 2 and 4 points on the scale for most attributes. Given the wide variability among consumers' scores, as revealed by the box-and-whisker plots, a hierarchical cluster analysis was carried out separately for Spain and Italy to investigate in-depth the importance of each mandarin attribute for consumers to repurchase. A great variability in consumers' response has been also described when they tasted mandarin samples and were asked to score liking (Tarancón *et al.*, 2020).

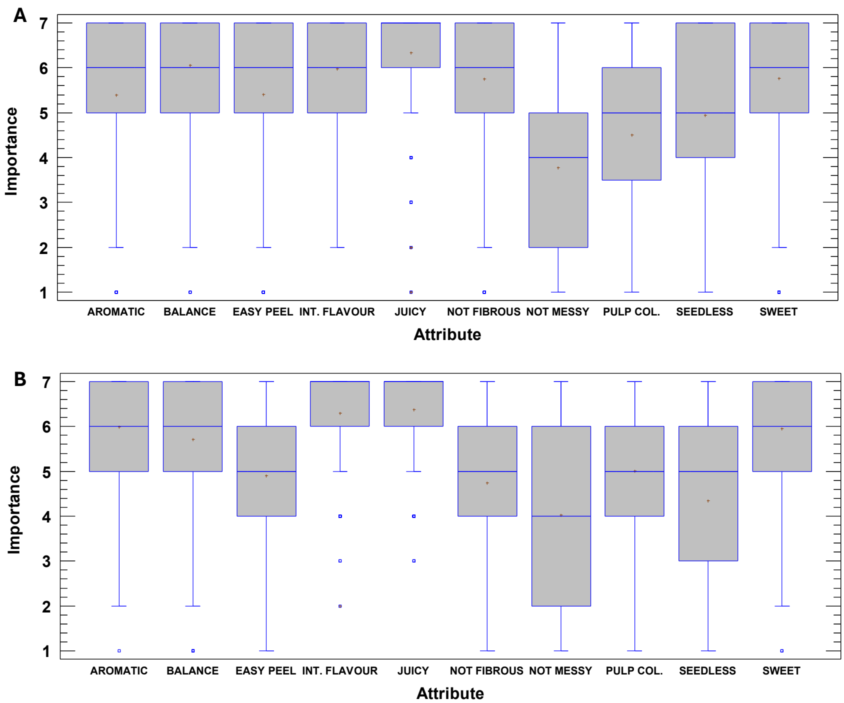

Figure 1. Box-and-wisher plots of descriptive statistics for importance scores for repurchase given to different mandarin attributes by Spanish (A) and Italian consumers (B). The lower and upper edges of a box represent the first (Q1) and third (Q3) quartile, while the line and cross within the box show the median and the mean, respectively. The whiskers represent the lower and upper extremes of the data.

Hierarchical Cluster Analysis allowed to identify two different patterns with regard to the importance of each mandarin attribute among consumers in both countries, Spain and Italy (Figure 2). Hierarchical Cluster Analysis has been previously reported as a powerful tool to identify different preference patterns among fruit consumers (Bonany *et al.*, 2014; Giménez-Sanchis *et al.*, 2021).

Between the two groups of consumers identified in Spain, Cluster 1 (C1) included 41% of consumers and Cluster 2 (C2) 59% (Figure 2A). The two groups of consumers identified in Italy were made up of similar number of consumers (48% Cluster 1, 52% Cluster 2) (Figure 2A).

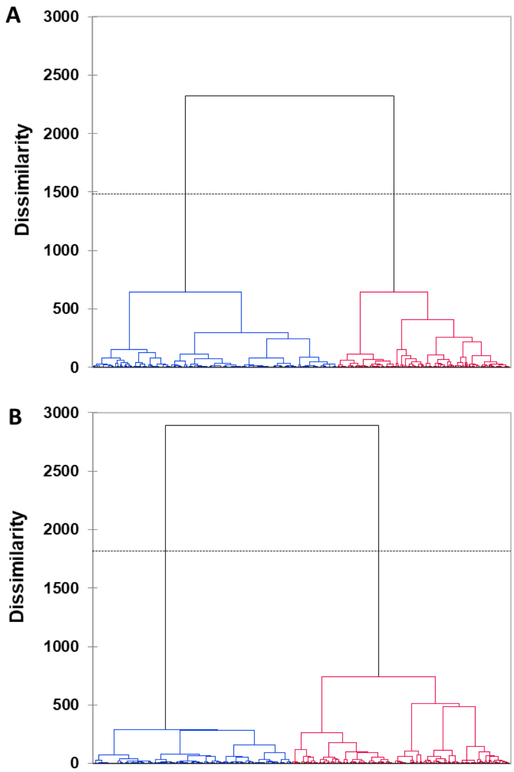

Figure 2. Dendrograms obtained from HCA applied to dataset of importance for repurchase scores from Spanish (A) and Italian (B) consumers.

Once identified the different groups of consumers in each country, ANOVA analysis was performed having into account importance scores data of each of them (Figure 3).

This analysis revealed similar patterns in both countries. Thus, consumers in Cluster 1 in Spain (41% of participants) and Cluster 2 in Italy (52% of participants) reported that the most important attributes that mandarins should have to buy them again are: ‘sweet’, ‘balance sweet-acid’, ‘intense flavour’, ‘juicy’ and ‘not fibrous’. For these consumers, other attributes that are mainly perceived with the hands and sight (easy peeling, not messy when eating, pulp colour) and ‘seedless’ were not important aspects. Therefore, we named them “Consumers focused on mouth perceptions”. The other group of consumers from each country, i.e., Cluster 2 in Spain (59% of participants) and Cluster 1 in Italy (48% of participants) were named “Multisensory consumers”, as they stated that both the attributes perceived in mouth and those related to texture and visual aspect are important.

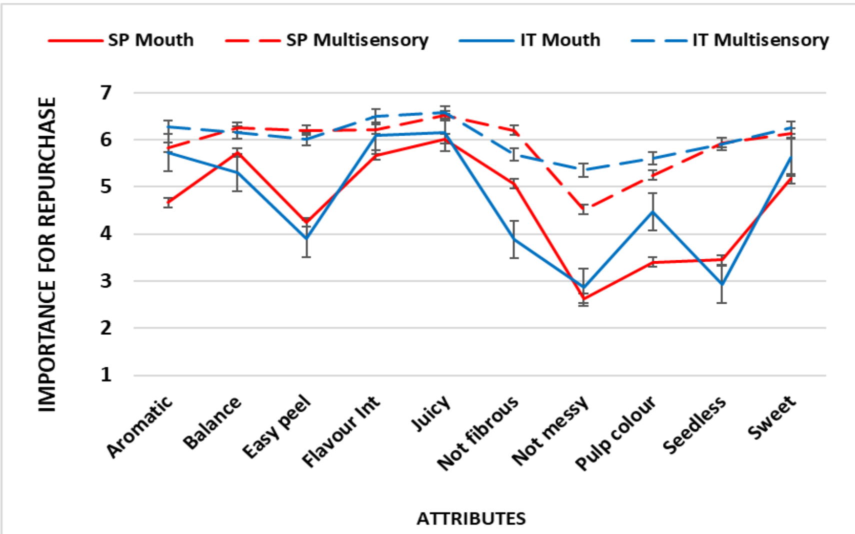

Figure 3. Mean scores of importance for repurchase given to different mandarin attributes by Spanish (SP) and Italian (IT) consumers included in the two clusters identified in each country. Error bars represent the LSD test (p-value=0.05).

Our data revealed that mouth perceptions are important for both types of consumers, “Consumers focused on mouth perceptions” and

“Multisensory consumers”, while convenience attributes are relevant only for the second group. The relevance of taste and flavour related attributes has been previously reported by Tarancón *et al.* (2020) who after giving different mandarin cultivars to Spanish consumers for evaluation, found that attributes perceived in mouth such as ‘juicy’, ‘aromatic’, ‘intense flavour’ and ‘sweet’ are the main drivers of liking. Along these lines, Lado *et al.* (2021) identified mandarin flavour and juiciness as the most important characteristics underlying Uruguayan consumers’ purchase decisions. Previous studies conducted in the USA had reported that sweetness and flavour are key drivers of consumers’ willingness to purchase mandarins (Bi *et al.*, 2014; Kurzer *et al.*, 2019).

Italian consumers gave more importance to ‘aromatic’ and ‘pulp colour’ attributes and less importance to ‘not fibrous’ than the Spanish ones. Pulp colour of mandarins is not among the main mandarin drivers of liking, but it has recently been shown to be a key factor for consumer purchase intention in several countries (Tarancón *et al.*, 2021a).

In addition to the importance of these results for breeders to design high quality mandarins, our findings are also very relevant for quality control. As described for acidity and juiciness (Morales *et al.*, 2020), mandarin aroma and taste intensity may decrease significantly during cold storage (Tarancón *et al.*, 2021b). Therefore, special attention must be paid to these attributes when commercializing fruit after long conservation as consumers’ satisfaction may be compromised.

Our results also revealed that the attribute ‘not messy when eating’ was the less important one for both groups of consumers.

4. Conclusions

The main conclusions of this study are:

- Two kinds of mandarin consumers were identified in Spain and Italy: “Consumers focused on mouth perceptions” and “Multisensory consumers”.
- ‘Sweet’, ‘balance sweet-acid’, ‘intense flavour’, ‘juicy’ and ‘not fibrous’ are very important attributes for consumers’ repurchase intention of both

groups.

- ‘Easy peeling’, ‘seedless’ and ‘pulp colour’ are relevant attributes mainly for the “Multisensory consumers” group.
- The percentage of “Multisensory consumers” was higher in Spain than in Italy.
- Italian consumers gave more importance to ‘aromatic’ and ‘pulp colour’ attributes and less importance to ‘not fibrous’ than the Spanish ones.

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CHAPTER II

Check-All-That-Apply Questions Including the Ideal Product as a Tool for Selecting Varieties in Breeding Programs. A Case Study with Mandarins

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Abstract

Obtaining superior quality varieties is one of the main objectives of fruit breeding programs worldwide. In this study, we investigate employing check-all-that-apply (CATA) questions, which include the ideal product, as a method to select new varieties according to consumer organoleptic quality requirements. To this end, mandarin cultivars were used as a case study. Four new cultivars from the IVIA breeding program ('Pri-88', 'Pri-89', 'Pri-90' and 'Tri-707'), and two commercial cultivars related to them ('Clemenules' and 'Oronules'), were evaluated by a consumer panel using the CATA questions method, which include their ideal product. Our results reveal that this method is a useful tool for selecting varieties based on consumer descriptions of their sensory properties in relation to those of their ideal cultivar. This allows the different consumer preference patterns and differences in sample perceptions to be taken into account. A penalty analysis was performed, including the ideal product, to identify the "must-have" attributes; i.e., those that consumers included in their ideal mandarin description and, whenever present in samples, they significantly increased acceptance. For the mandarins herein evaluated, these attributes are "refreshing taste", "very aromatic", "sweet", "juicy", "very intense taste", "sour" and "not very fibrous". The fruit characteristics that contributed to improve the quality of the new varieties vs. the original varieties were also identified. 'Pri-89' and 'Tri-707', obtained from 'Clemenules', came closer to consumers' ideal variety, because besides the aforementioned "must-have" attributes, these mandarins have small segments. 'Pri-90' implied improvement in relation to 'Oronules' and is an appropriate variety for those consumers sensitive to bitterness and who like mandarins with a certain level of sourness.

Keywords: sensory; ideal product; penalty analysis; consumer; liking; quality improvement

1. Introduction

In the last few decades, numerous breeding programs have been established all around the world in fruit-producing countries. In many cases, these programs have focused on improving crop agronomic characteristics, such as adapting grapevine fruit to high temperatures (Bigard *et al.*, 2018) or improving disease resistance in citrus fruit (Wang *et al.*, 2019). In parallel to these agronomic objectives, obtaining new varieties that satisfy consumer convenience and sensory quality requirements has always been in breeders' minds. Clear examples of convenience-based breeding are obtaining seedless varieties of watermelon (Liu *et al.*, 2009) and citrus fruit (Ali *et al.*, 2013), or easy-peeling citrus fruit varieties (Yu *et al.*, 2021) and tomato (Dick, 2009). Although the evaluation of these traits is relatively easy, determining to what extent new varieties satisfy consumers' sensory quality demand may be more complicated. Moreover, exploring characteristics of new spontaneously generated varieties can be especially interesting because some can be more desirable than those of original varieties (Koornneef *et al.*, 2004; Besada *et al.*, 2017). For decades, the selection of new varieties for fruit quality has been based mainly on evaluating physicochemical properties (Suzuki *et al.*, 2006; Cantin *et al.*, 2009; Nunoo *et al.*, 2014). However, when only physico-chemical information is available, consumer responses are difficult to predict. For this reason, the inclusion of sensory studies as a step in the selection process of new varieties has gradually become essential (Predieri *et al.*, 2006; Lado *et al.*, 2010).

Initially, the approach to describe the sensory attributes that characterize new varieties was by means of trained panels (Hampson, 2000; Vaysse *et al.* 2005). However, this methodology has certain drawbacks, like the time and resources needed to train assessors and the fact that some of the information produced by them may be irrelevant for consumers (Varela and Ares, 2012). Thus, new sensory attributes description techniques have been developed to be used with a consumer-based approach (Varela and Ares, 2012). Of them all, check-all-that-apply (CATA) questions have gained ground in recent years as a reliable and quicker alternative to trained assessor panels (Lado *et al.*, 2010; Ares and Jaeger, 2015; Ares *et* *al.*, 2017). CATA questions consist of a list of words or phrases from which consumers should select all the attributes that they consider appropriate to describe samples (Varela and Ares, 2012; Ares and Jaeger, 2015). In a recent study, Maheeka *et al.* (2021) reported that consumer and expert CATA panels are not interchangeable and panel type should be chosen in accordance with the study objective. CATA questions with consumer panels have proven to be a useful tool for the characterization of new fruit varieties, such as strawberries (Lado *et al.*, 2010) and citrus fruit (Tarancón *et al.*, 2020), and obtained information is more relevant from a commercial point of view. In such studies, the combination of CATA questions and consumer preference evaluations allows the main drivers of liking to be identified (Tarancón *et al.*, 2020).

Moreover, several studies have claimed the usefulness of using CATA questions including the Ideal Product (IP) description for optimizing formulations of elaborated products and their sensory profile, in order to come close to consumers' IP (Tarancón *et al.*, 2020; Oliveira *et al.*, 2017). In this approach, after completing the CATA questions for each evaluated sample, consumers are asked to use the same list of attributes to define their IP (Ares *et al.*, 2011, 2014; Bruzzone *et al.*, 2015; Oliveira *et al.*, 2017). Initially, the aim of this practice is to identify how product characteristics differ from consumers' IP to reformulate the product (Ares *et al.*, 2017). As far as we know, including an IP description in this quite novel methodology of CATA questions has never been used during the process of evaluating new varieties. Based on our expertise in breeding (Aleza *et al.*, 2010, 2012) and new variety evaluations (Besada *et al.*, 2017; Tarancón *et al.*, 2020, 2021), we believe that it would be very useful to include this procedure in breeding programs.

On the one hand, it can help to understand consumer preferences based on the characteristics that new varieties share with consumers' IP. On the other hand, it can allow breeders to select varieties as starting material for future obtainments based on the specific characteristics that make them interesting.

In this context, the objective of this study was to assay CATA questions, including the ideal product description, as a tool for evaluating new varieties. To do so, citrus fruit varieties were selected as a case study because citrus is the most grown fruit tree crop worldwide (FAO, 2021). We specifically applied this methodology to select the new early-season mandarin varieties preselected in the breeding program hosted by the Valencian Institute of Agriculture Research (IVIA, Spain). In addition to the new varieties, commercial cultivars related to them that ripen at the same time of the season were also included in the study.

2. Materials and Methods

2.1. Samples

In the present study, six early-season mandarin varieties were evaluated: 'Tri-707', 'Pri-88','Pri-89', 'Pri-90', 'Clemenules' and 'Oronules'. As shown in Table 1, three of them ('Tri-707', 'Pri-88' and 'Pri-89') are new mandarin cultivars developed in the IVIA breeding program and the IVIA-Industry program, whose main objective is to obtain unseeded and superior quality early-season varieties. The new variety 'Pri-90' was spontaneously generated from cv. Oronules in a grower orchard. As it has drawn breeders' attention, it is being studied in the program. 'Clemenules' and 'Oronules' were included in this study as the main varieties from which the new ones derived. These two commercial varieties, which spontaneously originated more than 40 years ago, are also early varieties.

Table 1. Origin of varieties.

Variety	Origin
Tri-707	Triploid hybrid obtained by open pollination of cv. Clemenules 4x
Pri-88	Triploid hybrid obtained by controlled pollination of cv. Clemenules 4x (♀ parental) and Satsuma mandarin (♂ parental)
Pri-89	Triploid hybrid obtained by controlled pollination of cv. Clemenules 4x (♀ parental) and Satsuma hybrid (♂ parental)
Pri-90	Spontaneous mutation of clementine cv. Oronules
Clemenules	Spontaneous mutation of clementine cv. Fina
Oronules	Spontaneous mutation of clementine cv. Fina

By mid-October, commercial varieties were obtained upon their arrival at a commercial packing house in Valencia (the FONTESTAD S.A. Company, Valencia, Spain), while the new cultivars were harvested from the IVIA's experimental orchards. All the fruit were transported to the IVIA's Postharvest Department for the physico-chemical analysis and consumers' sensory evaluations.

2.2. Physico-Chemical Evaluation

The following physico-chemical parameters were evaluated: colour, firmness, titratable acidity (TA), total soluble solids (TSS), juice yield (JY) and maturity index (MI). Peel colour was measured by a Minolta colorimeter (model CR- 300; Minolta Co. Ltd., Osaka, Japan) using 20 fruits per variety and two measurements were taken in the equatorial zone of each fruit. The mean lightness (L), red-green (a) and yellow-blue (b) Hunter parameter values were calculated for each fruit and expressed as a colour index (CI = 1000 a/Lb) (Jiménez-Cuesta *et al.*, 1981).

Firmness measurements were taken by an Instron Universal Testing Machine (model 3343, Instron Ltd., Buckinghamshire, UK) on 20 fruits per variety. The results were expressed as the percentage of millimetres of fruit deformation that resulted from 10 N pressure, applied by a 3.5 cm plunger on the longitudinal axis at constant speed.

For the TSS and TA, four samples of five fruit each per treatment were squeezed into an electric juice extractor with a rotating head and were determined following the procedure described by Morales *et al.* (2020). Maturity Index (MI) was calculated as TSS/TA. Firstly, the juice yield was measured and expressed as a percentage, calculated by dividing the volume of juice by the total fruit weight.

2.3. Sensory Study

In total, 134 consumers participated in the study. Consumers were recruited based on their mandarin consumption frequency (at least once a week during the season) and their interest in participating. Participants were aged between 19 and 58 years, and the male/female ratio (%) was 47/53.

The evaluations were carried out in a standardized test room (ISO 8589; ISO 2007). The fruits were hand-peeled and each sample was composed of four segments of the same mandarin (presented together). Samples were coded with three-digit random numbers and were presented monadically following a Williams' Latin square design, and consumers were provided with water to cleanse palate between samples. Consumers were instructed to separate the segments before tasting, and were asked to firstly score their overall liking using a 9-point hedonic scale anchored at 1-"dislike very much" and 9-"like very much", and then to indicate their purchase intention on a 5-point scale ranging from 1-"I definitely would not buy" to 5-"I definitely would buy". Next, they were asked to answer the CATA questions with 20 terms related to the sensory characteristics of mandarins. After testing each of the six samples, consumers were asked to complete the CATA questions to describe their ideal mandarin.

The descriptors included on the CATA list were initially selected according to previous research (Goldenberg *et al.*, 2017; Maheeka *et al.*, 2021). Then a group of six semi-trained panellists evaluated the samples to adapt the initial list to their specific characteristics. On the final list, attributes were ordered in such a way that they were likely to be perceived. The selected terms were: stain hands when eating, intense odour when separating segments, small segments, big segments, bitter, very intense taste, not very aromatic, very aromatic, not very sour, sour, very sour, refreshing taste, tasteless/dull, not very sweet, sweet, very sweet, fibrous, not very fibrous, juicy and juiceless (Figure S1).

Finally, when consumers had finished their mandarin assessments, they answered demographic questions about the gender, age and frequency of mandarin/orange consumption during the season. The response options for fruit consumption were as follows: "Almost every day", "At least twice weekly" or "Less than twice weekly".

The protocol and procedures used in this study were revised by the scientific directorate of Valencian Institute for Agricultural Research, which stated a waiver consent. All articles from the Declaration of Helsinki and the 2016/679 EU Regulation on the protection of natural persons regarding the processing of personal data and on the free movement of such data were met. The experimental procedure was explained and a written consent indicating voluntary participation was obtained from each participant prior the beginning of the study.

2.4. Statistical Analysis

One-way ANOVA was used to evaluate the difference in the physico-chemical parameters among cultivars. Significant differences between means were determined by calculating the Least Significant Difference test ($p \leq 0.05$).

The Kruskall–Wallis test followed by Dunn's multiple comparisons test were applied to evaluate difference in acceptance scores among cultivars ($p \leq 0.05$).

A hierarchical cluster analysis (HCA) was performed with the liking data (6 columns x 134 rows) to identify groups of consumers with similar preference patterns. Euclidean distances (dissimilarity), Ward's techniques (agglomeration method) and automatic truncation were selected for this analysis. The number of groups was selected by automatic truncation minimizing entropy. Smaller values of overall entropy indicate less disorder, which indicates a better clustering.

The frequencies of citation of each attribute in the CATA question were determined for each sample. The non-parametric Cochran's Q test was performed on the raw binary CATA data to determine significance among samples for each sensory attribute ($p \leq 0.05$). In order to assess the relation between CATA responses and acceptance scores, a Multiple Factor Analysis (MFA) was performed on the acceptability data of the two clusters, and the CATA attributes frequency of mentioning were also included as supplementary variables.

Penalty analysis of the CATA data including the ideal, was used to determine the attributes with impact on acceptability. For each attribute, for those consumers who selected it in the ideal, the difference in average liking of those mandarins having the attribute and those mandarins not having the attribute was calculated. Those attributes showing a significantly ($\alpha = 0.05$) higher acceptability when the attribute was present were considered “must have” attributes. For each attribute, for those consumers who did not select it in the ideal, the difference in average liking of those mandarins having the attribute and those mandarins not having the attribute was calculated. Those attributes showing a significantly ($\alpha = 0.05$) lower acceptability when the attribute was present were considered “must NOT have” attributes.

All the calculations were carried out with the XLSTAT software (version 2019, Addinsoft Inc. New York, NY, USA).

3. Results and Discussion

3.1. Physico-Chemical Parameters

Of the citrus fruit’s physico-chemical parameters, external colour, MI and juice yield are especially important for EU countries because they are included in the regulation for exporting and marketing citrus fruit within the European Union (UNECE, 2017). Thus, the minimum required MI and JY values are 6.5 and 33% for satsuma mandarins, 7 and 40% for clementines, and 7.5 and 33% for other mandarins or hybrids, respectively. However, the regulation does not specify any minimum colour index values because it only refers to the variety’s typical colour: ‘colour must be typical of the variety on at least one third of the fruit surface’.

In this study, mandarins ‘Oronules’ and ‘Pri-90’ were highlighted for presenting a homogenous orange-coloured skin with CI values of +16 and + 14 at harvest time, respectively, while the part of the skin surface of the other varieties was still green (CI < +4). In this study, external colour was not considered a quality-limiting factor for harvesting because previous studies performed in our department (personal communication) have demonstrated that the four evaluated new varieties adequately respond to the degreening treatment habitually applied to early varieties to enhance colour change. Moreover, a recent study, which evaluated the degreening treatment effect on a range of mandarin and orange varieties, demonstrated that this treatment does not modify the sensory properties that consumers perceive (Morales *et al.*, 2020).

The determination of the physico-chemical parameters linked with internal quality revealed that all six varieties obtained the minimum required MI (Table 2). The highest MI, with values of 12.9 and 13.6, were respectively determined in 'Pri-90' and 'Tri-707'. The other cultivars had similar MI values to one another, which ranged between 9.6 and 11.5. As previously explained, MI is the ratio between TSS and acid concentration, and it is worth mentioning the new variety 'Pri-89', which, despite being that with the highest TSS (13.7 °Brix), presented the lowest MI due to its high acidity level (1.43 g citric ac./100 mL).

Table 2. Physico-chemical parameters of the six mandarin cultivars.

Cultivars	Firmness (%def)	TSS[1] (°Brix)	TA[2] (g citric ac./100mL)	MI[3] (TSS/Ac)	JY[4] (%juice)	Weight (g)
Pri-88	2.2 a	10.6 b	1.05 c	10.1 a	31.9 a	219.7 f
Pri-89	3.8 b	13.7 e	1.43 d	9.6 a	47.0 c	107.8 c
Pri-90	4.6 c	11.2 c	0.87 ab	12.9 c	40.1 b	123.0 d
Tri-707	5.4 d	12.7 d	0.93 b	13.6 c	46.2 c	58.8 a
Oronules	3.8 b	10.7 bc	0.93 b	11.5 b	43.5 b	140.0 e
Clemenules	5.8 d	9.2 a	0.83 a	11.1 b	47.2 c	88.4 b

[1]TSS-total soluble solids, [2]TA- titratable acidity, [3]MI-maturity index, [4]JY-juice yield. Different letters in the same column indicate significant difference among cultivars according to LSD test ($p \leq 0.05$).

The minimum required JY values were met for all the evaluated varieties, except 'Pri-88', which had 31.87% JY. The other varieties obtained JY values over 40%. Regarding size and texture, the 'Pri-88' mandarins were the firmest (2.22% deformation) and the biggest (220 g), while the 'Tri-707' variety produced the smallest fruit with a mean weight value of 59g.

3.2. Preference Patterns and Purchase Intention

The mean liking scores and the percentage of consumers who stated that they would buy each mandarin variety are shown in Table 3. The Global column includes the results by taking into account the total dataset. Similar liking scores (around 6) were determined for three new varieties ('Pri-89', 'Pri-90' and 'Tri-707') and for 'Oronules'. The commercial cultivar 'Clemenules' and 'Pri-88' obtained liking scores below 5.

For most samples, liking scores showed wide dispersion (according to the histograms and whisker-box plots; data not shown), which indicates distinct patterns in consumer responses to the different varieties. Previous studies that have evaluated new varieties of mandarin (Tarancón *et al.*, 2020) and other fruit (Ares *et al.*, 2014), reveal that consumer preferences usually respond to different patterns, because not all people like similarly. A hierarchical cluster analysis (HCA) was performed to further study the data on this aspect, and two different consumer groups, were identified based on their preferences. Cluster 1 (Cl1) was made up of 61 consumers (46%) and Cluster 2 (Cl2) included 73 consumers (54%). Significant differences were detected in the acceptance scores for varieties in both clusters (Table 3). For the Cluster 1 consumers, the preferred mandarins were ‘Tri-707’ and ‘Pri-89’ (mean liking value of 7.5 and 6.8, respectively) and the least preferred one was ‘Pri-88’ with a liking value of 5. The other three varieties (‘Pri-90’, ‘Oronules’ and ‘Clemenules’) obtained intermediate liking values between 6 and 6.4.

Table 3. Mean liking scores and purchase intention of all consumers (Global) and from cluster 1 (Cl1) and cluster 2 (Cl2) consumers.

	Liking[1]			Purchase Intention (%)[2]		
Cultivar	**Global**	**Cl 1**	**Cl 2**	**Global**	**Cl 1**	**Cl 2**
Pri-88	4.1 a	5.0 a	3.4 a	17.9	26.2	11.0
Pri-89	6.1 c	6.8 c	5.5 b	53.7	62.3	47.9
Pri-90	6.1 c	6.1 b	6.3 c	50.7	47.5	54.8
Tri-707	6.4 c	7.5 d	5.5 b	56.7	82.0	37.0
Oronules	6.1 c	6.4 b	5.7 bc	50.0	39.3	45.2
Clemenules	4.8 b	6.1 b	3.8 a	21.6	55.7	8.2

[1]Liking was evaluated on a 9-point hedonic scale; scores not sharing letters within each column were significantly different ($p \leq 0.05$) according to Kruskal-Wallis test. [2]Purchase intention is expressed as the percentage of consumers that responded that they were willing to buy (probably would buy + definitely would buy).

A different preference pattern was observed for the Cluster 2 consumers, who generally scored the varieties with lower values than Cluster 1, except ‘Pri-90’, which was their most preferred mandarin. They disliked samples 'Pri-88' and 'Clemenules' (both with mean liking values below 4). Varieties 'Tri-707' and 'Pri-89', i.e., those preferred by the Cluster 1 consumers, received intermediate values by the Cluster 2 consumers.

In agreement with preference, a higher percentage of the Cluster 1 consumers stated that they would purchase 'Tri-707' and 'Pri-89' (82% and 62%, respectively), and a low percentage of consumers (26%) indicated that they would buy 'Pri-88'. In Cluster 2, 55% of the consumers would buy sample 'Pri-90', and very few (11% and 8%) would buy 'Pri-88' and 'Clemenules'.

Analysis of demographic data from Cluster 1 and 2 consumers revealed a not significant influence of gender, age or consumption frequency in their pattern of liking.

3.3. Consumers' Sensory Descriptions of Varieties and Their Ideal Mandarin Variety

The sensory description of the mandarin varieties from the CATA questions, which include the ideal variety, was studied independently for both consumer clusters. After determining the frequency of mention for each attribute (Tables S1 and S2), a correspondence analysis (CA) was performed (Figure 1A, B). In both cases, the first and second dimension explained around 80% of the variability.

The CA plot for the Cluster 1 consumers (Figure 1A) showed that variety 'Tri-707' came very close to the ideal mandarin on the left side of the space. This closeness in allocation meant that this group of consumers found most of the properties of their ideal mandarin in 'Tri-707', such as "juicy", "sweet", "sour", "small segments", "very aromatic", "intense odour when peeling" and "refreshing taste". This similarity between the description of the 'Tri-707' mandarins and the ideal one explained why this variety was that which the Cluster 1 consumers liked the most (Table 3).

If we pay the attention to the first dimension, which explained most variability, we observed that variety 'Pri-89' came quite close to the IP, along with 'Tri-707', for sharing characteristics of "very intense taste", "sour", "juicy", "very sweet" and "very aromatic". However, consumers described 'Pri-89' as sourer than their ideal mandarin and 'Tri-707'.

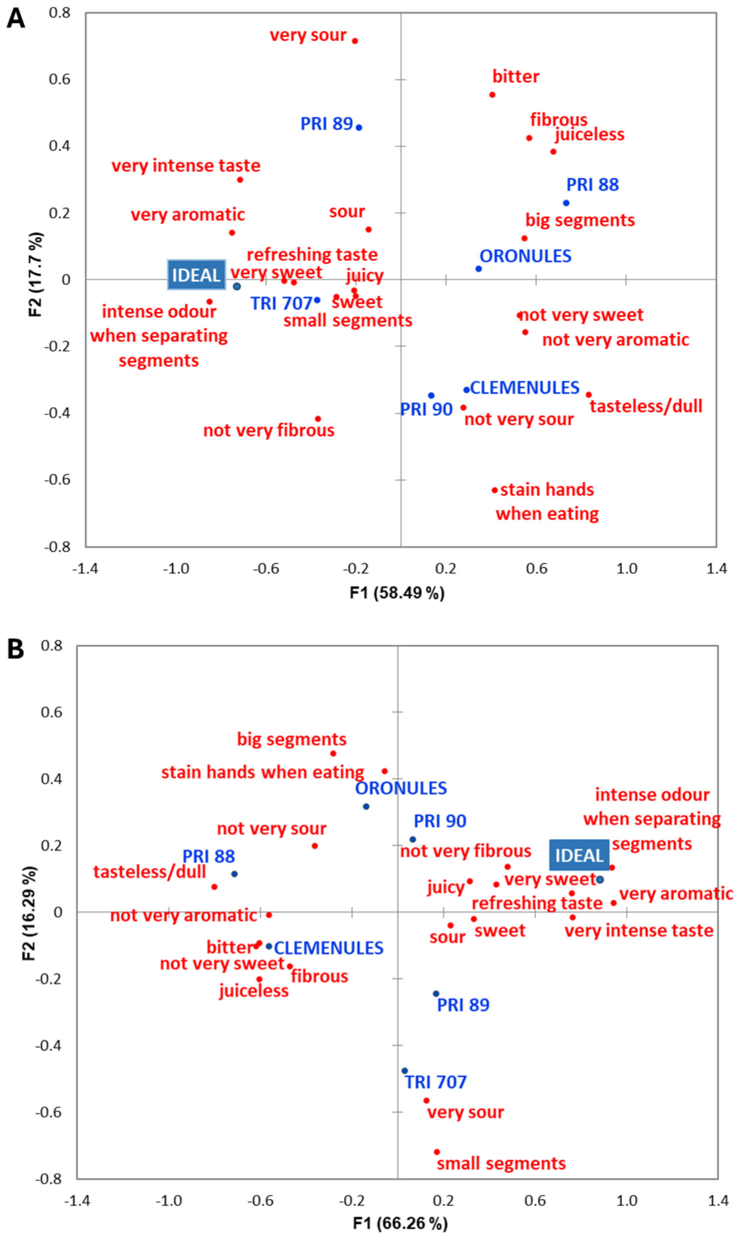

Figure 1. Correspondence analysis for Cluster 1 (A) and Cluster 2 (B).

In the upper right side of the plot, ‘Pri-88’ was the variety that moved the furthest away from the IP and was associated mainly with the terms “big segments”, “bitter”, “fibrous” and “juiceless”. Consumer perceptions of the attributes did not match their ideal mandarin, which would explain why this variety obtained the lowest liking scores (Table 3). This characterization also agreed with the physico-chemical data as ‘Pri-88’ was the biggest variety with the lowest juice yield percentage

Finally, varieties ‘Oronules’, ‘Pri-90’ and ‘Clemenules’, which obtained similar liking scores (6.1–6.4), lay between the ‘Pri-88’ and consumers’ ideal mandarin on the first dimension. These three varieties shared characteristics with the ideal one, but also with ‘Pri-88’. The main difference in them was that consumers perceived more sourness in ‘Oronules’ compared to ‘Pri-90’ and ‘Clemenules’, and the latter stained hands when eating.

Figure 1B shows the results of the cluster analysis performed with the data corresponding to the participants from Cluster 2. In this case, the plot revealed that none of the varieties came very close to the ideal one, which agrees with the lower liking scores detected in Cluster 2 compared to Cluster 1 (Table 3).

‘Pri-88’ and ‘Clemenules’ were located furthest away from the IP and were associated with the terms “not very aromatic”, “not very sweet”, “tasteless/dull”, “not very sour” and “fibrous”. In these two varieties, consumers did not find the characteristics that they wished to perceive in their ideal mandarin, which was why they disliked the samples and scored them with values lower than 5 (Table 3).

Cultivars ‘Oronules’, ‘Pri-90’, ‘Pri-89’ and ‘Tri-707’ were located more closely to the IP and, among them, two different groups were detected. The first group, which came closer to the IP on the second dimension, comprised ‘Oronules’ and ‘Pri-90’. To a greater or lesser extent, these cultivars shared most of the characteristics of Cluster 2 consumers’ ideal mandarin, mainly “juicy”, “sweet”, “sour”, “refreshing taste” and “not very fibrous”. However, these varieties presented characteristics like “big segments”, “not very sour”, and “stain hands when eating”, which make them differ from the IP. 'Pri-89' and 'Tri-707' formed the second group, which obtained negative values on the second dimension. These varieties shared some characteristics with the ideal mandarin, such as "sweet", "sour", "juicy", "very intense taste" and "very aromatic". The separation between these cultivars and the ideal mandarin was mainly due mainly to the fact that consumers used the terms "very sour" and "small segments" to describe them.

A global view of the two plots revealed three results worth mentioning. The first one is the relevance of the information obtained by consumer segmentation. Application of this technique to sensory data is quite recent, and it has been mainly based on 'emotional' response to different products (Jaeger *et al.*, 2019; Nijman *et al.*, 2019; Pierguidi *et al.*, 2020; Low *et al.*, 2021). In the mentioned studies, the evaluated products (beer, tea-break, snacks, etc.) showed relevant differences in organoleptic properties among the evaluated samples. In the present study, a priori, accused differences among samples were not expected, as the cultivars under evaluation are related to each other. However, our results showed that consumer segmentation is a powerful tool to obtain a deeper understanding of consumer behaviour, even when there are small differences among samples.

The second result is that all the new varieties except 'Pri-88' shared more characteristics with the ideal mandarin than the two commercial varieties from which they originated. The ideal product concept refers to the standard of perfection, i.e., the consumers' description of the characteristics that a mandarin should have to be perfect. Therefore, our results indicate that the objective of the breeding program of obtaining superior quality varieties is being achieved. The exception was 'Pri-88', which was initially preselected for its big size fruit, among other reasons. However, our results revealed that consumers do not like this characteristic.

The third one to highlight is that despite showing different preference patterns for the evaluated varieties, Clusters 1 and 2 similarly described their ideal mandarin. Thus, in both cases (Figure 1A, B), the attributes used to describe the ideal mandarin were: "intense odour when separating segments", "very aromatic", "refreshing taste", "very sweet", "sweet" "very intense taste", "sour", "juicy" and "not very fibrous". To better understand the preference differences between the two groups, the relation between the description of sensory characteristics and the liking values for each cluster was studied.

3.4. Relations between Acceptability and the Sensory Description of the Mandarin Varieties

First by combining CATA questions and liking data, a penalty analysis was used to determine the relevance of each attribute that consumers included, or not, in their ideal mandarin (Figure 2).

Figure 2A, C shows the "must-have" attributes that corresponded to those included in consumers' ideal mandarin and significantly ($p < 0.05$) increased consumer acceptability when they found the attribute in a mandarin variety (compared to those cases in which that attribute was not selected). For both clusters, "refreshing taste" and "very aromatic" were the most relevant attributes. Around 70% of all the consumers included these attributes in their ideal mandarin. When they tasted the mandarins and found these attributes, their average acceptability was around 2 points higher than for those mandarins in which these attributes were not selected. Being juicy, not very fibrous and very tasty were also characteristics that increased consumer acceptability. The only difference observed between both clusters was that having small segments was relevant only for Cluster 1 and "sourness" had a much stronger impact on Cluster 2 with an increased consumer acceptability of 1.9 compared to Cluster 1 (0.8).

Figure 2B, D shows the "must-NOT-have" attributes that corresponded to those that were not included in the consumers' ideal mandarin, and which significantly ($p < 0.05$) decreased average acceptability when consumers found the attribute in a mandarin (compared to those cases in which the attribute was not selected). For both clusters, attributes "tasteless/dull", "juiceless" and "not very sweet" had a strong impact. When one of these attributes was used to describe a mandarin, average acceptability decreased by 1.5 to 2 points. Having slight aroma and being fibrous also decreased acceptability. The differences between clusters lay in big segments being a negative attribute only for the Cluster 1 consumers, while being "bitter" and "not very sour" significantly impacted acceptability, but only for the Cluster 2 consumers.

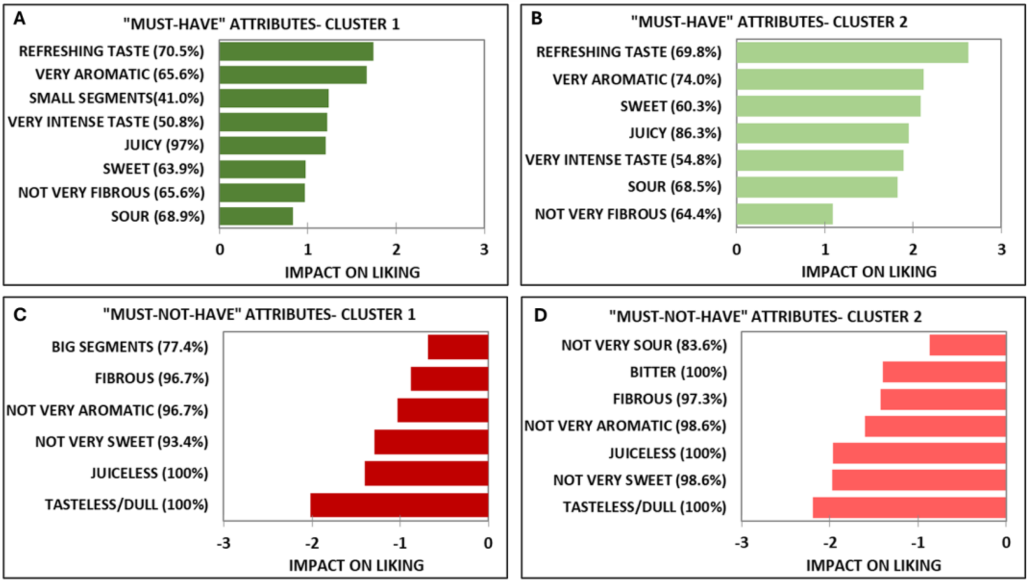

Figure 2. Penalty analysis. Impact on the average liking scores of the "must-have" and "must-NOT-have" attributes for Cluster 1 (A, C) and Cluster 2 (B, D) consumers. Only the attributes with a significant impact on liking ($p<0.05$) were included. For each attribute, the percentage of consumers who selected it for their ideal mandarin was indicated (see brackets).

In short, some differences between both clusters were found for those attributes that impacted mandarin acceptability. Segment size was relevant for the Cluster 1 consumers, who penalized big segments and liked small segments in mandarins. Sourness and bitterness were relevant for the Cluster 2 consumers, who penalized samples when they were slightly sour and bitter. However, the more relevant attributes that impacted acceptability were the same for both clusters.

To ascertain if a different use of descriptors between clusters could explain preference differences, an MFA was used to compare the mandarin sensory descriptions of the two consumer clusters in relation to acceptability (Figure 3). The samples in the plot were distributed according to the acceptability of both clusters. The position of attributes (included as supplementary variables) revealed the similarities and differences in the way that groups of consumers used descriptors to describe the six studied varieties. The position of some attributes came close for Clusters 1 and 2, which indicates that both groups of consumers similarly used them. This was the case for the appearance and texture attributes, such as “not very fibrous”, “stain hands when eating”, “big segments”, “small segments”, “juiceless” and “juicy”.

However, the attributes related to taste and aroma, especially those with a positive impact on acceptability like “refreshing taste”, “very intense taste”, “sweet”, and “very aromatic”, were differently positioned for Clusters 1 and 2. In each case, they went close to the corresponding acceptability variable. This meant that the samples described with these attributes in Cluster 1 were the preferred ones (‘Tri-707’ and ‘Pri-89’). The same can be stated for Cluster 2, for which these attributes were used mainly to describe ‘Pri-90’ and ‘Oronules’. This result falls in line with that reported by Ares *et al*. (2014) in a study about commercial apple cultivars. Those authors found that consumers’ use of CATA terms was markedly affected by their preferences, and mainly for terms referring to odour and flavour.

It is known that a genetic background has a very marked effect on volatile fruit composition (Besada *et al.*, 2017; Sánchez *et al.*, 2012). Thus, for the mandarin cultivars herein studied, as ‘Tri-707’ and ‘Pri-89’ both descend from ‘Clemenules’, they may have a similar volatile profile, but one that differs from that of ‘Oronules’ to some extent and its spontaneous mutation ‘Pri-90’.

Differences in volatile profile would result in specific odour and flavour characteristics that would explain the different preference patterns obtained for Clusters 1 and 2 in relation to these varieties. Odour and flavour descriptions provided by a trained panel would help to clarify to what extent varieties have characteristics that differ from one another (Baviera-Puig *et al.*, 2021). A gas chromatography analysis would be also a useful tool to this end (Besada *et al.*, 2013).

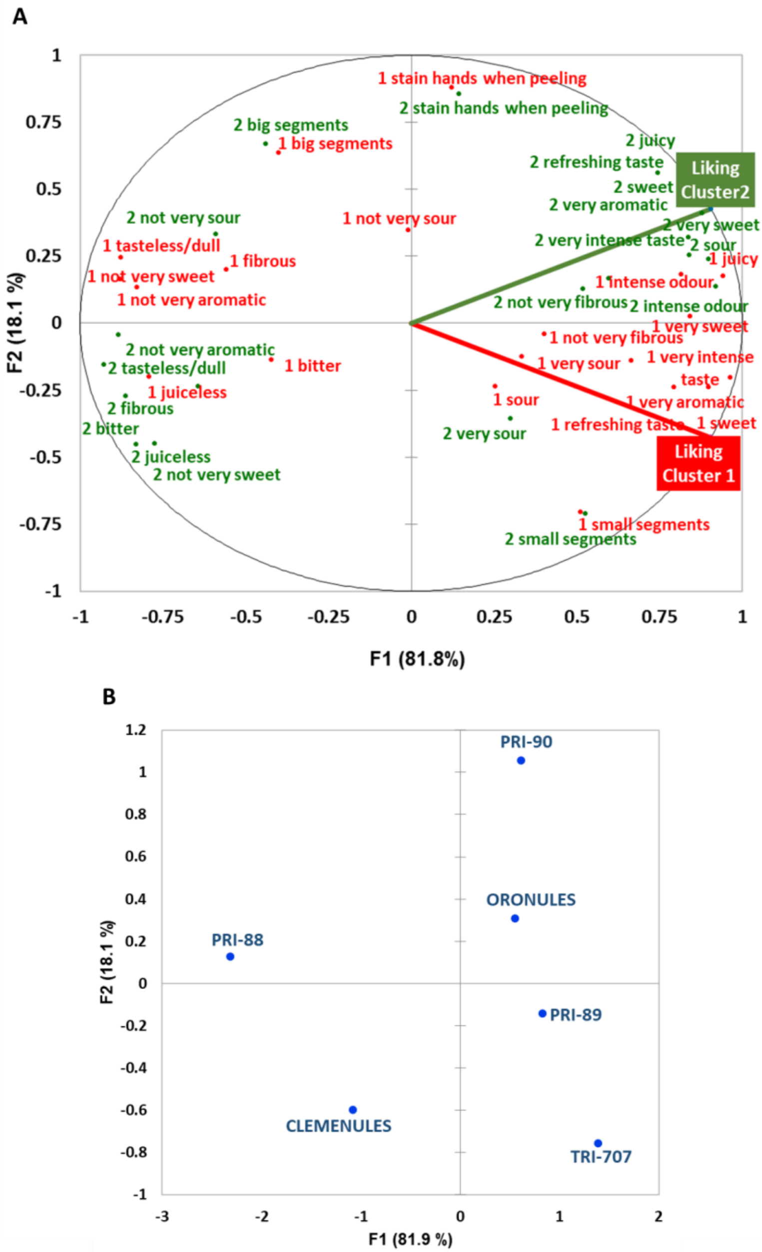

Figure 3. MFA of the CATA question considering liking scores for each of the two identified clusters as active datasets and attributes selected to describe the samples and the ideal product. (A) Vocabulary and liking (Cluster 1: red font; Cluster 2: green font). (B) Representation of samples.

In agreement with what the penalty analysis found, attributes “sour”, “bitter” and “not very sour” were also placed differently for Clusters 1 and 2 but were related to acceptability only in Cluster 2. Cluster 1 did not much differentiate samples according to bitterness or sourness, which could be due to consumers being less sensitive to this taste stimulus or because they paid less attention to them. However, the Cluster 2 consumers used the term ‘sour’ to describe the two samples that they liked the most (‘Pri-90’ and ‘Oronules’), and two attributes that were not desirable (“bitter” and “not very sour”) to describe the two mandarins that obtained the lowest liking scores (‘Pri-88’ and ‘Clemenules’). It is known that the perception of sensitiveness to bitterness vastly varies among people (Risso *et al.*, 2014). The Cluster 2 consumers’ greater sensitiveness to bitterness would explain why this group gave a much lower score to ‘Pri-88’ and ‘Clemenules’ than the Cluster 1 consumers, who also used this attribute to describe these two varieties, but to a much lesser extent.

4. Conclusions

Our results showed that CATA questions, which include the “ideal” description, is a useful tool for selecting varieties in breeding programs. It allows new varieties to be selected based on consumers’ descriptions of their sensory characteristics in relation to their ideal cultivar and take into account consumers’ different preference patterns and differences in sample perceptions. It also allows those attributes that have contributed to improve the quality of new varieties *versus* original varieties to be identified.

Moreover, this method allows identifying “must-have” and “must-not-have” attributes, which is very valuable information for breeders. Undoubtedly, in the context of breeding programs, information revealed by CATA-questions including the Ideal is very useful not only to select varieties with some guarantee of success but also for breeders to set future objectives.

The herein presented case study of mandarins identified two consumer groups according to their preference pattern, i.e., the liking scores of samples. However, for all the consumers, “refreshing taste”, “very aromatic”, “sweet”, “juicy”, “very intense taste”, “sour” and “not very fibrous" were the attributes that mandarins must have to obtain good liking scores. These desirable attributes were detected more in 'Pri-89', 'Tri-707' and 'Pri-90' than in their progenitors 'Clemenules' and 'Oronules'. Therefore, these attributes can explain the increased consumer liking associated with quality improvement. More specifically, 'Pri-89' and 'Tri-707', obtained from 'Clemenules', came closer to the consumers' ideal cultivars because, besides the aforementioned characteristics, these mandarins have small segments. 'Pri-90' implied quality improvement in relation to 'Oronules' and is an appropriate variety for those consumers sensitive to bitterness and who like mandarins with a certain level of sourness.

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Supplementary Material

☐ Big segments	☐ Bitter
☐ Intense odour when separating segments	☐ Tasteless/dull
☐ Stain hands when eating	☐ Not very sour
☐ Small segments	☐ Sour
☐ Very aromatic	☐ Very sour
☐ Not very aromatic	☐ Fibrous
☐ Refreshing taste	☐ Juiceless
☐ Not very sweet	☐ Juicy
☐ Sweet	☐ Not fibrous (easy to chew)
☐ Very sweet	☐ Others :
☐ Very intense taste	

Figure S1. List of attributes included in the CATA questions.

Table S1. Frequency of mention of consumers from Cluster 1 (61 consumers, 46% of the total number of consumers).

Attributes	PRI-88	PRI-89	PRI-90	TRI-707	CLEMENULES	ORONULES	IDEAL
big segments	46	17	29	3	12	52	14
intense odour when separating segments	0	8	8	6	3	3	32
stain hands when peeling	3	1	15	1	3	6	1
small segments	6	24	9	52	18	2	25
very aromatic	2	18	8	15	3	6	40
not very aromatic	31	13	22	12	34	26	2
refreshing taste	11	21	20	26	17	22	43
not very sweet	29	15	20	8	31	21	4
sweet	13	24	22	34	18	29	39
very sweet	2	10	10	12	2	4	17
very intense taste	1	21	6	10	2	6	31
bitter	12	10	2	5	3	5	1
tasteless/dull	23	1	15	1	22	10	0
not very sour	20	5	34	25	26	25	9
sour	24	32	17	24	17	24	42
very sour	3	21	3	4	3	4	5
fibrous	52	33	15	13	13	33	2
juiceless	16	6	1	5	6	6	0
juicy	18	37	37	35	28	37	59
not very fibrous	2	8	25	21	24	11	40

Table S2. Frequency of mention of consumers from Cluster 2 (73 consumers, 54% of the total number of consumers).

Attributes	PRI-88	PRI-89	PRI-90	TRI-707	CLEMENULES	ORONULES	IDEAL
big segments	53	24	37	2	20	59	22
intense odour when separating segments	1	8	6	6	3	7	40
stain hands when peeling	4	1	13	3	2	5	3
small segments	7	24	10	57	20	6	27
very aromatic	0	13	16	13	2	5	54
not very aromatic	46	18	32	25	47	26	1
refreshing taste	8	24	32	22	9	25	51
not very sweet	46	18	20	30	56	31	1
sweet	10	34	37	30	8	28	44
very sweet	2	8	7	6	1	5	24
very intense taste	2	24	11	7	2	8	40
bitter	19	8	5	9	11	8	0
tasteless/dull	42	4	14	12	40	17	0
not very sour	31	7	26	17	28	31	12
sour	20	33	32	33	17	29	50
very sour	4	23	3	8	7	2	7
fibrous	61	40	24	37	31	28	2
juiceless	15	10	5	7	15	5	0
juicy	13	26	47	32	21	36	63
not very fibrous	3	11	16	12	17	19	47

CHAPTER III

Effect of Pulp Pigmentation Intensity on Consumer Acceptance of New Blood Mandarins: A Cross-Cultural Study in Spain and Italy

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Abstract

One of the current objectives of different citrus breeding programmes is obtaining new pigmented mandarins. This study investigates to what extent consumer preferences, expectations and purchase intention are affected by the appearance of new mandarins, specifically pulp pigmentation intensity. Four hundred consumers from both Italy and Spain (800 in total) participated in the study. In each country, half were informed about the healthy properties of the anthocyanins responsible for red pulp colouration, while the other half were not. Italians more readily accepted new mandarin varieties than Spaniards, which was linked to them being more familiar with blood oranges. In Italy, both slight- and medium-pigmented mandarins were well-accepted. Spanish consumers preferred the slight-pigmented variety. The most intense pigmented varieties were not well-accepted in either country. Health-related information positively affected Spaniards' consumer response but did not modify that of Italians. A halo effect was detected in Spain, where health-related information positively affected mandarin appearance liking and its expected taste liking. Consumer perception that new varieties were unnatural was identified as a consumption barrier, mainly in Spain. Blood orange familiarity and health claims are revealed as conditioning factors for consumer response to pigmented mandarins. Interventions should be made to inform consumers that these varieties are obtained by conventional breeding and not by transgenic technology. Future studies should evaluate consumer response to other sensory characteristics, such as odour, taste and texture.

Keywords: health claim; familiarity; natural; first choice; purchase intention; consumption barrier

1. Introduction

In recent years, mandarin production has significantly increased, while other citrus fruits such as orange have remained stable (FAO, 2022). Mandarins have a unique flavour that has attracted consumers' attention worldwide (Goldenberg *et al.*, 2017). One of the reasons for their good position in markets has been breeding seedless and easy-to-peel (Aleza *et al.*, 2010; Yu *et al.*, 2021). Thus, breeding programmes have been key for mandarin success in markets by providing the consumer with more convenient fruit of high sensory quality (Tarancón *et al.*, 2020, 2021a; Giménez-Sanchis *et al.*, 2021).

One of the current objectives of mandarin breeding programmes is responding to the increasing demand for healthier foods. Thus, breeding programmes have focused on obtaining new red-pigmented varieties by crossing classic mandarin with blood orange varieties such as 'Moro' or 'Tarocco' (Rapisarda *et al.*, 2009; Caruso *et al.*, 2020). These blood mandarin hybrids are rich in anthocyanins, which confer them their reddish colouration (Rapisarda *et al.*, 2008, 2009). Anthocyanins are well-known for their antioxidant properties. They have been reported to help to prevent diabetes, heart disease and viral activity, and have promising anticancer properties (Buscemi *et al.*, 2012; Grosso *et al.*, 2013). Indeed, the anthocyanin content of pigmented mandarins enhances their healthy character compared to traditional unpigmented ones, since the high antioxidant capacity of anthocyanins helps to neutralise the free radicals generated in different biological processes, thus exerting a protective effect against oxidative damage at the cellular level (Buscemi *et al.*, 2012; Grosso *et al.*, 2013).

The first red mandarin breeding programme was established in Italy (Recupero *et al.*, 2005), where most blood oranges originate and have a long-standing tradition in markets. In fact, a recent study with data from four countries (Italy, Spain, China and Mexico) has revealed Italy as the country with the greatest consumption of blood oranges, where 76% of the study's participants stated being habitual consumers of this kind of orange (Giménez-Sanchis *et al.*, 2022).

More recently, another programme to obtain pigmented mandarins has been established in Spain (Navarro, 2005), where blood oranges are currently being introduced on markets and the consumption rate is still low at around 17% (Giménez-Sanchis *et al.*, 2022). A range of new red varieties has been bred in the last few years at the Valencian Institute for Agricultural Research (IVIA) (Valencia, Spain) and is being evaluated.

As previously mentioned, anthocyanins are responsible for the red colouring of pigmented citrus fruit flesh. This means that the more intense the pulp pigmentation, the higher the concentration of healthy compounds (Cebadera-Miranda *et al.*, 2019; Tarancón *et al.*, 2022). A priori, it could be thought that the more intensely pigmented new varieties are, the better they will be from a commercial point of view. However, it has been shown that the presence of unfamiliar colours in food may result in consumers mistakenly anticipating their properties, which might negatively impact product acceptance. This effect has been observed in vegetables with atypical colours such as blue potatoes (Pakki *et al.*, 2016) and red carrots (Schifferstein *et al.*, 2019). In new kiwifruit and apple varieties, the presence of red colouration on the flesh has led consumers to consider the product to be unnatural (Jaeger and Harker, 2005; Zanetti *et al.*, 2020). So, despite the red pigmentation of new mandarin varieties being linked with healthier properties, it may negatively impact consumer perception and choice. This possibility means having to conduct studies to anticipate consumer response to these new mandarin varieties that present an unusual aspect.

Consumer studies such as those mentioned above may reveal valuable information for variety selection processes in breeding programmes. Moreover, identifying consumption barriers can help the industry to take measures to mitigate them. Indeed, nutrition and health claims have proven effective techniques for providing information to consumers that can help them to make better choices (Ares *et al.*, 2009; Sabbe *et al.*, 2009; Vidigal *et al*., 2011).

It is important to consider that all consumer response-related aspects are influenced by their cultural background (Galmarini *et al.*, 2013; Giménez-Sanchis *et al.*, 2022; Tarancón *et al.*, 2021b). Cross-cultural studies allow differing patterns among consumer populations to be identified, which can help breeders and industries to adapt their products depending on their destination market. According to Jamir *et al.* (2020), familiarity with similar products may be crucial for explaining cultural differences in a new product's acceptance.

Taking all this into account, the objective of this study was to evaluate if the pulp pigmentation intensity of new red mandarin varieties affects consumer preferences, expectations and purchase intentions. The study was conducted in Italy and Spain to evaluate the effect of consumer familiarity with other pigmented citrus fruit, i.e., blood oranges. It also investigated the effect on participants' decisions of providing them with information about the healthy properties of pigmented mandarins.

2. Materials and Methods

2.1. Consumer Sample

Four hundred people of each nationality, all of legal age, participated in this study. Spanish participants were recruited in Valencia (Spain) and its surroundings, while Italian participants were recruited in Palermo (Sicily) and its surroundings. They were recruited by several approaches: student mailing lists of the two institutions, intercepting pedestrians in the street to invite them to participate in the study, and snowball recruitment (word-of-mouth) through which participants were requested to invite family, friends and/or colleagues to participate. The inclusion criterion was to be a consumer of citrus fruit (at least once a week during the season). The proportion of men and women was similar in both countries, with around 60% women. Participants were aged between 18 and 62 years in Spain, and between 18 and 60 in Italy. Recruitment was conducted in both countries to obtain a balanced percentage of young adults (18-30 years), middle-aged adults (31-50 years) and adults aged over 50 years.

Participation was entirely voluntary. Participants were asked to complete a questionnaire in the Sensory Rooms of the IVIA (Spain) and at the *Università degli Studi di Palermo* (Italy). The questionnaire was designed online and was accessed via tablets. Consent indicating voluntary participation was obtained from all the participants. To this end, at the beginning of the questionnaire, they were shown the following statement: “If you agree to participate and us to employ your answers for this study, please click next”. Once the participants had finalised the questionnaire, they received a gift for taking part.

2.2. Samples

Four images of mandarins were used (Figure 1). One was a classic mandarin with orange pulp. The other three were images of red-pigmented mandarins with different degrees of pulp pigmentation intensity (slight, medium and intense). The objective of this study was to evaluate the pulp pigmentation effect. Therefore, to avoid bias due to external appearance, images were edited in such a way that the three pigmented varieties looked very similar externally.

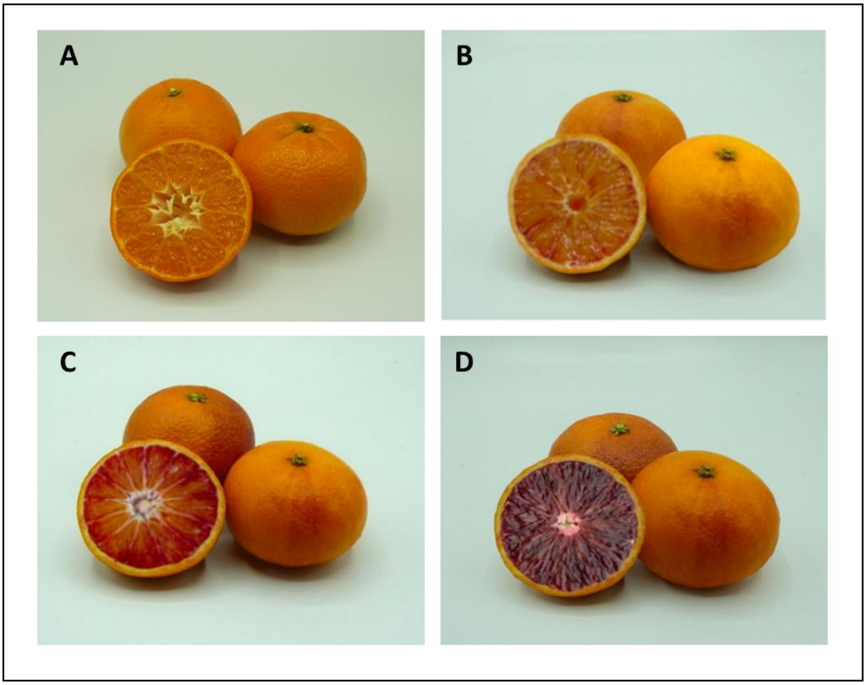

Figure 1. Pictures of normal (A), slight (B), medium (C) and intense pigmented (D) mandarins shown in the questionnaire.

2.3. Questionnaire Design

2.3.1. Presentation of the Study

After obtaining the participants' consent, the study methodology was explained to them. This section served to divide the participants into "Uninformed" and "Informed" ones so that of the 400 Spanish and 400 Italian participants, half were informed about the health properties of pigmented mandarins and half were not.

For this reason, the study was presented in two different ways. On the one hand, the Uninformed group was shown the following text: "We are going to show you images of four mandarin varieties. Of them, you will see new varieties with red flesh. We want to know your opinion. Please answer the questions we ask you in each case". On the other hand, the Informed group received information about the healthy properties of the pigmented varieties: "We are going to show you images of four mandarin varieties. Of them, you will see new varieties with red flesh. These varieties have natural antioxidant compounds that give them their reddish colouring. We want to know your opinion. Please answer the questions we ask you in each case".

The order in which images were presented was balanced to avoid any order effect.

2.3.2. Consumer Choice

The participants were shown the four mandarin images at the same time and were asked about their first and last choices using the following questions: (1) "Which of these mandarins would you choose?"; (2) "Which of these mandarins would you not choose? (Which would be your last option)". To answer them, they used a drop-down box with the four mandarin options.

2.3.3. Naturalness and Healthiness Perception

In this part of the questionnaire, consumers were presented with images of the four mandarins one at a time. For each mandarin, the participants' perception of naturalness and healthiness was investigated. To this end, the participants were asked to indicate their level of agreement or disagreement with the following statements on a 7-point Likert scale (1 = "Not at all"; 7 = "Very much") (Joshi *et al.*, 2015): 'I think these mandarins are natural', and 'I think eating these mandarins is good for my health'.

2.3.4. Acceptance and Purchase Intention

After responding to the question about the naturalness and healthiness perceptions, the participants were asked to score their liking of both appearance ("How much do you like the way these mandarins look?") and expected taste ("How much do you think you would like these mandarins?") on a 7-point hedonic scale (1 = "dislike/will dislike very much" and 7 = "like/will like very much") (Lawless *et al.*, 2010).

They were also asked about their purchase intention ("Would you buy these mandarins?"). The participants answered on a 5-point scale ranging from 1- "I would not buy them" to 5- "I would definitely buy them" (Wright *et al.*, 2007).

In the last questionnaire section, consumers' familiarity with blood oranges was investigated. For this matter, consumers were asked about their level of blood orange consumption using three response options: "I have never tried them", "I have tried them, but I do not usually eat them" and "I usually eat them when they are available on the market". Those participants who selected the first and second options were classified as "Unfamiliar" since they were not habitual consumers, while those who selected the third option were classified as "Familiar". Finally, consumers were asked about their gender and age.

The final questionnaire was originally produced in Spanish by the Spanish research group and underwent a pilot trial with consumers (n = 10) to check for errors, ambiguity, the logical flow of questions and completion time. It was subsequently translated into Italian by a bilingual researcher.

The protocol and procedures used in this study were revised by the Scientific Directorate of the IVIA, which stated waiver consent. All the articles of the Declaration of Helsinki and EU 2016/679 Regulation on the protection of natural persons regarding personal data processing and on the free movement of such data were met.

2.4. Data Analysis

To analyse the data from the choice tests and the purchase intention question, the percentage of consumers who selected each response option was calculated. The comparison of the k-proportion test (procedure of Marascuilo and the chi-squared test, $p < 0.05$) was used to compare the frequency of choice of the different mandarins.

For each country and consumer type (Informed, Uninformed), ANOVA analyses were performed to evaluate the effect of pulp pigmentation intensity on the naturalness and healthiness perceptions, and on the liking of both appearance and expected taste ($p < 0.05$). ANOVAs ($p < 0.05$) were also carried out to determine the effect of the participants' age, provided information, nationality and familiarity with blood oranges on consumer response. All the analyses were performed with Statgraphics Centurion XVI.

3. Results

3.1. Consumer Choice

Figure 2 shows the results of consumers' first mandarin choice. Irrespective of the country and the Uninformed or Informed condition, the first choice of most consumers was the normal mandarin. However, a clear effect of nationality, information and pulp pigmentation was observed when analysing the results in detail. When Spanish consumers were Uninformed, 28% of them indicated any of the pigmented mandarins as their first choice, and the slight-pigmented variety was the most frequently selected of them. When information about content of antioxidants compounds was provided, 10% more Spaniards chose pigmented mandarins as their first option, which resulted in around 40% of consumers selecting any of the new varieties. The slight-pigmented mandarin remained the preferred one by the Informed consumers.

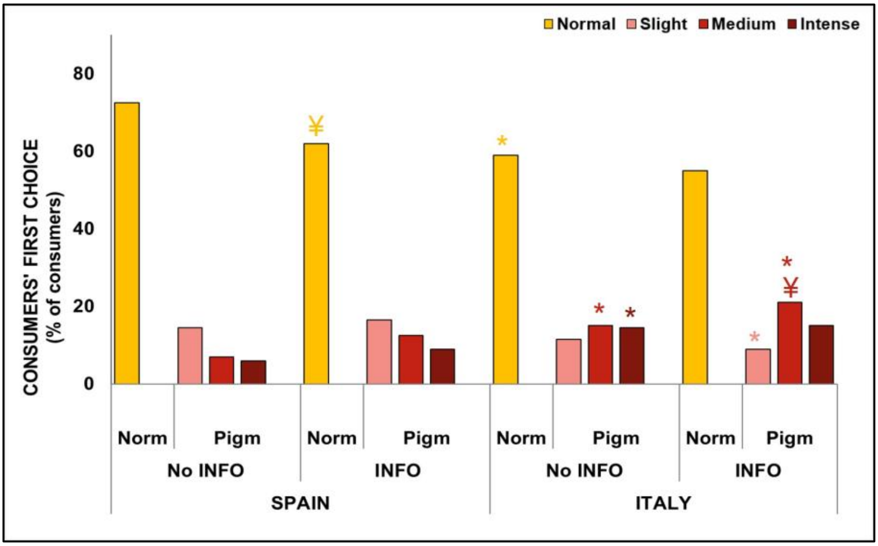

Figure 2. Percentage of consumers from Spain and Italy who indicated each mandarin type (normal-, slight-, medium- and intense-pigmented varieties) as their first choice. The Informed consumers (Info) received information about the antioxidant properties of the pigmented mandarins. ¥ indicates significant differences between the percentage of informed and uninformed participants in each of the countries who selected a specific type of mandarin. *Indicates significant differences between the percentage of Italians and Spaniards who selected a specific type of mandarin under the same conditions and information.

A different pattern was detected in Italy. For the Uninformed condition, more Italians (40%) reported pigmented mandarins as their first option than Spaniards (28%). Italians showed more interest in the more intensely pigmented varieties compared to Spaniards, and the effect of providing information was not so marked in Italy. The percentage of Informed consumers who chose any of the pigmented mandarins very slightly increased with respect to the Uninformed ones and was mainly associated with the medium-pigmented mandarins being selected more.

When Spanish consumers were asked to indicate which mandarin would be their last option under the Uninformed conditions, most (60%) selected the most intense-pigmented variety (Figure 3). The least frequently mentioned pigmented mandarins were the medium-pigmented ones (18%), followed closely by the slight-pigmented ones (20%). The provided information had a slight effect on consumers' last choice. Thus, knowing about anthocyanin's healthy properties led to the percentage of consumers who selected the most pigmented variety as their last option to be slightly lower.

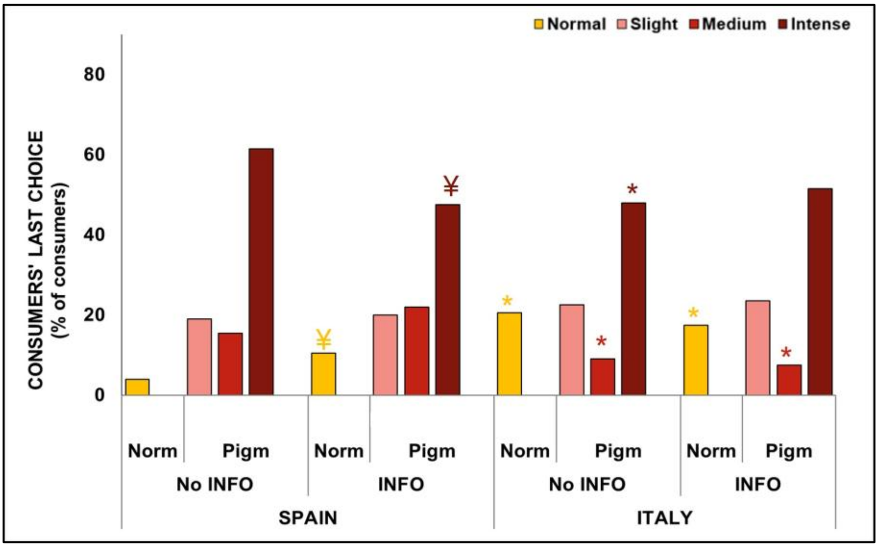

Figure 3. Percentage of consumers from Spain and Italy who indicated each mandarin type (normal-, slight-, medium- and intense-pigmented varieties) as their last choice. The Informed consumers (Info) received information about the antioxidant properties of the pigmented mandarins. ¥ indicates significant differences between the percentage of informed and uninformed participants in each of the countries who selected a specific type of mandarin. * Indicates significant differences between the percentage of Italians and Spaniards who selected a specific type of mandarin under the same condition of information.

For Italy, the provided information had no effect on their last mandarin choice. The percentage of Italians who indicated the normal variety as their last choice was higher (20%) than in Spain (4%). Of the pigmented varieties, the most intense-pigmented one was by far the most frequently indicated as the last option, which is in concordance with that detected in Spain. Once again, the medium-pigmented variety was highlighted for being the least frequently chosen one. In fact, less than 10% of the Italian Informed consumers indicated this variety as their last option, which is an even percentage lower than that observed for normal mandarins.

3.2. Purchase Intention (PI)

Figure 4 shows the results of consumers' purchase intention (PI) depending on mandarin pigmentation under the Informed and Uninformed conditions. When Spanish consumers did not receive information about the properties of the new varieties, they indicated a higher PI for the normal cultivar (83% were willing to buy and 9% perhaps would buy) than for the pigmented varieties. Of the pigmented ones, Spanish consumers were most interested in the slightly-pigmented mandarins, with 68% of consumers indicating that they would buy them or perhaps would do so. The medium- and intense-pigmented varieties were less interesting to consumers.

Informing consumers about the beneficial properties of pigmented mandarins led to an increase in Spaniards' PI. This information resulted in around 20% more consumers showing an interest in buying the three pigmented varieties. The pattern of pulp pigmentation preferences remained unchanged for the Uninformed ones, i.e., the slight- pigmented variety was that with the highest PI, followed by the medium- and the intense-pigmented ones.

When the test was performed in Italy with the Uninformed consumers, the PI for the pigmented varieties was higher in all three cases compared to the results for Spain. Italians showed more interest in the slight- and medium-pigmented varieties, with PI at around 80% (60% willing to buy and 20% perhaps willing). In fact, PI was similar to that of the normal variety. They indicated lower PI for the intense-pigmented variety.

Unlike what was observed in Spain, the information provided about new varieties had no noticeable effect on the Italian consumer PI.

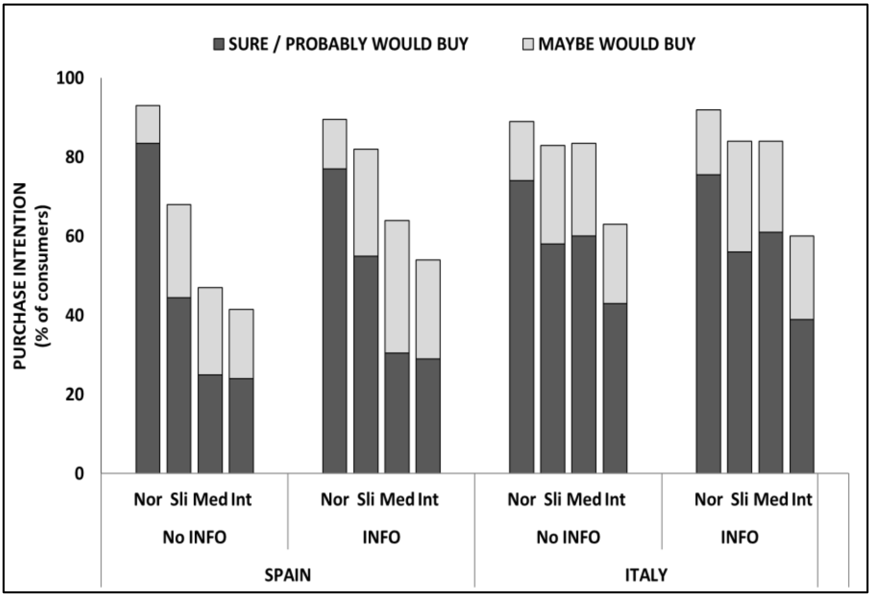

Figure 4. Purchase intention of Spaniards and Italians for mandarins with different pulp pigmentation intensities (normal, slight, medium and intense). The Informed consumers (Info) received information about the antioxidant properties of the pigmented mandarins.

3.3. Consumer Perception and Expected Liking

Figure 5 shows consumers responses when asked to score on a Likert scale the variety's 'naturalness' and 'healthiness'. Acceptance based on liking mandarins' appearance and their expected taste when eaten is also shown. As Figure 5A illustrates, the Spanish Uninformed group perceived pigmented varieties to be less natural and healthy than normal ones, especially those with the more intense pigmentation. Regarding their appearance acceptance and their expected taste liking, the pigmented varieties also obtained lower scores than the normal variety. Clear differences were revealed among the new varieties depending on the degree of pigmentation because the more intense pigmentation was, the less attractive its appearance and the less they expected to like their taste. Of the three pigmented ones, the slight-pigmented mandarin was highlighted by being the most visually liked and generated the better expected taste.

When Spanish consumers were informed about the beneficial properties of the pigmented varieties, their ‘healthy’ perception significantly increased for the three red mandarins. The liking scores for appearance and expected taste also rose in association with the provided information (Figure 5B).

The results from the test performed in Italy with the Uninformed consumers (Figure 5C) showed that they gave higher scores for the pigmented varieties compared to Spaniards (Figure 5A). Thus, the scores of the three pigmented varieties came closer to those of the normal one in Italy than in Spain. Another remarkable difference to appear between countries was that Italians gave the same scores to the slight- and medium-pigmented varieties for all the evaluated aspects, which was not observed in Spain. Interestingly enough, Italians considered that irrespective of pigmentation intensity, the three red varieties were equally healthy. The main effect of pigmentation intensity was detected for the acceptance evaluation, as the intense-pigmented variety was the least liked in terms of appearance and was also expected to be the least liked when eaten.

When information about the antioxidant properties of anthocyanins was provided to the Italian consumers, no remarkable changes in their perception and expectations of the different varieties were observed (Figure 5D).

A comparison between the data from the Informed consumers from Spain (Figure 5B) and Italy (Figure 5D) revealed differences in how they perceived the medium-pigmented variety. For all the evaluated aspects, Italians gave higher scores than Spaniards.

A more thorough analysis of the data to determine if participants’ perception depended on their age revealed that irrespective of the country and conditions, there were no differences among age groups for any of the four evaluated (data not shown).

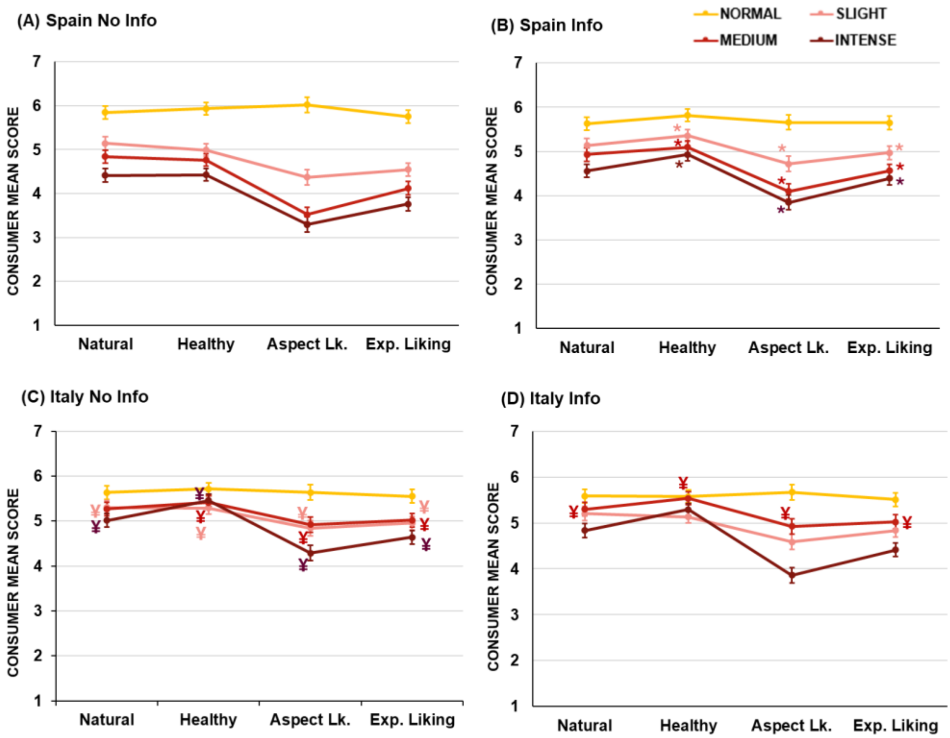

Figure 5. The mean scores of consumer perceptions of the natural and healthy characters (7-point Likert scale), and the liking of appearance and expected taste liking (7-point hedonic scale), of mandarins with different pigmentation intensities. In the four figures, vertical bars depict the LSD intervals when evaluating the pigmentation intensity effect on the scores for each aspect. * Denotes significant differences between the Uninformed and Informed consumers from a specific country, i.e., when comparing the results between Fig. A and B, and between Fig. C and D. ¥ Indicates significant differences between Spaniards and Italians under a specific condition (Informed or Uninformed), i.e. when comparing the results of Fig. A and C and those of Fig. B and D (LSD test, p-value=0.05).

As our results seemed to corroborate the initial hypothesis of a cultural effect associated with Italians being more familiar with blood oranges possibly resulting in a more positive response, we wished to go one step further and evaluate the effect of familiarity those consumers who stated not normally eating blood oranges and those who indicated that they did; that is, the ‘Unfamiliar’ and ‘Familiar’ participants from each country (Table 1). The first result worth mentioning about this regard was the percentage of participants from each country who were familiar with blood oranges: 16% in Spain and 72% in Italy. This result confirmed the interest in including these two countries in the study to evaluate the familiarity effect at the country level.

Familiarity with blood oranges did not affect Spaniards’ evaluation of traditional unpigmented mandarins (Table 1). However, the Italians who were familiar with blood oranges gave a lower liking score for the appearance of traditional mandarins than those who were Unfamiliar. When evaluating the slight-pigmented mandarin, the effect of familiarity became evident mainly for the Uninformed consumers. In both countries, the scores for naturalness, and the liking of both appearance and expected taste, given by the Unfamiliar consumers were significantly lower than those received from the Familiar one. Besides, familiarity also had an effect on Italians’ healthiness perception.

The familiarity effect was more evident in the evaluation of the medium- and intense-pigmented varieties. The scores given by the Unfamiliar consumers for the four evaluated aspects were affected by familiarity, and in such a way that the Unfamiliar consumers with blood oranges perceived red mandarins to be less natural and healthy, and they liked their appearance less and gave a lower liking score to expected taste than the Familiar consumers. A similar pattern was detected in the evaluation made by the Informed consumers.

Table 1. Effect of familiarity with blood oranges on consumer perceptions of the natural and healthy character, and on the liking of appearance and expected taste (Ex. Liking), of mandarins with different pigmentation intensities. *Indicates significant differences between the Familiar and Unfamiliar consumers for a specific pigmentation intensity according to the LSD test (p-value=0.05).

			NORMAL		**SLIGHT**		**MEDIUM**		**INTENSE**	
			Fam.	**Unf.**	**Fam.**	**Unf.**	**Fam.**	**Unf.**	**Fam.**	**Unf.**
SP	**NO INFO**	Natural	6.1	5.8	5.7	5.0*	5.8	4.6*	5.2	4.2*
		Healthy	6.1	5.9	5.3	4.9	5.6	4.6*	5.1	4.3*
		Aspect L.	6.1	6.0	5.1	4.2*	4.9	3.2*	4.8	3.0*
		Ex. Liking	6.0	5.7	5.1	4.4*	5.4	3.8*	4.9	3.5*
	INFO	Natural	5.7	5.6	5.4	5.1	5.6	4.8*	5.3	4.5*
		Healthy	5.9	5.8	5.1	5.4	5.5	5.0	5.3	4.9
		Aspect L.	5.8	5.6	4.5	4.8	4.4	4.0	4.7	3.7*
		Ex. Liking	5.9	5.6	4.8	5.0	5.3	4.5*	5.5	4.2*
IT	**NO INFO**	Natural	5.7	5.5	5.4	5.0*	5.4	4.9*	5.2	4.6*
		Healthy	5.7	5.8	5.4	5.0*	5.6	5.0*	5.7	4.9*
		Aspect L.	5.5	6.0*	5.0	4.5*	5.2	4.3*	4.6	3.4*
		Ex. Liking	5.5	5.7	5.1	4.5*	5.3	4.2*	5.1	3.5*
	INFO	Natural	5.6	5.6	5.2	5.3	5.3	5.2	4.9	4.7
		Healthy	5.6	5.6	5.1	5.1	5.6	5.3	5.4	4.9*
		Aspect L.	5.5	6.1*	4.7	4.3	5.2	4.2*	4.3	2.7*
		Ex. Liking	5.4	5.7	5.0	4.5*	5.4	4.1*	4.8	3.4*

4. Discussion

Sight is the first sense that helps consumers to anticipate fruit quality. Thus, fruit appearance is a decisive factor for consumer decisions to purchase or not. In recent years, several studies have evaluated the effect of different visual aspects, such as external colouration, gloss, skin dehydration or pulp colour (green/orange colour), on consumer expectations and their choice of traditional mandarins (Joshi *et al.*, 2015; Tarancón *et al.*, 2021a, 2021b). Most of these studies, which confirm the importance that consumers attach to fruit appearance, have been based on fruit images because their use allows responses to be obtained from more consumers and cross-cultural evaluations can be made (Tarancón *et al.*, 2021b; Giménez-Sanchis *et al.*, 2022). Furthermore, editing images can be very useful for evaluating specific fruit appearance aspects (Wright and MacRae, 2007; Lawless *et al.*, 2010).

In the present study, the images of pigmented mandarins were edited in such a way that the main difference among varieties was flesh colour. So, we could be sure that the differences among samples were linked with the influence of pulp pigmentation on consumer responses, and without any bias due to other factors, such as fruit size, shape or presence of skin defects.

4.1. Uninformed Consumers' Preferences and Familiarity Effect

Research works have demonstrated that new food with unfamiliar colouring can trigger two types of reactions. Some consumers may be attracted to the new product through their curiosity and desire to try it (Zanetti *et al.*, 2020). Another possible reaction is that atypical colours lead consumers to distrust the product by them generating erroneous expectations, and even rejecting it (Schifferstein *et al.*, 2019). The latter reaction is related to the 'neophobia' concept, i.e., the tendency to avoid new food. This tendency seems to originate from the primitive instinct of surviving, which leads humans to avoid unfamiliar foods that can be potentially dangerous. More recently, it has been observed that neophobia can also be generated from the fear of food tasting unpleasant, which creates bad experiences (Siddiqui *et al.*, 2022).

The results of this study revealed both types of reactions. A significant percentage of participants (28% in Spain and 40% in Italy) indicated one of the pigmented mandarins as their first choice, which implies a positive response to this new product. Conversely, those participants who took a more conservative or neophobic aptitude chose traditional orange-coloured mandarins.

One of the starting hypotheses of this study was that Italians' familiarity with blood oranges could result in a more positive response to new mandarin varieties compared to Spaniards, who are not as familiar with pigmented oranges. Our results corroborated this hypothesis because Italians showed more interest in the pigmented mandarins than the Spaniards. Italians liked the slight- and medium-pigmented varieties, while Spaniards were interested mainly in the slight-pigmented mandarins.

The differences and similarities in Spaniards' and Italians' responses were, to a great extent, explained by their perceptions of naturalness and healthiness, and their scores to both appearance liking and taste expected liking. According to previous studies, all these aspects are strongly affected by familiarity (Paakki *et al.*, 2016; Schifferstein *et al.*, 2019).

The data from Spain revealed that the more pigmented samples were, the least natural and healthy they were perceived. It would seem, therefore, that the more different the variety from the familiar orange-coloured mandarins, the more Spaniards had doubts about their origin and healthiness. Furthermore, a link was detected between the 'natural' and 'healthy' perceptions, and between them and the liking of appearance and expected taste liking in such a way that the mandarins that Spaniards perceived as being less natural were those that received the lowest scores for being perceived as healthy, and for appearance liking and expected taste liking.

Naturalness has been revealed as an important aspect for consumers from different countries (Román *et al.*, 2017), and the interrelation of the naturalness perception with other relevant product perception aspects has been previously observed. Sulistyawati *et al.* (2019) reported a link between the healthiness, nutrition, naturalness and taste perception of dried mango after exploring the response of consumers from different countries in the early product development stage. Moreover, the effect of unfamiliar colourations negatively affected consumers' perceptions of aspects such as naturalness, healthiness or expected acceptance, as herein observed. This falls in line with that reported by Paakki *et al.* (2016), who found that consumers ranked salads made with familiar colour potatoes better for attributes such as healthy, natural and appetising than the same salads containing blue potatoes. In the same line, Schifferstein *et al.* (2019) observed that consumers perceived carrots with atypical colours as being less healthy and attractive than traditional orange-coloured ones.

According to previous studies on novel fruit with atypical colours, such as kiwis and apples with red pulp, people's concern about them being developed by transgenic technology is the main reason why these fruits were perceived as unnatural (Jaeger and Harker, 2005; Zanetti *et al.*, 2020). In a recent study in which Brazilian consumers were asked to evoke the first four words that come to their minds when viewing photos of the blood orange 'Cara Cara', terms related to genetic modifications and 'artificial' received 30% of mentions (Ávila *et al.*, 2020). In line with the cited studies, our results revealed that consumers mistrusted the naturalness of the new varieties, which may act as a barrier to consuming them, mainly in Spain. Thus, interventions should be made to explain to consumers that these varieties were obtained by conventional breeding rather than by transgenic technology. According to Jaeger and Harker (2005), informing consumers that a novel fruit is not genetically modified may positively impact their willingness to pay. Likewise, Schirmacher *et al.* (2022) reported that adding a 'natural claim' to yoghurt increases PI, which is mediated by brand trust and product attitude. So, one possible intervention to overcome the unnatural perception barrier would be to include a 'natural red pulp' claim on new mandarin packaging or on supermarket panels.

The data from Italy showed that the greater familiarity of its consumers with blood oranges was reflected by their increased perception that the new pigmented mandarins were natural and healthy. Irrespective of pulp pigmentation intensity, the three new varieties obtained the same scores for these two attributes. However, the most pigmented variety was highlighted for being that which obtained lower scores for liking appearance and expected taste liking. A sensory study conducted by Simons *et al.* (2019), in which consumers evaluated a range of blood orange varieties, showed that the lighter-coloured ones (e.g., 'Cara Cara', 'Tarocco', 'Boukhobza' and 'Shahani') were liked by most consumers. They also presented fewer undesirable sensory characteristics, such as bitterness and sourness, than the most pigmented ones. This could explain why Italian consumers did not expect to like the intense-pigmented variety. Italians were likely to have eaten intense-pigmented blood oranges, and given their association with oranges' taste, these consumers expected undesirable attributes in the most pigmented mandarins.

Before closing this section, it is worth mentioning that the positive effect of blood orange familiarity on consumer response to new mandarins was also corroborated by the participants from each country. Blood orange familiarity strongly affected preferences from not only a cross-cultural perspective but also for individuals.

4.2. Effect of Information on Consumer Preferences

In this study, Spaniards and Italians showed very different responses to the provided health-related information. This information has contributed to increasing Spaniards' perception of the 'healthy' aspect of the new varieties. Moreover, a halo effect was detected, in which the healthiness perception created a favourable impression that increased consumers' liking for the appearance and how much consumers expected to like fruit when tasting the fruit. On the contrary, there were no differences in Italy between the Informed and Uninformed consumers. This result indicates that the provided information was already known by Italians but was new to Spaniards.

The halo effect that the health-related information had on the Spanish consumers who participated in this study has been previously observed when adding health claims on the package of different products. In a study carried out by Fernan *et al.* (2018), the inclusion of 'protein' claims on energy bar labels led consumers to believe that the quantity of other non-claimed nutrients was also bigger. In a similar way, Stoltze *et al.* (2021) demonstrated that the presence of fibre-related claims on breakfast cereal packages led people to make misleading generalisations that cereals were more natural, had higher vitamin content and were of better global quality. There are also reports that show that the inclusion of health claims on packages may have a positive effect on consumer liking when tasting a product (Wansink *et al.*, 2006; Sabbe *et al.,* 2009; McSweeney, 2022).

Different studies into novel foods have shown that the positive effect of adding health benefits information on consumer product perceptions may result in a higher PI and more willingness to pay (Barauskaite *et al.*, 2018; Kallas *et al.*, 2019; Biondi and Camanzi, 2020; Tuorila and Hartmann, 2020; Goulart *et al.*, 2021). It has been demonstrated that the nutritional information and health perception of novel foods can reduce food neophobia, especially for those consumers who are more health conscious (Hartmann *et al.*, 2018; Tuorila and Hartmann, 2020). In agreement with these studies, our data showed that health-related information enhanced Spaniards' inclination to try new mandarins, as reflected by their better PI and a higher percentage of participants who selected them as their first choice. Our results are in line with those reported for blood oranges by Ávila *et al.* (2020) who identified dissemination of the nutritional benefits of the blood varieties as the main characteristic that should be considered in marketing strategies to promote consumption.

Overall, our data indicate that providing Spanish consumers with information about the healthy properties of new varieties moves their response closer to that of Italians. The long-standing tradition of blood oranges in Italy explains Italians being more aware of the link between red citrus fruit pulp pigmentation and their health benefits. The creation of the IGP 'Arancia Rossa di Sicilia' in 1994 to regulate the commercialisation of the blood oranges grown in Sicily, and to promote their benefits and consumption, has played a crucial role (Arancia Rossa di Sicilia, 2022).

It is worth highlighting that natural perception was the only aspect of all those evaluated that was not affected positively by the health information halo detected in Spain. As previously indicated, our results reveal the need to make specific interventions to overcome the unnatural perception barrier.

5. Conclusions

This study offers the first insights into consumer responses to the appearance of new pigmented mandarins, which may be used to create practical interventions that allow this novel fruit to be more readily accepted.

Our study corroborates that cultural background affects how food products are perceived and, thus, consumer behaviour towards them. Blood orange familiarity is revealed as a conditioning factor for consumer response to pigmented mandarins.

The herein obtained information will be useful for both breeders and the citrus industry. The identified consumer rejection of the most intense pigmented varieties can help breeders to set their objectives as regards colouration. Industry can use this information for marketing purposes. From a commercial point of view, a better understanding of how consumer perceptions differ between groups will help traders to implement the most effective interventions according to specific markets.

Consumer perceptions that new varieties are unnatural have been identified as the main barrier. Interventions should be made to inform consumers that these varieties have been obtained by conventional breeding, which is expected to positively influence their response. Evaluating the effect of such information and exploring the best way to convey it to consumers are interesting topics for future studies.

At this point, it is necessary to evaluate consumer responses to other sensory characteristics, such as odour, taste and texture, because the ultimate consumer acceptance of pigmented mandarins will depend on all perceptions, and not only those captured by the eye. Thus, sensory studies should include a selection criterion in current breeding programmes.

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CHAPTER IV

Understanding Blood versus Blond Orange Consumption: A Cross-Cultural Study in Four Countries

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Abstract

Understanding consumer perceptions and attitudes to specific fruit is key information for not only increasing fruit consumption, but also for marketing reasons. It may also give clues to breeders to set quality objectives. This study explores different aspects that help to explain blood vs. blond orange consumption: availability and consumption habit, satisfaction attributes, facilitators and consumption barriers, consumption contexts, expectations and purchase intention. The study was conducted in China, Mexico, Spain and Italy, where citrus fruit consumers were invited to respond an online questionnaire. Our results revealed Italy as the country with the highest availability and consumption of blood oranges, followed by China, Mexico and Spain. “Liking” and “healthy properties” were the most important reasons for consumption irrespective of orange type, but certain differences among countries were detected in secondary reasons. In all the countries, “juicy” was the most relevant attribute for consumer satisfaction, followed by flavour/taste attributes. “Aromatic” and “unfibrous” were substantial requirements for Italians and Chinese, while Spaniards attached importance to the blood oranges colour. Regarding consumption contexts, “eat with salt or chilli powder” was specific for Mexico, while “to improve health”, “as a gift” or “at a restaurant” were contexts mainly cited in China. Despite taste preferences for other fruit being the main consumption barrier in all the countries for both orange types, the relevance of other barriers depended on culture and orange type. Mexican participants seemed to take a more neophobic attitude to blood oranges, while “inconvenient” was reported as a barrier for consuming blond ones in Spain and China. We conclude that blond and blood oranges can co-exist on markets at a high consumption rate, as in Italy. Specific interventions are needed in other countries because consumer attitudes to oranges, mainly blood ones, depend on culture.

Keywords: consumption contexts; barriers; facilitators; expectations; purchase intention; organoleptic properties

1. Introduction

Among fruit species, citrus fruit represents the major fruit tree crop in the world, with 10 million of harvested hectares and global estimated production close to 158 million tons (FAO, 2022). In quantity terms, orange (*Citrus sinensis* (L.) Osbeck) production is the most important of the main citrus species (close to 4 million of harvested hectares and 75.5 million tons of production). However, orange production has remained relative stable in the last 10 years, while production of other citrus fruit such as mandarins has remarkably increased.

One strategy recently adopted by the citrus industry to build momentum on the oranges market is the promotion of blood oranges. Consumers' awareness about the effect of food on their health has substantially increased in the last decade (Bradford *et al.*, 2021; Carl *et al.*, 2021), and in these oranges with flashy pigmented pulp, the citrus industry has found an opportunity to respond to consumer demand for healthier food. Blood orange varieties are highlighted, in relation to blond ones, for their high content of the bioactive compound anthocyanin, which confers fruit red pulp colouration (Lo Piero, 2015; Fallico *et al.*, 2017). Anthocyanin may help to prevent diabetes, heart disease and viral activity, and has been shown to have promising anticancer properties (Grosso *et al.*, 2013).

Traditionally, blood oranges have been mainly cultivated in Sicily, where environmental conditions promote fruit colouration (Zarbà and Pulvirenti, 2006), and have been commercialised all over Italy, where they are well-placed on markets. Currently, the popularity of these oranges is growing worldwide (Simons *et al.*, 2019). Breeding programmes that focus on blood varieties have been established (Rapisarda *et al.*, 2009; Russo *et al.*, 2015), and the number of research works about their physico-chemical and sensory properties is growing all around the world (Habibi and Ramezanian, 2017; Lu *et al.*, 2018; Cebadera-Miranda *et al.*, 2019; Simons *et al.*, 2019; Ávila *et al.*, 2020; Modica *et al.*, 2022).

Consumers' experience of orange purchases must be inevitably affected by the expansion of the offer with red-pigmented varieties that, in certain countries, may be a novelty. Two recent studies have approached consumers' acceptance of blond and blood oranges based on organoleptic properties. Ávila *et al.* (2020) after performing an acceptance test with 100 Brazilian volunteers reported strong acceptance and positive perception of consumers in relation to the most sensory attributes of blood oranges. According to the results from a study including 224 Californian people (152 adults and 72 children) (Simons *et al.*, 2019), lighter coloured varieties, such as "Cara Cara", "Tarocco", "Boukhobza" and "Shahani", were liked by the majority of consumers, and were low in less desirable sensory characteristics, such as bitterness and sourness, than the most pigmented ones.

Despite the indubitable value of sensory studies, many consumer experience aspects cannot be measured or described by them (Kurzer *et al.*, 2019). Thus, aspects other than sensory perception that affect consumers' consumption experience need to be investigated. Along these lines, after performing a word association test with Brazilian consumers, Ávila *et al.* (2020) reported that they showed mainly positive attitudes to blood oranges. In a study in which Californian adults and children gave their views of oranges versus mandarins, consumers showed an interest in varieties that can be identified by their aspect, such as the blood orange "Cara Cara" (Kurzer *et al.*, 2019).

Overall, the few existing works on this matter show that blood oranges represent a promising category for the citrus industry and warrant further investigation (Simons *et al.*, 2019). In the current context of markets internationalisation, to maintain the blond orange market position and to successfully introduce blood varieties, a better understanding of their consumption is needed. Therefore, it is necessary to investigate not only consumer expectations with regard to quality, but also other aspects of consumption experiences, such as when, why and why not (Scholliers, 2015) each orange type is consumed.

It is also important to bear in mind that all these aspects of consumers responses are strongly influenced by the cultural background (Chung *et al.*, 2012; Galmarini *et al.*, 2013; Tarancón *et al.*, 2021a) which may, in some cases, be linked with the level of familiarity with similar products (Jamir *et al.*, 2020). Cross-cultural studies allow differing patterns among consumer populations to be identified, which leads to a better understanding of global market sand can help breeders and industry to adapt their product depending on its destination market (Galmarini *et al.*, 2013). In other commodities, acquiring knowledge from consumers about different aspects associated with consumption has proven to be key for production purposes and developing communication strategies (Dibsdall *et al.*, 2003; Sijtsema *et al.*, 2012; Ergönül and Ergönül, 2015; Phan and Chambers, 2016).

Studying consumer attitudes to a product, while covering a range of different aspects, usually implies the need to combine different types of questions in the same questionnaire. This is the case of using multiple-choice questions and Likert scales, which are useful tools to collect a range of information in cross-cultural studies (Martins *et al.*, 2022). Multiple-choice questions allow participants to check multiple responses for a list of options and have been used to evaluate, among others, consumption contexts (Tarancón *et al.*, 2021b), label information required by consumers (Fernández-Serrano *et a*l., 2020) or consumer behaviour with regard to food storage at home (Wucher *et al.*, 2020). Likert scales are appropriate for evaluating intensities of different aspects, such as sustainability perception (Fernández-Serrano *et al.*, 2022), food neophobia (Dovey *et al.*, 2008) or the importance of food characteristics for choices in different contexts (Verain *et al.*, 2020).

With all this in mind, and to gain a deeper understanding of consumers' consumption of blond and blood oranges, this study investigates their market availability, the importance of different sensory attributes for consumer satisfaction, and consumption contexts and consumption barriers in four different countries: China, Mexico, Spain and Italy. Of these countries, China, Mexico and Spain (each one situated in a different continent) are the world's first, fourth and sixth largest citrus fruit producer countries, respectively. Italy, located in Europe such as Spain, occupies thirteenth position, but it stands out for its long-standing tradition in blood orange production and consumption.

2. Materials and Methods

2.1.Consumer Sample

Six hundred and five people of each nationality, all of legal age, participated in this study. Sampling was carried by members of all the four participating institutions (Valencian Institute for Agricultural Research from Spain, the *Università degli Studi di Palermo* from Italy, the *Universidad Autónoma Metropolitana* from Mexico and the China National Institute of Standardization). Participants were recruited by several approaches: street surveys using a tablet device or a QR with a link to the survey, and student mailing lists of the four institutions. Snowball recruitment (word-of-mouth) was also adopted using interpersonal relations and connections among consumers to reach a large number of participants. Thus, participants were requested to invite family, friends and/or colleagues to participate by forwarding the online survey link. Only the people who reported consuming citrus fruit (whether fresh or otherwise) at least once a month during the commercial season were invited to participate. Consent indicating voluntary participation was obtained from each participant at the beginning of the questionnaire. They were shown the following statement: “If you agree to participate and allow us to use your answers for this study, please click next”.

Table 1 shows the demographic information of each nationality. The recruitment of participants was carried out with the aim to obtain a similar percentage of responses from young adults (18–30 years old), middle-aged adults (31–50 years old) and late-aged adults (more than 50 years old). Moreover, the proportion of men and women was similar in all four countries, with a percentage of women between 58–60%. In educational attainment terms, the proportion of respondents with a bachelor’s degree was higher among the four nationalities and was the equivalent to 65–74%.

Table 1. Demographic data of the participants from each country expressed as a percentage.

		Spain	Italy	China	Mexico
Gender	Male	37.9 %	39.5 %	42.3 %	31.7 %
	Female	62.1%	60.5 %	57.7 %	68.3 %
Age	18-30	31.7 %	20.7 %	33.4 %	37.2 %
	31-50	37.5 %	39.3 %	33.4 %	42.3 %
	>50	30.7 %	40.0 %	33.2 %	20.5 %
Consumption	Almost every day	52.0 %	52.4 %	30.0 %	20.2 %
	2 or 3 times a week	30.4 %	25.7 %	29.7 %	44.8 %
	2 or 3 times a month	17.6 %	21.8 %	40.3 %	35.0 %
Education	Elementary	6.0 %	0.2 %	3.0 %	4.0 %
	Secondary	20.2 %	29.9 %	31.4 %	23.8 %
	Bachelor's degree	73.8 %	69.9 %	65.5 %	72.2%

2.2. Survey Design

This study was based on an online questionnaire via the Google Forms platform (Google LLC, Mountain View, CA, USA) in the case of Spain, Italy and Mexico, and via Wenjuanxing (Changsha Ranxing Information Technology Co., Ltd, China) in the case of China. The online survey included different sections that were answered by the participants in relation to blond and blood oranges.

In order to contextualise the participants, pictures of blond and blood oranges were shown to them before they answered the questions (Figure S1). To avoid possible bias due to the presentation order, half the consumers were firstly shown blond oranges and their corresponding questions, while the other half started the questionnaire with blood oranges.

Section 1. Availability and consumption

In the first section, consumers were asked about the availability of oranges in their usual shopping place and their consumption habit by means of a multiple-choice question. The provided options were: (1) “They are available where I usually buy fruit. I usually consume them”; (2) “They are available where I usually buy fruit, but I don’t usually consume them”; (3) “They are not available where I usually buy fruit”. The participants were then guided to the second questionnaire section, which depended on the response they gave to the initial question, and according to the scheme shown in Figure 1 and Table S1.

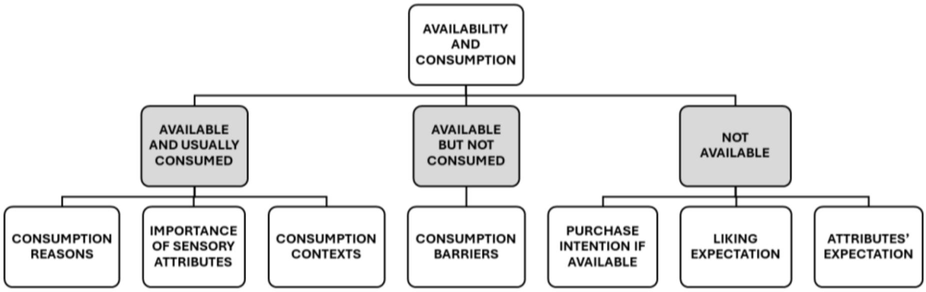

Figure 1. Scheme of the main questionnaire sections.

Section 2A. Reasons to consume, sensory attributes’ importance and consumption context

The consumers who stated that blond/blood oranges are available in their shopping place and they usually buy them, were then requested to answer three questions in which we investigated the reasons to consume, sensory attributes’ importance and the consumption contexts of the two orange types.

In order to know the main reasons for consuming blond and blood oranges, the participants were asked the following question: “What are your main reasons for consuming these oranges (blond oranges/blood oranges)? Check all that apply”. The multiple-choice list of the possible answers included: “I like them a lot”, “Because of their healthy properties”, “Because of their affordable price compared to other fruit”, “They last longer than other fruit” and “They are more convenient (easier to carry, to eat, etc.) than other fruit”. The participants had the possibility of adding another reason for consuming them with the option “others”.

After that, this group of participants was requested to answer the following question: “Imagine that you are eating the oranges (blond orange/blood orange) that you have just bought. How important are the following characteristics to buy them again?” They had to rate the importance of certain sensory attributes to buy the fruit again using a 7-point Likert scale, where 1 = not important at all, 7 = very important (Galati *et al.*, 2019). The attributes to evaluate were: “being seedless”, “being easy to peel”, “not being messy when eating”, “being sweet”, “a balance between sweetness and acidity”, “being aromatic”, “having intense flavour”, “being juicy”, “not being fibrous (easy to chew)” and “having an intense pulp colour”.

After rating the sensory properties, the participants were asked about the main consumption contexts by means of the following multiple-choice question: “In which of these contexts do you consume or do you think it is appropriate to consume these oranges (blond oranges/blood oranges)? Check all that apply”. The provided options were: “when I am trying to lose weight”, “as a gift”, “when I want to improve my health”, “as part of breakfast”, “as a snack at home”, “as a snack when I’m not at home (at work, university, travelling, at a picnic, etc.)”, “In a school lunchbox”, “as dessert for lunch or dinner at home”, “at a restaurant”, “to make fresh juice or smoothies”, “to make cocktails”, “as a cooking ingredient (for salad, baking, sauces, etc.)”, “to eat with salt or chilli powder” or “to eat with sugar”. They also had the possibility of adding another possible consumption context through the option “others”.

Section 2B. Consumption barriers.

The participants who indicated that they do not usually consume fruit, even though it is available in their regular purchase place, were requested to indicate their main purchase barriers by answering the multiple-choice question that follows: “What are the main reasons for you not consuming this kind of oranges (blond oranges/blood oranges)? Check all that apply”. The available options were: “I prefer the taste of other fruit”, “I have never tasted it”, “I have tasted it, but don’t like it”, ‘It’s too expensive”, “I think they are not natural", "I don't like the way it looks", "I don't know how to eat/prepare it" and "I think they are not safe". They had the possibility of adding another reason through the option "others".

Section 2C. Liking expectations and purchase intention.

Finally, the consumers for whom fruit is not available in their shopping place had to answer a different set of questions.

They were asked about their expected liking and purchase intention if fruit were available. The questions were formulated as follows: "If these oranges were available where you usually buy fruit, would you buy them?" and "How much do you expect to like this fruit?". The scales to respond were on a 5-point scale ranging from 1 - "I definitely would not buy" to 5 - "I definitely would buy" and on a 7-point hedonic scale where 1 = I will dislike it very much and 7 = "I will like it very much", respectively.

Further, to know their perception and expectations about fruit, they were requested to answer the next multiple-choice question: "What do you think about this fruit? Check all that apply". The response options were the following: "it's very healthy", "it's natural", "it's not safe for eating", "it's appealing", "it's a new fruit", "it comes from another country", "taste will be similar to other citrus fruit", "taste will be surprising", "it will be sweet", "it will be acid", "it will be bitter" and "it will be more expensive than other fruit". Participants had the option to add more reasons through the option "others".

The lists of options presented to the participants in the different multiple-choice questions (reason for consumption, sensory attributes, barriers, contexts and expectations) were preliminarily created by the Spanish research group based on the previous literature about citrus and other fruit (Onwezen and Bartels, 2011; Sijtsema *etal.*, 2012; Phan and Chambers, 2016; Silvestri *et al.*, 2016; Ingrassia *et al.*, 2017; Iofrida *et al.*, 2019; Jaeger *et al.*, 2019; Di Vita *et al.*, 2020; Fernández-Serrano *et al.*, 2020; Tarancón *et al.*, 2020, 2021a, 2021b; Verain *et al.*, 2020; Giménez-Sanchis *et al.*, 2021; Kasprzak *et al.*, 2021) (Table S2). The initial lists, which covered the most relevant options according to the existing literature, were completed by performing a test run with 10 consumers to add any missing option relevant for them. The lists generated in Spain were checked and completed by researchers of the other three countries and then pre-tests were carried out with the consumers from the different countries to add country-specific options. Thus, for example, in the contexts section, options "to eat with salt or chilli powder" and "as a gift" were added by request from Mexican and Chinese consumers, respectively. The presentation order of the list terms was balanced among consumers to avoid bias.

The last questionnaire section (Section 3) was about demographic data: gender, age and level of education.

The final questionnaire was originally generated in Spanish by the Spanish research group and underwent a pilot trial with consumers (n = 10) to check for errors, ambiguity, logical flow of questions and completion time. The questionnaire was directly sent to Mexico where it was pretested with 10 consumers.

In parallel, a professional translator translated the questionnaire into English before sending it to Italy and China. The native members of the participating research institutions with good knowledge in English translated the questionnaire into Italian and Chinese. The questionnaires in each language were then carefully assessed and back-translated into English by different researchers from each country to ensure that the meaning of the original content was maintained and the four versions were consistent with one and other. Before releasing the final questionnaire, it was pretested in Italy and China with 10 consumers.

The protocol and procedures used in this study were revised by the scientific directorate of the Valencian Institute for Agricultural Research, which stated a waiver consent. All articles from the Declaration of Helsinki and the 2016/679 EU Regulation on the protection of natural persons regarding the processing of personal data and on the free movement of such data were met.

2.3. Statistical Analysis

To analyse the data, different non-parametric tests were performed. The comparison of k-proportion test (procedure of Marascuilo and the chi-squared test, $p < 0.05$) was used to compare the availability and consumption data. The two-proportion z-test ($p < 0.05$) was used to compare the frequencies of mentioning of reasons to purchase in each country depending on the orange type. The Kruskall–Wallis test followed by Dunn's multiple comparisons test were applied to evaluate for each country and orange type the differences in importance for repurchase among attributes ($p < 0.05$).

A correspondence analysis (CA) was carried out with the percentages of participants from each country who mentioned each consumption context (of the participants who stated consuming each orange type), purchase barrier (of those who stated not consuming) and expectations (of those who stated not having orange availability) to visualise the more relevant differences among the four countries and between the two orange types. All the analyses were performed with XLStat 2019 (Addinsoft, Paris, France).

3. Results and Discussion

Availability has been reported as a prime factor for determining the consumption of specific fruits (Konopacka *et al.*, 2010). Thus, a question about blond and blood oranges availability in the habitual fruit shopping place and consumption habit was the starting point of our questionnaire. Then depending on the response to the initial question, the participants were guided to different sections to investigate some of the main aspects of consumer behaviour with regard to oranges: (a) reasons to consume, sensory attributes importance and consumption contexts; (b) barriers to consume; (c) expectations and purchase intention.

3.1. Availability and Consumption Habit

The results of the availability and consumption rate for blond and blood oranges are shown in Table 2. Blond oranges availability on markets was almost 100% in the four countries under study, except for China where 3% of the participants stated that blond oranges are not available where they usually buy fruit. The lowest percentage of consumption was also detected in this country. Seventy-four percent of the Chinese participants were habitual consumers of blond oranges. In Spain, Italy and Mexico, the consumption rate was higher than 85%.

The data about blood oranges revealed a very different picture compared to the blond ones. Italy was by far the country with the highest availability (98%) and the highest consumption rate (76%). China occupied second position for both availability (83.5%) and purchase rate (46%). In Mexico and Spain, availability was much lower because only 50% of the participants can buy blood oranges on their habitual markets. Of the four studied countries, the lowest consumption was detected by far in Spain, where only 17% usually consume blood oranges.

Our data revealed a similar pattern among countries for blond oranges, but a very different one for blood ones. The data from both kinds of oranges taken together confirmed that availability is a prime factor for the consumption of specific fruits, as previously reported by Konopacka *et al.* (2010).

Table 2. Percentage of participants from each country (ES-Spain, IT-Italy, MX-Mexico, CN-China) who stated that blond oranges/blood oranges were available in their habitual purchase place (Avail. column). The 'Cons.' column indicates the percentage of them who stated consuming oranges habitually, and 'Not Cons.' was the percentage who stated not consuming them.

	Blond oranges			**Blood oranges**		
	Avail.	Cons.	Not Cons.	Avail.	Cons.	Not Cons.
ES	99.2 b	85.5 b	13.7 b	48.6 a	17.2 a	31.4 b
IT	99.8 b	91.6 c	8.2 a	98.0 c	75.9 d	22.1 a
MX	99.8 b	89.6 bc	10.2 ab	50.7 a	34.2 b	16.5 a
CN	97.0 a	73.9 a	23.1 c	83.5 b	46.1 c	37.4 b

Different letters in the same column indicate significant differences among countries (comparison of k proportions-test, p-value = 0.05).

As explained in the Introduction, blood oranges are a traditional crop in Italy, which is the first growing country and the origin of these pigmented fruits (Zarbà and Pulvirenti, 2006). Accordingly, our data showed that both availability and consumption rate were much higher in this country than in the other three. Furthermore, the data indicate that Italy represents a scenario in which blond and blood oranges naturally co-exist on markets.

3.2. Reasons to Consume, Sensory Attributes' Importance and Consumption Context

3.2.1. Reasons to Consume

The relevance of identifying reasons to consume (facilitators) and fruit consumption barriers is well-known but, until now, it has been mostly approached by considering fruit to be a whole group of food rather than evaluating specific commodities (Ylitalo *et al.*, 2019; Kasprzak *et al.*, 2021).

In order to identify the main facilitators for orange consumption, we asked those participants who stated that they consume them habitually about the main reasons for doing so. Our data revealed that, irrespective of country, the core drivers to consume both blond and blood oranges are liking ("I like them a lot") and health benefits ("Because of their healthy properties") (Table 3).

Table 3. Percentage of participants from each country (of those who stated consuming each orange type habitually) who selected each reason to consume. Conven.-Convenience. Durab.-Durability.

Spain		Italy		Mexico		China	
Blond	**Blood**	**Blond**	**Blood**	**Blond**	**Blood**	**Blond**	**Blood**
Liking (84.4 %)*	Liking (72.1 %)	Liking (87.5 %)	Liking (85.4 %)	Liking (83.4 %)	Liking (85.0 %)	Liking (74.2 %)	Liking (73.7 %)
Health (68.3 %)*	Health (52.9 %)	Health (57.8 %)	Health (53.3 %)	Health (64.4 %)	Health (58.9 %)	Health (61.2 %)	Health (65.5 %)
Durab. (34.9 %)*	Durab. (21.2 %)	Price (11.3 %)*	Price (7.0 %)	Price (49.3 %)*	Price (39.6 %)	Price (37.7 %)*	Conven. (32.7 %)
Price (31.9 %)*	Conven. (18.3 %)	Conven. (9.5 %)	Conven. (6.3 %)	Conven. (46.3 %)*	Conven. (36.2 %)	Conven. (36.3 %)	Price (29.5 %)
Conven. (29.6 %)*	Price (8.7 %)	Durab. (8.5 %)*	Durab. (4.3%)	Durab. (36.2 %)*	Durab. (24.2 %)	Durab. (28.9 %)	Durab. (24.8 %)

*Indicates significant differences between blond and blood oranges (z test, p-value=0.05).

In Mexico and China, the frequency of mentioning the other three reasons was similar and ranged among 25–50%. After health benefits, the importance of reasons was as follows: price, convenience and durability. In Mexico, the percentage of consumers who cited all these three reasons was slightly higher for blond than for blood oranges. In China, only "affordable price" seemed to be slightly more important for buying blond than blood oranges.

The ranking of reasons for the Italian consumers was the same as in Mexico and China. However, it is important to highlight that the percentage of consumers who cited price, convenience and durability reasons was much lower in Italy. This result revealed that liking and healthy benefits are indisputably the most important reasons for Italians buying oranges. In this study, the data from Italians were obtained mainly but not exclusively from consumers living in a citrus-producing region (Sicily). The herein identification of liking and healthy benefits as core drivers for consumption is in accordance with previous studies performed in different areas around Italy (Ingrassia *et al.*, 2017; Iofrida *et al.*, 2019; Di Vita *et al.*, 2020). Thus, these studies revealed the "healthy identity" of citrus fruit and its pleasant taste as highly relevant attributes for Italians, while affordable price and ease of peeling were not relevant.

Finally, a different ranking order of the secondary reasons was detected in Spain, where consumers attached more importance to oranges' durability than in the other countries. In this country, the percentage of participants who selected all five reasons was higher for blond than for blood oranges. This difference was especially evident for "affordable price", which is explained by the fact that blood oranges in Spain are generally more expensive on markets that blond ones.

The results of this study support previous findings that have demonstrated the importance of healthiness and taste for consumer food choice (Roininen *et al.*, 1999; Ingrassia *et al.*, 2017; Iofrida *et al.*, 2019; Di Vita *et al.*, 2020). Along these lines, in a cross-cultural study that included several European countries, Onwezen and Bartels (2011) identified four consumer segments based on their reasons for choosing fruit and reported natural and healthy as the main reasons for one of the groups, while healthy and taste were the most important reasons for fruit choice in three of the four identified profiles.

Despite the consensus among countries about identifying liking and health as prime reasons for blond and blood orange consumption, our results show relevant differences among countries with regard to the other investigated reasons. So, the number of Italian consumers for whom price, convenience and durability were the reasons for choosing oranges was significantly smaller than in the other countries. Familiarity with the product did not seem to explain such differences because the result was equally observed for blond and blood oranges, while difference in familiarity among Italy and the other countries was detected only for blood ones. Therefore, the importance of the different reasons for food choice seems to be influenced by cultural factors beyond familiarity, as previously reported by Nielsen *et al.* (1998) and Prescott *et al.* (2002).

3.2.2. Sensory Attributes' Importance for Repurchase

One of the reasons for lower than recommended fruit consumption is that the fruit market does not always meet consumer expectations (Onwezen and Bartels, 2011). Therefore, providing markets with fruit that satisfy consumers could help with the challenge of increasing a global population's fruit consumption. To fulfil this objective, it is necessary to understand consumer expectations in terms of sensory properties while bearing in mind that such expectations may differ among populations from different countries (Galmarini *et al.*, 2013).

Consumer satisfaction is key for repurchasing a product (Hidayat *et al.*, 2020). Thus, in this study, we asked consumers to rate the importance of finding different sensory properties in each orange type in their repurchasing intention. The results of this question are shown in Table 4, in which the attributes are ranked according to their importance score. Mean values of importance scores are shown in detail in Table S3. As we can see, irrespective of country and orange type, consumers agreed that "juicy" was the most important attribute for their satisfaction.

Table 4. Ranking of sensory attributes' importance for consumer satisfaction after eating blond/blood oranges. Importance was scored in a 7-point scale (1—not important at all, 7—very important) by Spanish, Italian, Mexican and Chinese consumers (n = 605 in each country).

Spain		Italy		Mexico		China	
Blond	**Blood**	**Blond**	**Blood**	**Blond**	**Blood**	**Blond**	**Blood**
Juicy [a]	Juicy [a]	Juicy [a]	Juicy [a]	Juicy [a]	Juicy [a]	Juicy [a]	Juicy [a]
Flavour [b]	Sweet [ab]	Flavour [a]	Flavour [b]	Sweet [b]	Sweet [ab]	Sweet [ab]	Sweet [ab]
Balance [b]	Balance [ab]	Aromatic [b]	Aromatic [c]	Balance [bc]	Flavour [bc]	Unfibrous [b]	Aromatic [ab]
Sweet [c]	Flavour [ab]	Balance [b]	Sweet [c]	Flavour [c]	Balance [bc]	Aromatic [bc]	Unfibrous [ab]
Aromatic [d]	Aromatic [ab]	Sweet [b]	Balance [c]	Aromatic [d]	Aromatic [cd]	Flavour [bc]	Balance [ab]
Unfibrous [d]	Colour [bc]	Colour [c]	Colour [d]	Unfibrous [d]	Unfibrous [d]	Balance [bc]	Flavour [ab]
Easy Pe [e]	Unfibrous [c]	Unfibrous [d]	Unfibrous [e]	Colour [e]	Colour [d]	NoMessy [c]	NoMessy [bc]
Seedless [e]	Easy Pe [d]	Easy Pe [d]	Easy Pe [e]	Easy Pe [f]	Easy Pe [e]	Easy Pe [c]	Easy Pe [bc]
Colour [e]	Seedless [d]	Seedless [e]	Seedless [f]	NoMessy [g]	Seedless [f]	Seedless [d]	Seedless [c]
NoMessy [f]	NoMessy [e]	NoMessy [f]	NoMessy [g]	Seedless [g]	NoMessy [f]	Colour [e]	Colour [c]

Different letters in the same column indicate significant differences among attributes' importance (Kruskall–Wallis test, p-value = 0.05).

Spaniards and Mexicans agreed in considering that three attributes related to flavour/taste ("intense flavour", "sweet" and "balanced sweet-acid") were the most important attributes after "juicy" and reported "being aromatic" and "not being fibrous" as the next most important attributes. However, the Italian and Chinese found the "being aromatic" attribute to be equally important to those linked with taste/flavour. Smell has been previously reported as a relevant attribute for Italian consumers (Di Vita *et al.*, 2020). Furthermore, for Chinese consumers the texture attribute "unfibrous" was much more relevant than in other countries and was equally important as the flavour/taste and "aromatic" attributes.

"Being easy to peel", "being seedless" and "not being messy when eating" are attributes particularly linked with fruit convenience (Wu *et al.*, 2009; Kakade *et al.*, 2017; Kurzer *et al.*, 2019). In general, these three attributes were ranked together and were less important to consumers than the other attributes.

The attribute in which the consumers from the different countries differed the most in importance terms was "having an intense pulp colour". In Italy, Mexico and China, there were no differences between blond and blood oranges. For the Chinese consumers, it was the least important attribute, while for the Italian and Mexican consumers, it was more important than the group of convenience attributes ("being easy to peel", "being seedless" and "not being messy when eating"). Spain was the only country where clear differences were observed in the importance of colour between the two orange types because the Spaniards considered colour to be more important than the convenience attributes in blood oranges, but not in blond oranges.

These results fall in line with recent studies on mandarin fruit in which attributes, such as "juicy", "sweet" and "sour", were identified as drivers of liking for Spanish and Mexican consumers (Tarancón *et al.*, 2020, 2021a). By comparing the results of these two studies, which focused on mandarins, to the results herein obtained, it seems that "easy to peeling" is a more relevant attribute for mandarins than for oranges. Along these lines, after investigating what kind of sensory information consumers would like to receive when buying different types of fruit, Fernández-Serrano *et al.* (2020) found that the juicy, flavour/taste related attributes and easy peeling were the most relevant information for citrus fruit as a group.

Drivers of liking have been traditionally identified by means of sensory tests in which liking scores given by consumers are related to the sensory attributes perceived by a trained panel or consumers themselves after tasting fruit (Ares *et al.*, 2014; Tarancón *et al.*, 2020; Giménez-Sanchis *et al.*, 2021). In this study, evaluating the importance of different attributes by means of an online questionnaire filled in by consumers without testing any sample led to similar results to existing studies with sensory tests. Our approach based on an online questionnaire is very similar to that followed by Galmarini *et al.* (2013) to evaluate the attributes that define apple quality for Argentinean and French consumers. As in our case, those authors found some differences among countries, mainly in the importance of visual aspects, because they were more important for Argentinean consumers, while the French ones showed more interest in taste-related attributes.

To go one step further, we investigated if the satisfaction attributes depended on the gender and age of the participants. Results from ANOVA analysis are shown in Figures S2–S5. Regarding gender influence (Figures S2 and S3), it is worth to comment that a gender effect was detected in the importance that Italians and Mexicans gave to the three convenience attributes (easily peeling, no messy and seedless). Curiously enough, women from Italy gave less importance than men to these three attributes, while an opposite pattern was observed in Mexico.

As far as the participants age is concerned, the main effect was detected in Spain, where the youngest consumers (13–30 years old) gave lower importance scores to all sensory attributes except the flavour intensity and juicy (Figures S4 and S5).

3.2.3 Consumption Contexts

The role of consumption contexts in purchase is perhaps less obvious than that of facilitators or sensory properties, but there are reports showing that the final decision to buy or consume a particular food depends as much on the anticipated usage context as it does on intrinsic product properties (Giacalone, 2019). So recent studies on fruit have paid attention to it (Jaeger *et al.*, 2019; Tarancón *et al.*, 2021b).

For both orange types, the more frequently mentioned contexts for the Italian, Spanish and Mexican consumers were “as a dessert for lunch or dinner at home”, “as part of breakfast”, “as a snack at home” and “as a snack when I’m not at home (at work, university, travelling, at a picnic, etc.)” and “to make fresh juice or smoothies” (Table S4). For Chinese people, these five contexts were relevant, but “when I want to improve my health” was also among the most cited contexts and occupied second position.

For a profounder evaluation of the main similarities and differences among countries, a CA was performed (Figure 2). The first result of this analysis to highlight is that for the four countries, blond and blood oranges were very close in the CA space. This indicates that consumption contexts did not depend on oranges being blond or blood.

The Spain and Italy samples were allocated very close to one and other, and very close to the origin on the first dimension and with negative values on the second one. The contexts “as part of breakfast”, “as a dessert for lunch”, “to make fresh juice or smoothies”, “as a cooking ingredient” and “to eat with sugar” were characteristics of these two countries compared to Mexico and China. Consuming oranges or orange juice as part of the breakfast has been previously reported as a main consumption context in Spain and Italy (Di Vita *et al.*, 2020; Tarancón *et al.*, 2021b).

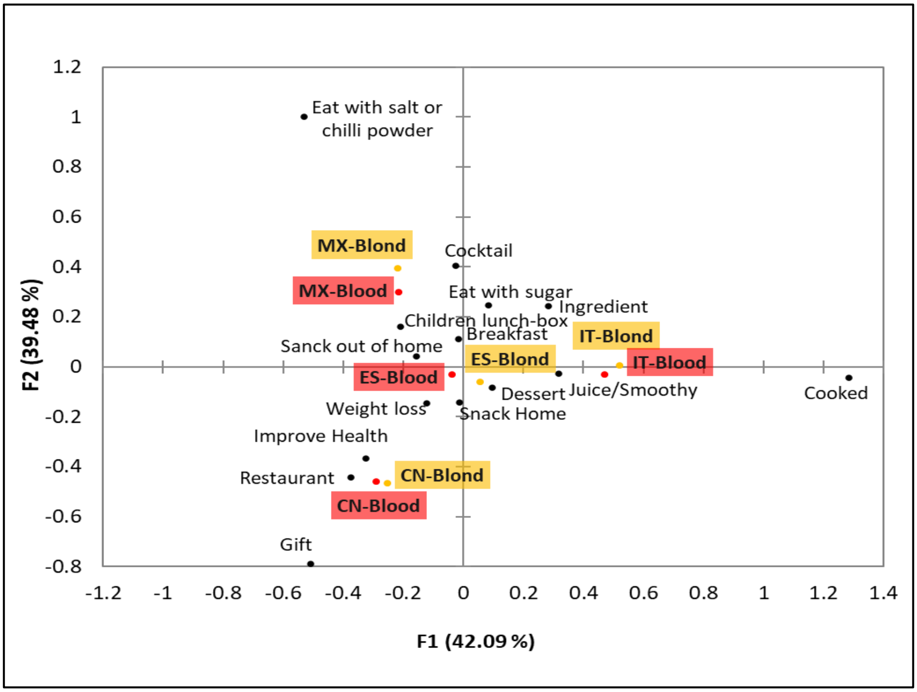

Figure 2. Correspondence analysis for the consumption contexts. The data used to perform the analysis were the percentage of participants (of those who stated consuming each orange type habitually) who selected each context. ES-Spain, IT-Italy, CN-China, MX-Mexico.

The Mexico samples gave positive values on the second coordinate and negative values on the first one. This position on the CA plot was explained by the contexts "to eat with salt or chilli powder" and "to make cocktails", which were mainly mentioned in this country compared to the other three. Finally, consumption of oranges in China was much more frequent "as a gift", "when I am trying to lose weight", "at a restaurant" and "when I want to improve my health".

Differences among countries in consumption contexts were more marked than those detected in facilitators for purchase or sensory attributes' importance. Thus, some contexts seemed specific for certain countries. This was the case of "with salt and chilli powder" in Mexico and "as a gift" in China.

According to Gutiérrez and Simon (2016), the main reason why spicy food was more frequently consumed in hot climate countries like Mexico in the past is because of the antibacterial characteristics of spices that contribute to people's health. However, these authors suggest that presently, people like spicy food not for its historical antimicrobial properties, but because it is rooted in their culture.

Tradition seems to also be linked with the Chinese habit of giving fruit as a gift. According to Vasil'eva and Chibisova (2017), to offer a live orange tree or its fruit as a gift is an ancient tradition in China, meaning a wish of happiness, wealth and success. In the same line, a traditional Chinese value is to regard food as ''medicine'' (Prescott *et al.*, 2002), which would explain why consumption contexts like "when I am trying to lose weight" and "when I want to improve my health" were more frequent in China than in Mexico or European countries.

3.3. Consumption Barriers

Barriers, as defined by Yeh *et al.* (2008), are factors that inhibit consumption. Identifying barriers is important for designing behaviour change interventions to increase the consumption of specific food (Nicklas *et al.*, 2013), and for industry to anticipate potential obstacles and mitigate their impact.

Table S5 shows the percentage of consumers who cited each consumption barrier. We can observe that the main barrier stated by all consumers, irrespective of country and orange type, was "I prefer other fruit". In Spain, Italy and Mexico, this barrier was by far the most cited one. However, in China, it was cited by fewer consumers, and was closely followed by the "Too expensive" barrier, mainly for blood oranges. Previous studies investigating fruit consumption barriers as a whole group of food have demonstrated that taste preferences for other products are among the common self-reported barriers to eat fruit (Livingstone *et al.*, 2020). Moreover, in a recent review about fruit and vegetable consumption in east and southeast Asia, Cheung *et al.* (2021) found that financial concerns stemming from high prices of fruit and vegetable are major barriers for their consumption.

It is important to clarify that "Because it is inconvenient" was not initially included in the list of barriers for the participants to check. However, as it was indicated by means of the "Others" option by a significant percentage of respondents from China and Spain, we decided to include it in the results.

Despite taste preferences being the most cited barrier in all the countries, the CA plot revealed certain differences in the relative importance of this and other barriers depending on culture and orange type (Figure 3). Thus, the samples for which taste preferences were by far the most important barrier were allocated on the left of the CA plot, while those samples for which the relative importance of this barrier was not as marked were allocated to the right of the space.

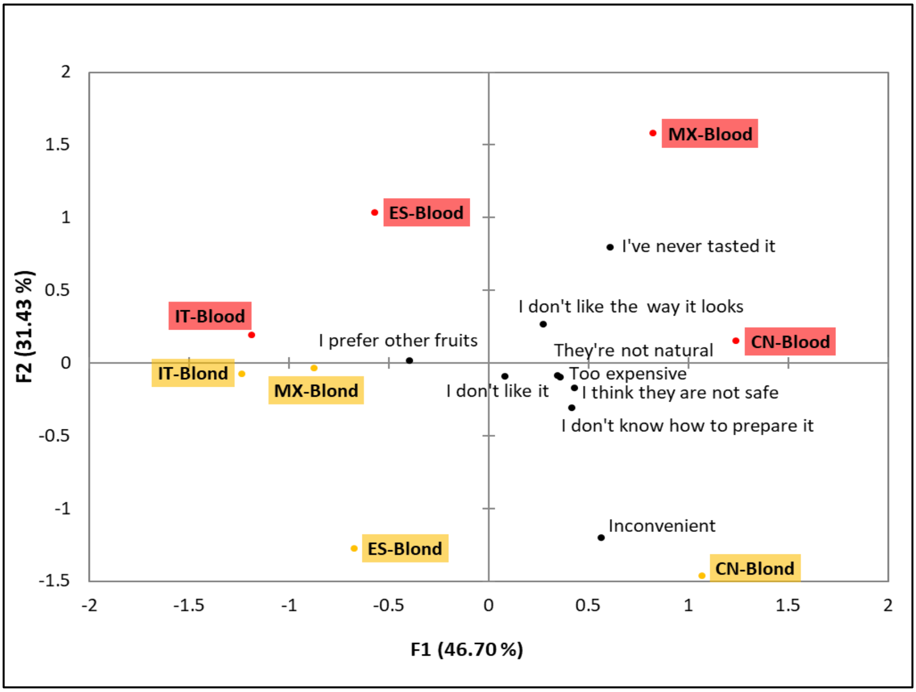

Figure 3. Correspondence analysis for the consumption barriers. The data used to perform the analysis were the percentage of participants (of those who stated not consuming each orange type habitually) who selected each barrier. ES—Spain, IT—Italy, CN—China and MX—Mexico.

Italy was the only country for which blond and blood oranges allocation on the CA plot was very close. Thus, taste preferences were indisputably the main barrier for both orange types in Italy.

Samples from Spain were also allocated in the left area of the plot but were separated along the second dimension. While "inconvenient" was a barrier for blond oranges, "I have never tasted" and "I don't like the way it looks" were the reasons reported by Spaniards to not consume blood ones.

Mexico was the country with the greatest distance between blond and blood oranges. In fact, each one was located on one side of the plot. Taste preferences were clearly the most relevant barrier for Mexicans to consume blond oranges, which was on the same line as in Italy. The blood orange sample from Mexico was allocated to the right of the plot together with both samples from China. Comparatively to other samples, the barrier "I prefer other fruits" was less frequently cited in these three cases. Barriers such as "it is too expensive", "I think they are not safe", "I don't know how to prepare it", "They are not natural", "I have never tasted" and "I don't like the way it looks" had greater relative importance in China and Mexico than in Italy and Spain, and mainly for blood oranges. "Inconvenient" was identified as a relevant barrier for blond orange consumption in China, as it was in Spain.

It is clear from the CA plot that familiarity had a marked influence on barriers for blood orange consumption. Thus, while taste preferences were the major barrier in Italy, "I have never tasted" and "I don't like the way it looks" were relevant barriers shared by the other three countries. In line with this, after evaluating consumers' response to carrots with atypical colours, Schifferstein *et al.* (2019) reported that consumers gave lower attractiveness ratings to the unfamiliar variants and suggested that at least at first glance consumers may be reluctant to try them. These authors also found that consumers may perceive unfamiliar coloured products as unnatural/artificial, which was the case of the Chinese and Mexicans in this study. This perception is related to the "food neophobia" concept, which is defined as the reluctance to eat unfamiliar food (Dovey *et al.*, 2012). This phenomenon has been associated with the omnivore's dilemma while searching for food: approaching novel foods or protecting oneself from potentially poisonous food, thus restricting one's diet (Armelagos, 2014; Damsbo-Svendsen *et al.*, 2017). This may also explain the perception of novel food as not safe.

With regard to blond oranges, the relevance of "inconvenient" as a barrier in Spain and China is noteworthy. As previously mentioned, this barrier was included by the participants from these two countries by means of the "Others" option. Some participants wrote down "more inconvenient than other citrus fruit", which gave us a clue to understand the cultural specificity of this barrier. In a recent study in which consumers were asked to group different fruit types based on characteristics that influenced their consumption context, oranges were included in the "Cutlery-needed fruit" (one-serving fruit that needs cutlery for the eating process), while mandarins were included in the "Easy-to-peel fruit" (one-serving fruit, easy to transport, that does not need to be peeled or is handy to peel) (Tarancón *et al.*, 2021b). This study clearly showed that consumers consider mandarins a more convenient citrus fruit than oranges. According to FAO statistics (FAO, 2022), mandarin production is much higher in China and Spain than in Italy and Mexico. Thus, our hypothesis in this regard is that Chinese and Spaniards are more familiar with mandarins, and they consider oranges a less convenient fruit because they instinctively compared it to mandarins.

3.4. Purchase Intention and Consumer Expectations

Schifferstein *et al.* (2019) reported that increasing the vegetables offer with varieties with unfamiliar aesthetic characteristics to the naïve consumer may lead them to uncertainty about what qualities and taste experiences to expect. Our objective with this questionnaire section was to investigate the expectations of unfamiliar consumers with the product due to lack of access to it on markets.

As explained in Section 3.1, orange availability was almost 100% for blond oranges in the four studied countries and for blood oranges in Italy. Therefore, the results shown in this section focused on blood oranges and on Spanish, Mexican and Chinese consumers. Other than expectations, we asked them about their purchasing intention if blood oranges were available in their habitual shopping place and how much they expected to like these oranges.

Figure 4 shows the distribution of blood orange expectations depending on the country. Mexican consumers defined blood oranges as a new product from another country and were somewhat suspicious of it ("I don't trust it"). According to Salgado *et al.* (2016), a strong attachment to their traditions is one of the main reasons for Mexican consumers rejecting new foods, especially adults.

Chinese people expected blood oranges to have a surprising flavour, while Spaniards reported mostly expectations not directly linked with sensory properties. They expected blood oranges to be natural, appetising and healthy. Both the Spanish and Chinese' consumers expected blood oranges to be an expensive product.

Despite the differences found in attribute expectations, when the participants were directly asked about how much they expected to like blood oranges, the expected liking was similar in Spain, China and Mexico, with values close to six (data not shown). Certain differences were, however, detected in purchasing intention. Thus, the percentage of participants who stated that they were willing to buy fruit if it was available on the market was 40% in Spain, and 60% in China and Mexico (Figure S6).

Therefore, Spain was not only the country with the lowest consumption rate of blood oranges (Table 1), but it was also where consumers had the lowest purchasing intention. A priori, "natural", "appetising" and "healthy", the attributes used by Spaniards to describe blood oranges, should have a positive impact on purchasing intention. It seems, therefore, that expectations of an expensive product may be the reason behind low purchase intention.

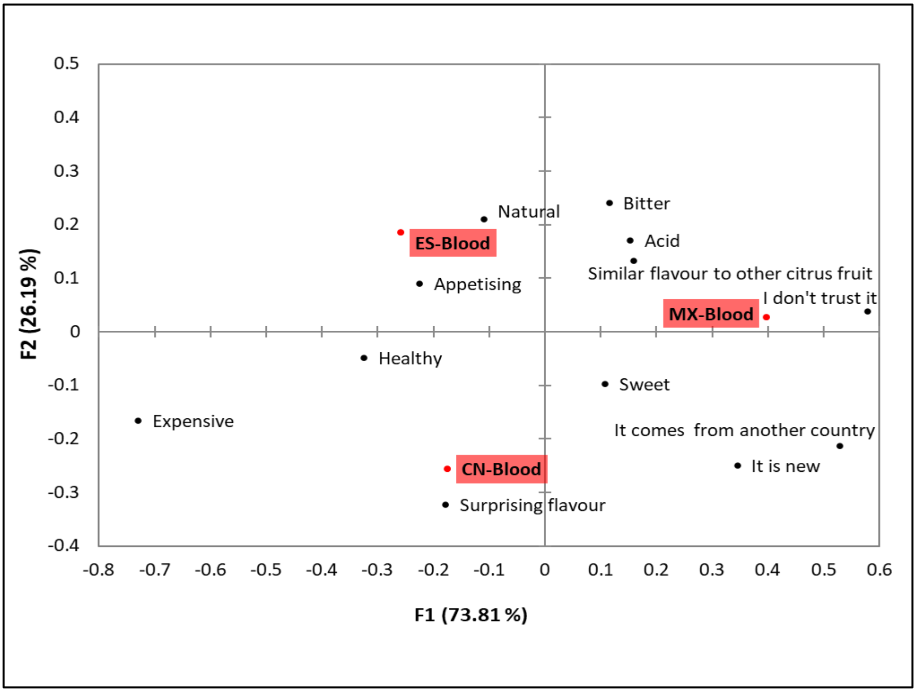

Figure 4. Correspondence analysis for consumers' expectations. The data used to perform the analysis were the percentage of participants (of those who stated that blood oranges are not available in their habitual shopping place) who selected each expectation. ES—Spain, CN—China and MX—Mexico

3.5. Strengths and Limitations of the Study, Practical Implications and Future Perspectives

Before closing this section, it is important to discuss the weaknesses and strengths of this study, the practical relevance of the information herein presented as well as the need of future studies.

In order to compare data from the different countries, we balanced consumer samples with regard to gender, age and level of education. All of them are factors that may have an influence on consumer response (Assari and Lankanari, 2018; Stea *et al.*, 2020). We decided not to ask about level of income because, in previous studies, we realised that the participants are reluctant to answer this question. However, level of income may have an influence on certain responses, mainly on the frequency of mentioning consumption barriers, such as “too expensive”. Thus, we cannot be sure if it is one of the reasons why this barrier was detected mainly in China.

It would have also been interesting to include a neophobia test at the end of our questionnaire because our results revealed certain cultural differences in this regard. Thus, the fact that the barrier “I have never tasted” and the expectation “I don‘t trust it” were more frequently mentioned in Mexico suggests that Mexicans take a more neophobic attitude to blood oranges compared to Chinese and Spaniards.

One of the strengths of this study is that it responds to a need for studies to approach the commercialisation and marketing aspects of specific fruit types rather than fruit in general (Briz *et al.*, 2008). By exploring consumers’ perceptions of blond and blood oranges, we obtained highly and directly applicable information. So, our findings have significant implications for the marketing and export activities of the citrus industry, particularly in a globalised world. Moreover, the information herein presented is very valuable for citrus breeders around the world to produce orange varieties that respond to consumers’ quality demand.

Another aspect to highlight of this study is its cross-cultural approach. corroborated that cultural background affects how food products are perceived and, thus, consumer behaviour towards it (Galmarini *et al.*, 2013; Jeong *et al.*, 2021). A better understanding of how consumers’ perceptions differ between groups will help traders to implement the most effective interventions depending on specific markets.

Our results revealed that Italy was the country where the position that blond and blood oranges occupy on the market seems ideal. Thus, both orange types co-exist on markets with a high consumption rate. The only reason why a low percentage of Italians do not consume oranges is because they have taste preferences for other fruit, which is normal and expected even in the best scenario for commercialising a specific fruit type.

Mexico and Spain were revealed as the countries with the lowest blood oranges consumption. According to our results, 60% of Mexican consumers for whom this fruit was unavailable could be potential buyers. Therefore, increasing blood oranges availability on Mexican markets can be a good intervention to increase their consumption. Moreover, the identification of "I've never tasted it" and "I don't like the way it looks" as relevant consumption barriers in this country suggests that interventions focusing on promoting people to taste fruit with tasting opportunities in fairs, supermarkets or schools may have a positive effect.

Blood oranges purchase intention was lower in Spain than in Mexico, and this seems to be related to people's expectations of expensive fruit. Thus, increasing availability in combination with promotional offers on markets may be a good option to reach out to a big group of Spanish consumers.

Blood oranges availability on Chinese markets was high with 83%. However, a high percentage of the consumers from this country stated not consuming them. Moreover, 20% of these people also stated that they did not consume blond oranges (Table 2). Thus, identifying consumption barriers would be a key result in this country. Interestingly enough, the Chinese participants pointed out "inconvenient" as a barrier for blond oranges consumption. Selling minimally processed, already peeled and segmented oranges could be a good option to overcome this barrier. As health-related characteristics of minimally processed oranges are retained during refrigerated storage (Plaza *et al.*, 2011), this product offers the convenience and health benefits required by Chinese consumers.

The relevance of unaffordable prices as a barrier in China suggests promotional offers on markets as a promising intervention to facilitate oranges consumption and to promote the blood orange varieties. Moreover, Liu *et al.* (2020) reported that establishing a trusted certification system would improve Chinese consumers' food safety evaluation and increase the demand for high-quality safe fruit on the Chinese domestic market. Moreover, adding claims about the "naturalness" of blood varieties and images of the product prepared to eat/drink may also have a positive impact in this country.

Apart from the cultural particularities, it is also worth mentioning the aspects that were common to all, irrespective of the country. The most important ones to highlight are the main reasons for purchasing oranges, which were identified as taste preferences and healthy properties in all four countries. Therefore, using labels that claim beneficial properties, such as high vitamin C, fibre or antioxidant compounds content, are likely to catch consumers' attention. This may be especially useful for promoting the strong points of blood oranges because these varieties are highlighted for their high content of antioxidant compounds and anthocyanins (Lo Piero, 2015). In a recent study, Tarancón *et al.* (2022) found a link between the rind pigmentation intensity and pulp anthocyanin content of "Sanguinelli", one of the most important blood orange varieties. These authors also reported that fruit colouration is linked with fruit sensory properties in such a way that the more intense the red pigmentation is, the more the perceived sweetness. Thus, rind colouration may become a nutritional and quality index for both marketers and consumers. Conveying this information to consumers may be especially relevant. Hence our data showed that Chinese consumers stand out for consuming fruit when they wish to improve their health ("to improve health" and "to lose weight" contexts) (Figure 2). However, they also highlighted disregarding fruit colour (Table 4). Thus, Chinese consumers must be informed about the relation between blood oranges pigmentation and their healthy properties.

Finally, our results may throw light on breeding programmes with regard to consumers' quality requirements. In the four evaluated countries, juicy was the most important attribute for consumer satisfaction, followed by flavour/taste attributes. Varieties with an intense aroma are specially indicated for the Italian and Chinese markets. Moreover, unfibrous varieties would be very much appreciated by Chinese consumers.

Future studies should investigate to what extent the sensory properties (other than the aspect) of blood oranges differ from those of the blond ones. This information would be useful not only for breeders but also for the citrus industry for marketing purposes.

By way of conclusion, we can state that blond and blood oranges can co-exist on markets at a high consumption rate, as in Italy. To achieve such a situation in other countries, specific approaches are needed because consumers' perception of oranges (reasons and barriers for purchasing, consumption contexts, expectations and purchase intention) depend on culture.

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Supplementary Material

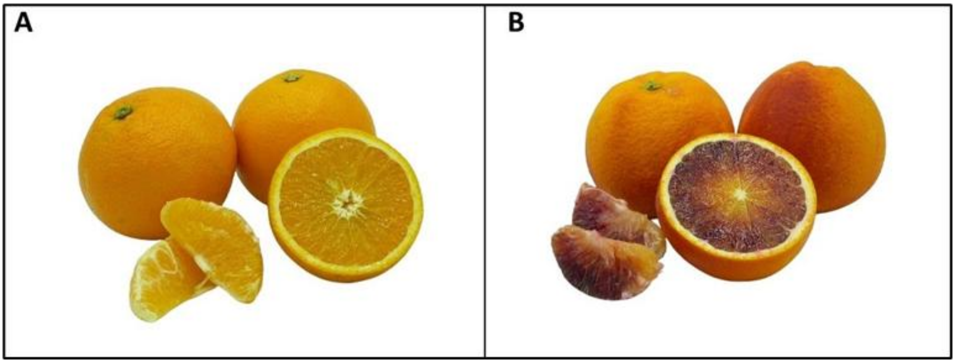

Figure S1. Pictures of blond (A) and blood (B) oranges shown in the questionnaire.

Table S1. Number of participants from each country who responded each section of the questionnaire.

Availability and consumption habit	Spain		Italy		Mexico		China	
	Blond	Blood	Blond	Blood	Blond	Blood	Blond	Blood
Available and usually consumed	517	104	554	459	542	207	447	279
Available but not consumed	83	190	50	134	62	100	140	226
Not available	5	311	1	12	1	298	18	100

Table S2. List of references used to create the preliminary lists of the questionnaire.

	References
Facilitators (Reasons to consume)	Onwezen and Bartels, 2011 Sijtsema *et al.*, 2012 Phan *et al.*, 2016 Iofrida *et al.*, 2019 Di Vita *et al.*, 2020 Kasprzak *et al.*, 2021 Ingrassia *et al.*, 2016
Sensory attributes	Iofrida *et al.*, 2019 Di Vita *et al.*, 2020 Fernández-Serrano *et al.*, 2020 Tarancón *et al.*, 2020 Tarancón *et al.*, 2021a Giménez-Sanchis *et al.*, 2021
Contexts	Jaeger *et al.*, 2019 Di Vita *et al.*, 2020 Verain *et al.*, 2020 Tarancón *et al.*, 2021b
Barriers	Sijtsema *et al.*, 2012 Silvestri *et al.*, 2016 Kasprzak *et al.*, 2021

Table S3. Ranking of sensory attributes' importance for consumer satisfaction after eating blond/ blood oranges. Importance was scored in a 7-point scale (1-not important at all, 7-very important) by Spanish, Italian, Mexican and Chinese consumers (n=605 in each country). Mean scores are shown in brackets.

Spain		Italy		Mexico		China	
Blond	**Blood**	**Blond**	**Blood**	**Blond**	**Blood**	**Blond**	**Blood**
Juicy (6.4)[a]	Juicy (6.2)[a]	Juicy (6.4)[a]	Juicy (6.5)[a]	Juicy (6.8)[a]	Juicy (6.7)[a]	Juicy (6.1)[a]	Juicy (6.2)[a]
Flavour (6.0)[b]	Sweet (5.9)[ab]	Flavour (6.3)[a]	Flavour (6.3)[a]	Sweet (6.4)[b]	Sweet (6.5)[ab]	Sweet (6.1)[ab]	Sweet (6.0)[ab]
Balance (6.0)[b]	Balance (5.8)[ab]	Aromatic (5.8)[b]	Aromatic (6.0)[c]	Balance (6.3)[bc]	Flavour (6.2)[bc]	Unfibrous (6.0)[b]	Aromatic (6.0)[ab]
Sweet (5.8)[c]	Flavour (5.8)[ab]	Balance (5.8)[b]	Sweet (5.8)[c]	Flavour (6.1)[c]	Balance (6.2)[bc]	Aromatic (5.9)[bc]	Unfibrous (6.0)[ab]
Aromatic (5.6)[d]	Aromatic (5.7)[ab]	Sweet (5.7)[b]	Balance (5.8)[c]	Aromatic (5.7)[d]	Aromatic (6.0)[cd]	Flavour (5.9)[bc]	Balance (6.0)[ab]
Unfibrous (5.6)[d]	Colour (5.5)[bc]	Colour (5.0)[c]	Colour (5.3)[d]	Unfibrous (5.7)[d]	Unfibrous (5.8)[d]	Balance (5.9)[bc]	Flavour (6.0)[ab]
Easy Pe (4.7)[e]	Unfibrous (5.3)[c]	Unfibrous (4.5)[d]	Unfibrous (4.6)[e]	Colour (5.4)[e]	Colour (5.8)[d]	No Messy (5.8)[c]	No Messy (5.9)[bc]
Seedless (4.6)[e]	Easy Pe (4.7)[d]	Easy Pe (4.4)[d]	Easy Pe (4.4)[e]	Easy Pe (5.0)[f]	Easy Pe (5.3)[e]	Easy Pe (5.7)[c]	Easy Pe (5.9)[bc]
Colour (4.6)[e]	Seedless (4.5)[d]	Seedless (4.0)[e]	Seedless (4.1)[f]	No Messy (3.9)[g]	Seedless (4.5)[f]	Seedless (5.4)[d]	Seedless (5.7)[c]
No Messy (3.6)[f]	No Messy (3.9)[e]	No Messy (3.7)[f]	No Messy (3.9)[g]	Seedless (3.8)[g]	No Messy (4.5)[f]	Colour (5.1)[e]	Colour (5.7)[c]

BLOND ORANGES

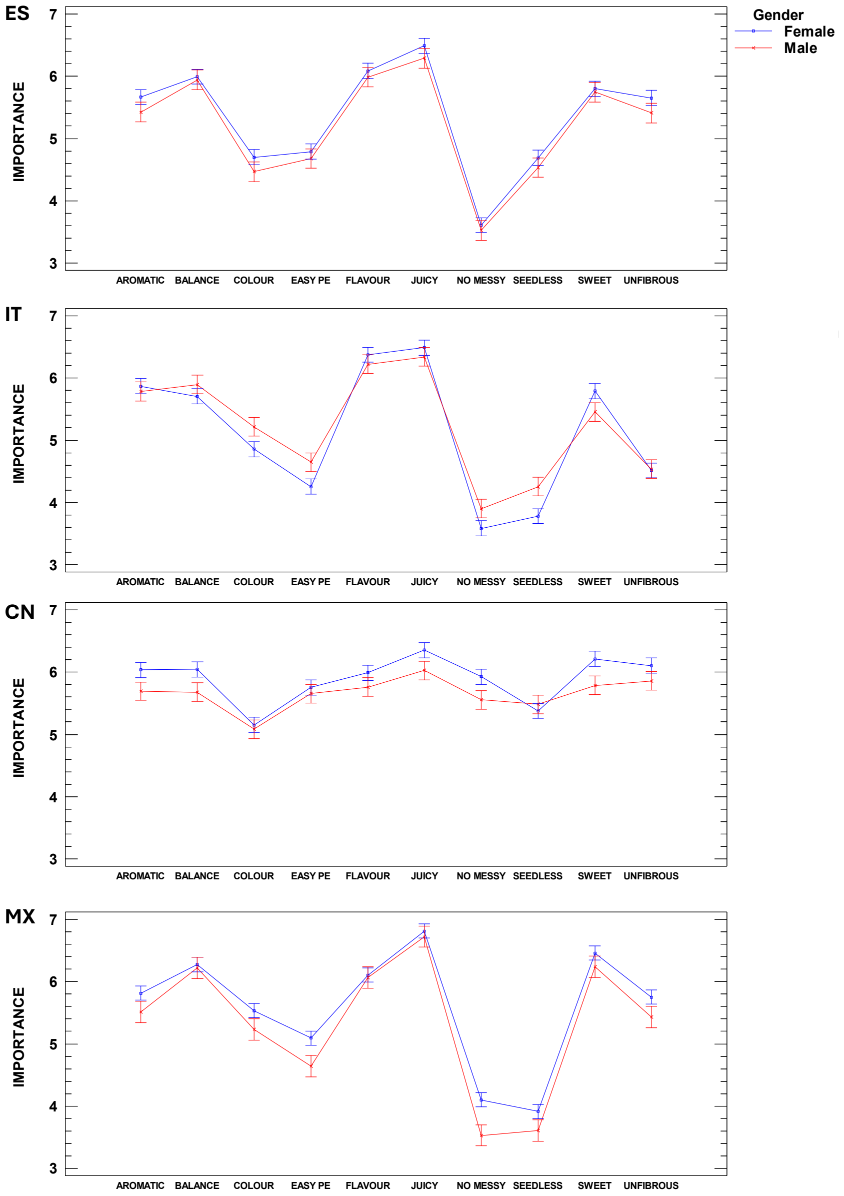

Figure S2. Effect of gender on the importance for satisfaction gave by consumers to different sensory attributes of blond oranges (1-not important at all, 7-very important). ES-Spain, IT-Italy, CN-China, MX-Mexico.

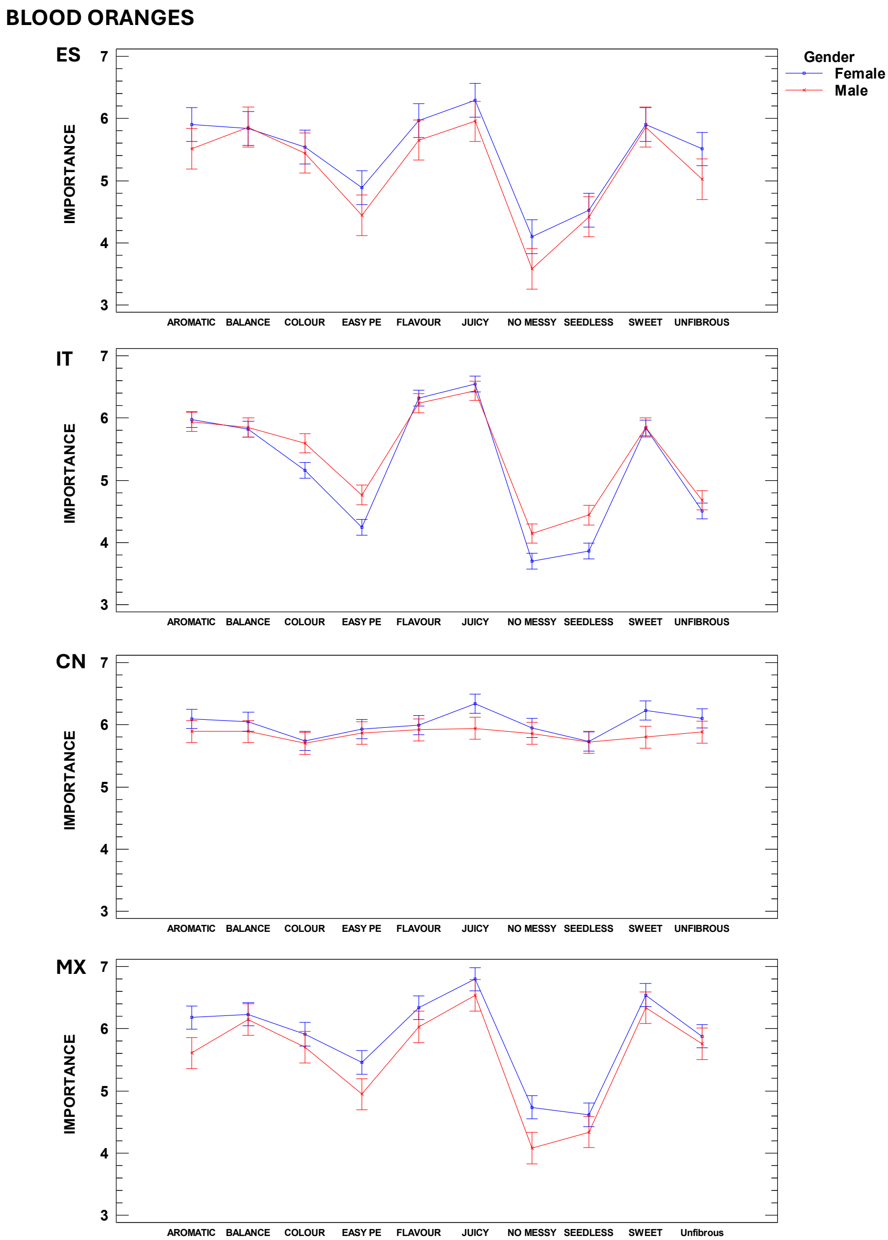

Figure S3. Effect of gender on the importance for satisfaction gave by consumers to different sensory attributes of blood oranges (1-not important at all, 7-very important). ES-Spain, IT-Italy, CN-China, MX-Mexico.

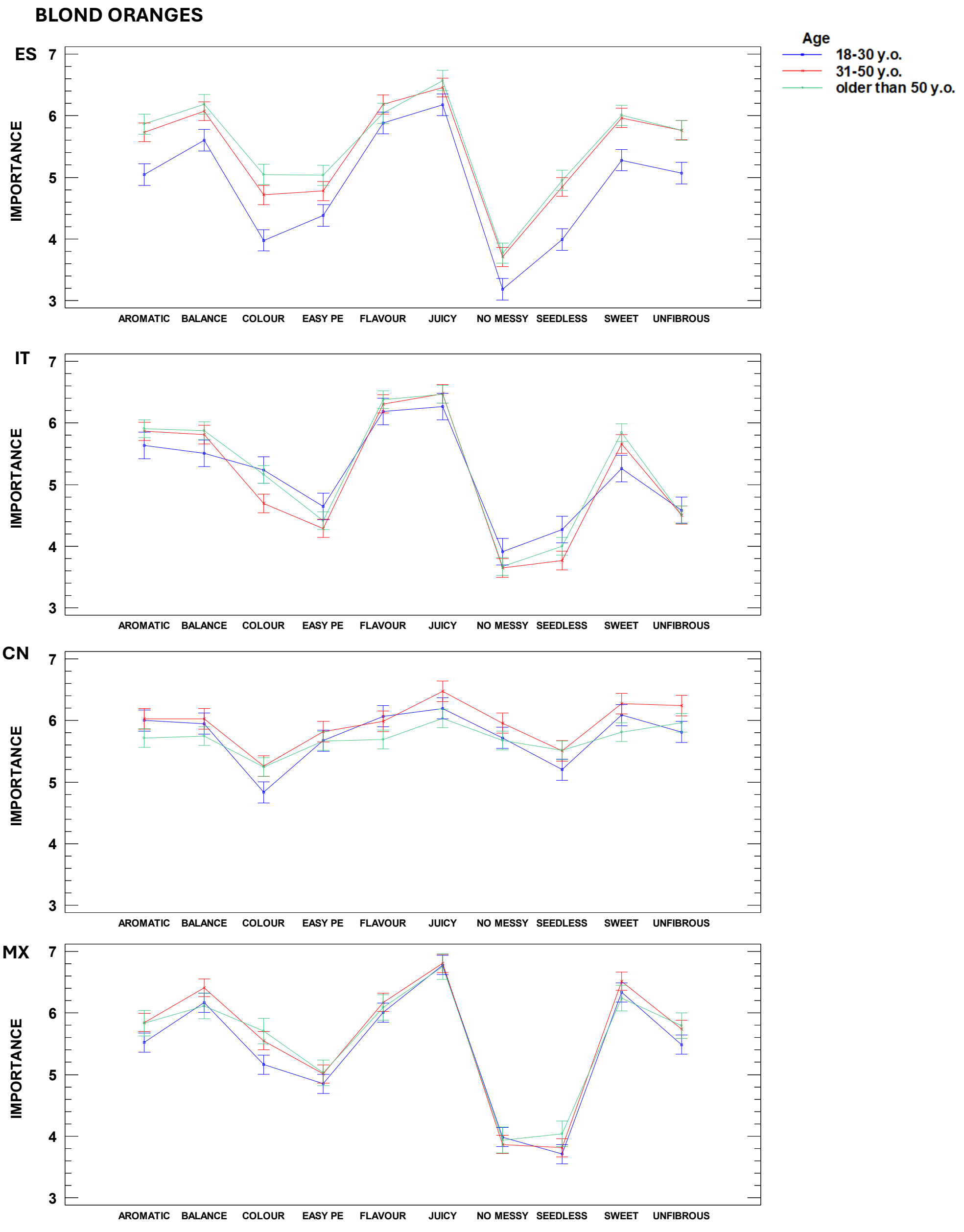

Figure S4. Effect of age range on the importance for satisfaction gave by consumers to different sensory attributes of blond oranges (1-not important at all, 7-very important). ES-Spain, IT-Italy, CN-China, MX-Mexico.

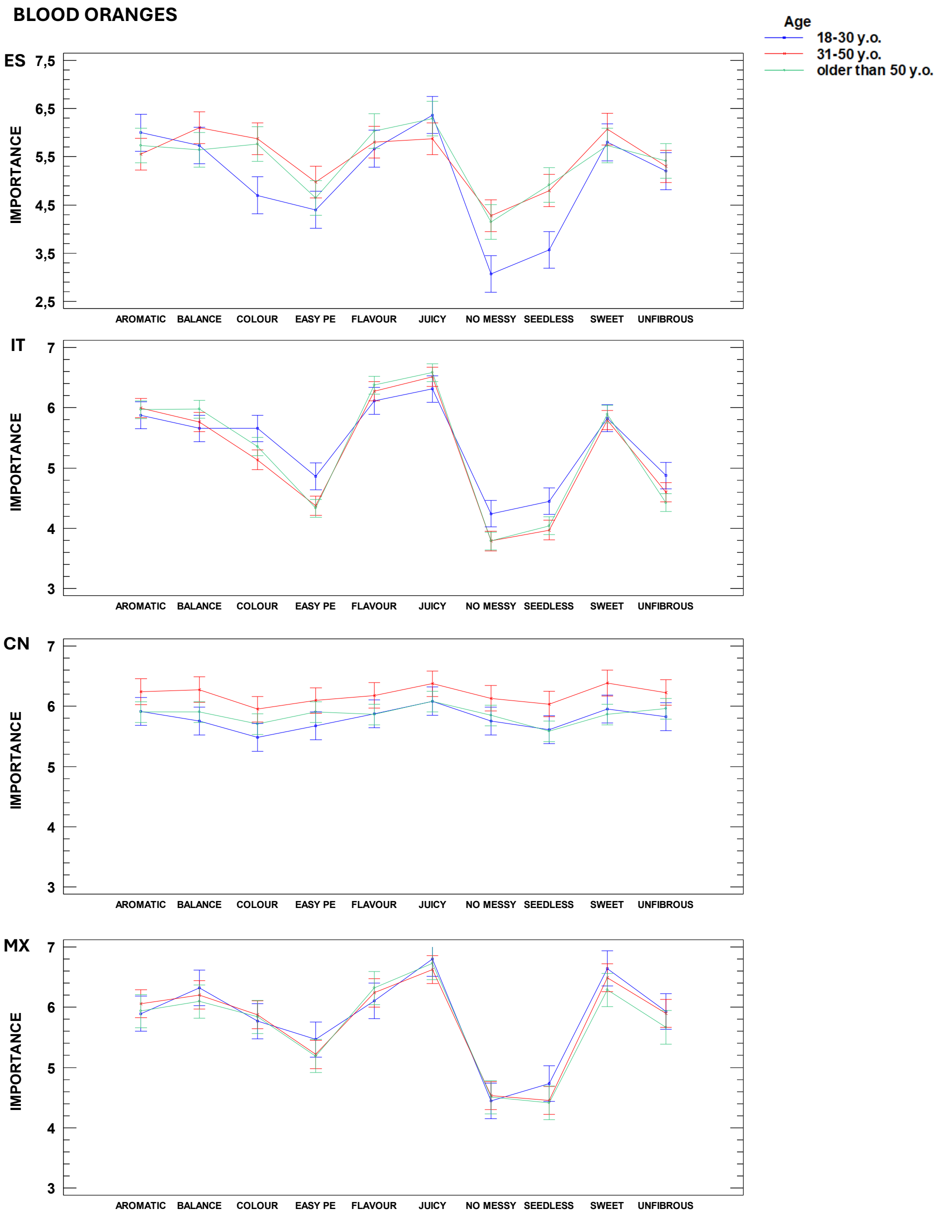

Figure S5. Effect of age range on the importance for satisfaction gave by consumers to different sensory attributes of blood oranges (1-not important at all, 7-very important). ES-Spain, IT-Italy, CN-China, MX-Mexico.

Table S4. Percentage of participants (of those who stated consuming each orange type habitually) from each country who selected each consumption context (continued on the following page).

Spain		Italy	
Blond	**Blood**	**Blond**	**Blood**
Dessert (77.9 %)	Breakfast (54.8 %)	Juice/Smoothy (68.1 %)	Juice/Smoothy (66.7 %)
Juice/Smoothy (70.0 %)	Dessert (51.0 %)	Snack home (63.5 %)	Snack home (64.9 %)
Breakfast (63.6 %)	Snack home (44.2 %)	Dessert (51.8 %)	Dessert (45.8 %)
Snack home (52.7 %)	Juice/Smoothy (26.9 %)	Cooked (40.6 %)	Breakfast (40.7 %)
Improve health (38.5 %)	Snack out home (26.9 %)	Ingredient (40.6 %)	Cooked (31.7 %)
Snack out home (38.1 %)	Improve health (25.0 %)	Breakfast (38.6 %)	Ingredient (31.7 %)
Ingredient (27.0 %)	Child lunch-box (20.2 %)	Snack out home (35.3 %)	Snack out home (31.0 %)
Child lunch-box (24.2 %)	Cocktail (17.3 %)	Improve health (34.2 %)	Improve health (28.1 %)
Cocktail (16.1 %)	Ingredient (15.4 %)	Cocktail (18.7 %)	Cocktail (12.8 %)
Restaurant (15.4 %)	Restaurant (8.7 %)	Child lunch-box (10.6 %)	Child lunch-box (9.0 %)
Weight loss (9.9 %)	Weight loss (7.7 %)	Weight loss (9.5 %)	Weight loss (7.0 %)
Sugar (7.9 %)	Gift (5.8 %)	Salt/chilli powder (4.7 %)	Gift (4.9 %)
Gift (7.5 %)	Sugar (4.8 %)	Sugar (4.2 %)	Salt/chilli powder (4.0 %)
Salt/chilli powder (2.6 %)	Salt/chilli powder (1.0%)	Gift (3.8 %)	Restaurant (3.6 %)
Cooked (1.7 %)	Cooked (1.0%)	Restaurant (3.6 %)	Sugar (2.7 %)

Table S4. Percentage of participants (of those who stated consuming each orange type habitually) from each country who selected each consumption context .

Mexico		China	
Blond	**Blood**	**Blond**	**Blood**
Snack home (75.1 %)	Snack home (66.2 %)	Snack home (71.3 %)	Snack home (65.8 %)
Snack out home (70.3 %)	Breakfast (65.7 %)	Improve health (46.4 %)	Improve health (42.1 %)
Breakfast (64.2 %)	Snack out home (63.3 %)	Snack out home (42.6 %)	Snack out home (39.2 %)
Salt/chilli powder (63.8 %)	Dessert (46.9 %)	Dessert (40.6 %)	Dessert (33.1 %)
Juice/Smoothy (49.6 %)	Salt/chilli powder (46.4 %)	Juice/Smoothy (32.1 %)	Breakfast (32.4 %)
Improve health (47.2 %)	Improve health (45.4 %)	Gift (30.3 %)	Gift (29.1 %)
Dessert (46.5 %)	Juice/Smoothy (37.7 %)	Breakfast (22.4 %)	Juice/Smoothy (25.2 %)
Ingredient (40.0 %)	Cocktail (34.8 %)	Weight loss (22.4 %)	Weight loss (24.5 %)
Cocktail (32.7 %)	Ingredient (31.4 %)	Restaurant (18.6 %)	Restaurant (17.3 %)
Child lunch-box (31.7 %)	Child lunch-box (29.0 %)	Child lunch-box (13.0 %)	Child lunch-box (12.2 %)
Weight loss (14.9 %)	Weight loss (16.4 %)	Ingredient (7.8 %)	Ingredient (8.6 %)
Restaurant (8.3 %)	Restaurant (11.6 %)	Cocktail (4.7 %)	Cocktail (5.0 %)
Gift (8.1 %)	Gift (8.7 %)	Cooked (3.1 %)	Cooked (1.8 %)
Sugar (5.9 %)	Sugar (5.3 %)	Salt/chilli powder (2.2 %)	Salt/chilli powder (1.4 %)
Cooked (3.1 %)	Cooked (3.4 %)	Sugar (1.1 %)	Sugar (0.7 %)

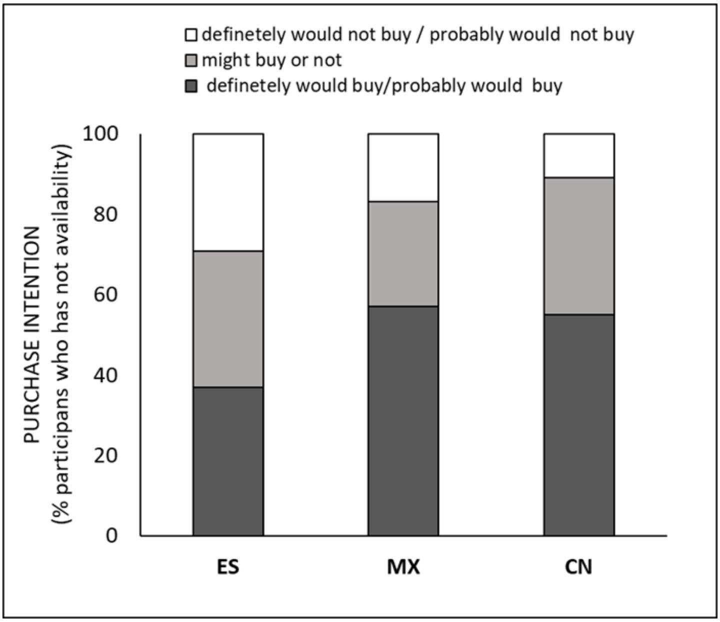

Figure S6. Purchase intention of those consumers for whom blood oranges are not available in their shopping place if oranges were available. ES-Spain, MX-Mexico, CN-China.

Table S5. Percentage of participants (of those who stated not consuming each orange type habitually) from each country who selected each consumption barrier (continued on the following page).

Spain		Italy	
Blond	**Blood**	**Blond**	**Blood**
I prefer other fruits (67.5 %)	I prefer other fruits (58.9 %)	I prefer other fruits (60.0 %)	I prefer other fruits (70.1 %)
I don't know how to prepare it (12.0 %)	I don't like it (17.4 %)	I don’t like it (12%)	I don't like it (16.4 %)
I don't like it (9.6 %)	I have never tasted it (13.2 %)	Too expensive (6.0 %)	Too expensive (9.0 %)
Inconvenient (8.4 %)	I don't like the aspect (7.4 %)	I don't like the aspect (4.0 %)	I don't like the aspect (6.0 %)
Too expensive (7.2 %)	Too expensive (6.8 %)	They are not natural (4.0 %)	They are not natural (3.0 %)
They are not natural (3.6 %)	I don't know how to prepare it (1.6 %)	I don't know how to prepare it (2.0 %)	I have never tasted it (2.2 %)
I don't like the aspect (1.2 %)	Inconvenient (0.0 %)	I think they are not safe (2.0 %)	I think they are not safe (0.7 %)
I have never tasted it (0.0 %)	I think they are not safe (0.0 %)	I have never tasted it (0.0 %)	I don't know how to prepare it (0.0 %)
I think they are not safe (0.0 %)	They are not natural (0.0 %)	Inconvenient (0.0 %)	Inconvenient (0.0 %)

Table S5. Percentage of participants (of those who stated not consuming each orange type habitually) from each country who selected each consumption barrier.

Mexico		China	
Blond	**Blood**	**Blond**	**Blood**
I prefer other fruits (61.3 %)	I prefer other fruits (48.0 %)	I prefer other fruits (36.4 %)	I prefer other fruits (31.9 %)
Too expensive (9.7 %)	I have never tasted it (28.0 %)	Too expensive (22.9 %)	Too expensive (30.1 %)
I don't like the aspect (8.1 %)	I don't like the aspect (15.0 %)	I don't like it (21.4 %)	I don't like it (27.9 %)
I think they are not safe (6.5 %)	Too expensive (13.0 %)	Inconvenient (15.7 %)	I have never tasted it (16.8 %)
They are not natural (4.8 %)	They are not natural (11.0 %)	I don't know how to prepare it (11.4 %)	I don't like the aspect (15.5 %)
I don't like it (3.2 %)	I don't know how to prepare it (8.0 %)	They are not natural (10.7 %)	I don't know how to prepare it (13.7 %)
I don't know how to prepare it (3.2 %)	I don't like it (6.0 %)	I don't like the aspect (10.0 %)	They are not natural (11.1 %)
I have never tasted it (0.0 %)	I think they are not safe (5.0 %)	I think they are not safe (9.3 %)	I think they are not safe (6.2 %)
Inconvenient (0.0 %)	Inconvenient (0.0 %)	I have never tasted it (5.7 %)	Inconvenient (0.0 %)

IV.2. BLOOD ORANGES' PHYSILOGY

CHAPTER V

Changes in the Sugars and Volatile Compounds Profiles Associated with Anthocyanin Accumulation in Oranges: Blood vs. Blond Varieties, and Slightly Pigmented vs. Intensely Pigmented Blood Fruit

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Abstract

Blood oranges accumulate anthocyanins in their peel and pulp, which give them their characteristic red colour. To investigate whether there is a specific volatile compounds profile for blood oranges, we compared the volatile composition of the blood varieties 'Moro', 'Tarocco Rosso' and 'Sanguinelli' to that of the blond ones 'Cadenera', 'Salustina' and 'Hamlin' using a non-targeted approach based on HS–SPME–GC–MS. Moreover, fruits from each blood orange variety were divided into slightly and intensely pigmented fruits, and individual sugars and anthocyanins were determined by liquid chromatography to investigate whether their profiles depend on the degree of pulp pigmentation. A total of 101 volatile compounds were identified in this study. Hierarchical Cluster Analysis, including all compounds, revealed that blood oranges have no unique volatiles profile that makes them more similar to one another than to the blond varieties. For blood orange varieties, our results corroborated that greater anthocyanin accumulation in the most intensely pigmented fruit is associated with higher sugar content (an increase of 0.8–2.3 % depending on the variety). Moreover, we reported for the first time that anthocyanin accumulation is also associated with changes in the volatiles profile. A Multifactor Analysis including data on volatiles, sugars and anthocyanin showed that sucrose is the sugar more strongly linked to anthocyanin accumulation. A group of volatile compounds, mainly esters (ethyl acetate, ethyl-2-methyl butanoate and ethyl-2-butenoate) along with ethanol, are present at increased levels, sometimes even doubling in the most intensely pigmented samples compared to the slightly pigmented ones. These results open the door to further investigate the possible metabolic link between sugars and anthocyanins accumulation and changes in volatile compounds.

Keywords: anthocyanins; volatiles; total soluble solids; blood orange; blond orange

1. Introduction

Blood oranges (*Citrus sinensis* (L.) Osbeck) are a group of oranges that differ from blond ones because of their high anthocyanin content, which confers the fruit its characteristic red colour (Lo Piero, 2015; Fallico *et al.*, 2017). Blood oranges have been traditionally grown in Sicily, where they originate and where environmental conditions favour the development of their reddish colour (Seminara *et al.*, 2023).

Anthocyanins are water-soluble compounds that belong to the flavonoid family and are responsible for the red and blue colouring of various plant species (Alappat and Alappat, 2020). These compounds also confer the fruit beneficial health properties because they have been shown to help prevent different illnesses, such as heart disease (Vendrame and Klimis-Zacas, 2019) or diabetes (Solverson, 2020). Of blood orange varieties, ‘Moro’ has been reported to have the highest anthocyanin content (Kafkas *et al.*, 2009), with concentrations over 200 mg/L (Rapisarda *et al.*, 2022).

In recent decades, consumers have become increasingly aware of the role that food plays in their health, and the citrus industry has seen pigmented oranges as an opportunity to meet the healthier food demand while widening the varietal citrus range. Thus, in the last few years, blood oranges’ popularity has grown and they are currently produced in countries all over the world, including Spain, China, Brazil and the USA (Magalhães *et al*., 2019; Simons *et al.*, 2019; Morales *et al.*, 2021; Chen *et al.*, 2023). Varieties ‘Moro’ and ‘Tarocco’, which originate from Italy, and ‘Sanguinelli’, which originates from Spain, are the main varieties grown in the Mediterranean region, and are also relevant in other countries’ citrus production (Seminara *et al.*, 2023).

Despite consumers showing more interest in products with health benefits, flavour liking is still the key factor for consuming oranges (Giménez-Sanchis *et al.*, 2022). Therefore, it is important to look in depth at the different aspects related to it. Fruit flavour depends on taste and aroma. Sweet, sour and bitter are the main tastes involved in fruit flavour. Aroma is associated with a wide variety of low-molecular-weight volatile compounds. Although both taste and aroma are well integrated into their contribution to overall flavour, volatile compounds are often considered to play a dominant role (Goff and Klee, 2006). Different studies have attempted to identify aroma compounds in *Citrus Sinensis* varieties. The major contributors to the aromatic characteristics of sweet oranges are monoterpenes, sesquiterpenes, hydrocarbons, alcohols, aldehydes, esters and ketones (Pan *et al.*, 2023), of which D-limonene is reported to be the most abundant aromatic compound in oranges (Rodríguez *et al.*, 2017; Kvittingen *et al.*, 2021).

Some studies have suggested that blood oranges have a characteristic volatile composition that differs from that of blond oranges (Näf *et al.*, 1996; Moufida and Marzouk, 2003). Conclusions drawn from such studies are based on comparing the volatile composition of only one blood orange variety to that of a blond one (Näf *et a*l., 1996) or to other citrus species (Moufida and Marzouk, 2003). The conclusion that these studies reach seems somewhat premature as it has been demonstrated that the volatile profile vastly differs among varieties, and even within the same species and group (González-Mas *et al.*, 2011). In 2006, Arena *et al*. went one step further and determined odour-active volatile compounds from two blood oranges ('Moro' and 'Tarocco') compared to two blond ones ('Washington Navel' and 'Valencia Late') by gas chromatography–olfactometry. These authors reported that certain volatile compounds, specifically methyl butanoate and ethyl octanoate, have concentrations at the odour-active level only in blood varieties. This result led them to conclude that blood oranges have their own characteristic aroma that distinguishes them from blond varieties. These are interesting results, but we believe that further research is needed before concluding about the specificity of blood orange aroma.

In addition to differences in the volatile profile, it has been reported that blood oranges exhibit other distinct characteristics compared to blond oranges. Rapisarda *et al.* (1998), after comparing a wide range of orange varieties, reported that hydroxycinnamic acids were more abundant in blood orange juice than in blond orange juice. This higher content of hydroxycinnamic acids in blood oranges was later associated with their tendency to accumulate malodorous vinylphenols during cold storage (Rapisarda *et al.*, 2001). More recently, Fabroni *et al.* (2020) demonstrated that the non-volatile compound putrescine increases during cold storage in blood oranges, correlating directly with the rise of anthocyanin pigmentation in these varieties. In contrast, putrescine levels remain constant in blond oranges stored at low temperatures. Fabroni *et al.* (2020) also reported that putrescine levels directly correlate with the accumulation of off-flavours in cold-stored ’Moro’ and ’Tarocco TDV’ pigmented fruit, indicating that its concentration significantly affects the sensory perception of the fruit.

On the other hand, Tarancón *et al*. (2022) evaluated the relation between rind colour and the internal characteristics of ‘Sanguinelli’ blood oranges at harvest time. At a given point in the season, fruits with different intensities of pigmentation, both external and internal, can be found on the tree. This is due to environmental factors such as exposure to low temperatures and sunlight, making the position in the tree canopy a determining factor (Lo Piero, 2015). They found that the more intense the rind pigmentation was, the more intense the internal pigmentation and the higher the total soluble solids content were. Using triangle tests, these authors demonstrated that consumers were able to differentiate intensely pigmented (In-P) samples from slightly pigmented (Sl-P) samples by blind taste. This result raises the question of whether the perceived differences are only associated with differences in the total soluble solids content, or if aroma compounds may also differ between the Sl-P and In-P fruit of the same variety and may, thus, contribute to different flavour perceptions.

While the study of Tarancón *et al.* (2022) is the only study that related anthocyanins and sugars at a specific point in the season, this relationship had already been previously observed both in preharvest and postharvest stages. On one hand, Yu *et al.*, (2020) after evaluating the evolution of ’Tarocco’ from day 200 to day 324 after flowering, found a positive correlation between the accumulation of fructose and glucose and that of anthocyanins throughout fruit maturation. On the other hand, Carmona *et al.* (2019) observed the activation of enzymes involved in sugar synthesis in parallel with the accumulation of anthocyanins during cold storage of the 'Moro' variety. In the review by Abdullah *et al.* (2018) about the sugar and light crosstalk in the induction of anthocyanin biosynthesis in fruits, the authors reported that sucrose significantly impacts anthocyanin accumulation through hormone interaction and triggers the transcript levels of many structural genes involved in anthocyanin biosynthesis.

After bearing all this in mind, we focused on fruit quality at harvest time and designed this study with two main objectives: 1) profoundly investigate whether there is a specific volatile compounds profile of blood oranges vs. blond oranges by evaluating the volatile composition of three varieties of each orange type; 2) investigate whether the sugars concentration and volatile composition of the fruit depend on the degree of pulp pigmentation for the same blood orange variety.

2. Materials and Methods

2.1. Samples

The fruit of three blood orange varieties ('Tarocco Rosso', 'Moro' and 'Sanguinelli') and three blond orange varieties ('Cadenera', 'Salustiana' and 'Hamlin') were harvested at commercial maturity stage on the same day (15 March 2021) from the orchard of the Citrus Germplasm Bank at the Valencian Institute for Agricultural Research (IVIA) in Valencia, Spain. Harvesting varieties at the same stage of ripeness is quite challenging since the sugar-acid relationship varies greatly, and the same maturity index (MI) can result from very different concentrations of sugars and acids. Therefore, a compromise solution was reached in this study. The harvest date was set at a time during the citrus season when all evaluated varieties were being commercially harvested. At the time of harvest, all varieties had a maturity index higher than the minimum required for the commercialization of oranges in the EU, which is 6.5 for both blood oranges and blond oranges (UNECE, 2023). For each variety, approximately 80 fruits were harvested around the canopy of two trees. Once in the laboratory, the fruits of the blood orange varieties were cut in half, and regardless of the colouration of their peel, they were classified into two groups based on the intensity of pulp pigmentation: slightly pigmented (Sl-P) and intensely pigmented (In-P) fruit. To classify the fruits as slightly or intensely pigmented, a previous step was performed in which 10 fruits from each variety were randomly selected, and individual juices were made. The a*/b* ratio (CIELAB) of each juice was determined, and the minimum (a*/b* min) and maximum (a*/b* max) values were identified. The distance between these two values was calculated and divided by four, thus obtaining a new value we will call Q4 [Q4 = (a*/b* max – a*/b* min)/4]. Fruits with a pigmentation lower than a*/b* min +Q4 were classified as slightly pigmented. Fruits with pigmentation higher than a*/b* max – Q4 were classified as intensely pigmented. Fruits with intermediate values were not used for the study. Thus, for example, for 'Tarocco' fruits, the a*/b* min =0.5, the a*/b* max =3.2, and Q4 =0.675. Therefore, fruits with juice colour a*/b* <1.175 were classified as slightly pigmented, and those with a*/ b* >2.525 as intensely pigmented.

Three juices (7 fruits per juice) were obtained from each blond variety and for each pigmentation intensity group in the case of the blood varieties (Figure 1).

Figure 1. Appearance of the intense-pigmented samples (left) and the slight-pigmented samples (right) of the juices taken from each blood orange variety.

The following physico-chemical parameters were evaluated in the fresh juice: juice colour, titratable acidity (TA), total soluble solids (TSS) and maturity index (MI). In addition, juice samples were stored at –80 °C to determine total anthocyanins, as well as the profiles of individual anthocyanins, sugars and volatile compounds.

2.2. Analytical Determinations

2.2.1. Physicochemical Parameters

Juice colour was measured using a Minolta colorimeter model CR-400 (Minolta Co. Ltd., Osaka, Japan) by calculating the a*/b* ratio (CIELAB), which is the colour index usually used for blood oranges (Buccheri *et al.*, 2015; Tarancón *et al.*, 2022). TSS and TA were determined according to the procedure described by Tarancón *et al.* (2022). MI was calculated as TSS/TA. The total content of anthocyanins was determined by the pH-differential method according to Rapisarda *et al.* (2000), which is based on the differences in absorbance at two different pH values (1.0 and 4.5) at 510 nm.

2.2.2. Anthocyanin Profile

Aliquots of 2 mL of the juice samples were taken and centrifuged (MiniSpin, Eppendorf SE, Hamburg, Germany) at 12,000 rpm for 30 min, the precipitate was discarded, and the supernatant was then filtered through 0.45 μm pore size membrane filters, diluted with water (1:1, v/v) and stored at –20 °C until analysis.

Individual anthocyanins were analysed by Ultra High-Performance Liquid Chromatography equipped with a Vanquish separation modules coupled to a TSQ Fortis Triple-Quadrupole Mass Spectrometer (UHPLC- QqQ-MS, Thermo Fisher Scientific, Madrid, Spain), using a Luna Omega 1.6 μm PS C18 (100 x 2,1 mm) column (Phenomenex, CA, USA). The mobile phase was composed of acetonitrile (A) and water with 5 % of formic acid (B). Chromatograms were recorded at 280, 350 and 550 nm absorbance. The column temperature was maintained at 35 °C, and the autosampler was kept at 4 °C. The gradient started at 10 % A, ramped to 100 % A at 4 min, retuned to 10 % A at 5 min, then held at 10 % A until 10 min. The flow rate was of 0.3 mL/min and the injection volume was 1 μL. Mass analysis was performed in a full scan from 400 to 650 m/z, with an electrospray ionization source in positive mode. Chromeleon 7.3 chromatography data system software was used for data treatment. Compounds were identified by comparing their retention times, UV–Vis spectra, and mass spectral data with authentic standards. Concentrations were determined using an external calibration curve with delphinidin-3-O-glucoside (Dp-3-glu, RT =3.12 min, [MH] +465 m/z) and cyanidin-3-O-glucoside (Cy-3-glu, RT =3.32 min, [MH] +449 m/z). Due to the unavailability of cyanidin-3-(6”malonyl)-O-glucoside (Cy- 3-6”mal, RT =3.61 min, [MH] +535 m/z) and cyanidin-3-(6”dioxalyl)-O-glucoside (Cy-3-6”diox, RT =3.75 min, [MH] +593 m/z) standards, their calibration curves were constructed using Cy-3-glu as external standard, and Cy-3-6”mal and Cy-3-6”diox were quantified as Cy-3-glu equivalents. Dp-3-glu and Cy-3-glu chloride standards were obtained from Cymit Química S.L. (Barcelona, Spain), and all solvents used were of UHLC-mass spectrometry grade and purchased from Scharlab S.L. (Barcelona, Spain). Three replicates per sample were analysed. The results were expressed as mg/L.

2.2.3. Sugar Profile

A juice sample of 1.2 mL was centrifuged (Eppendorf 5425 centrifuge; Eppendorf, Hamburg, Germany, Spain) for 30 min at 12,000 rpm. Precipitate was discarded, and the supernatant was filtered through a 0.45 μm nylon filter and analysed using an HPLC system equipped with a Waters 515 HPLC pump (Waters, Barcelona, Spain). A Waters 2414 refractive index detector, a 5 μm Tracer Carbohydrates column (250 mm × 4.5 mm) (Teknokroma, Barcelona, Spain), and a 20 μL Rheodyne loop injector were used for the sugar analysis. The mobile phase was consisted of acetonitrile and water (75:25) at a flow rate of 1.0 mL/min. Fructose, glucose and sucrose sugars were identified by comparing their retention time with standards and were quantified using an external calibration curve. The Empower 2 software (Waters, Spain) was used for data processing. Standards were purchased from Sigma (Sigma Co., Barcelona, Spain). Three replicates per sample were analysed. The results were expressed as g/L.

2.2.4. Volatile Organic Compound Profile

Juice samples were thawed at 27.5 °C for fifteen minutes right before analysis and then were subjected to headspace solid phase microextraction (HS-SPME). Extraction was carried out using 2 mL of sample into a 5 mL crimp cap headspace vial. A 65-μm PDMS/DVB (Supelco, PA, USA) fibre was used. Preincubation and extraction were done for 10 and 40 min, respectively, at 70°C. Desorption was performed at 250 °C for 1 min in splitless mode.

Volatile organic compounds trapped on the fibre were analysed by GC–MS using an autosampler COMBI PAL CTC Analytics (Zwingen, Switzerland), a 6890 N Gas Chromatograph (Agilent Technologies, CA, USA) and a 5975B Inert XL Mass Selective Detector (Agilent), equipped with an Agilent J&W Scientific DB-5 ms fused silica capillary column (5 %-phenyl-95 %-dimethylpolysiloxane as stationary phase, 60m length, 0.25 mm i.d., and 1 μm thickness film). Oven temperature conditions were 40 °C for 2 min, 5°C/min ramp until 250 °C and then held isothermally 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 35–220; seven scans per second). Chromatograms and spectra were recorded and processed using the Enhanced ChemStation software for GC–MS (Agilent). For the majority of the compounds, the identification was based on the consistency of GC retention time and Mass Spectra information with that of a customized library which have been generated using analytical grade commercial compounds (Sigma-Aldrich Química, Madrid, Spain). For the few compounds not included in the customized library, identification was based on the comparison between the MS for each putative compound with those of the NIST Version 2.3 Mass Spectral library. The peak area for each compound was calculated.

2.2.5. Data Processing and Statistical Analysis

Physico-chemical data were subjected to analyses of variance (ANOVA). Multiple comparison between means from different varieties was done using the Ducan's test (p-value ≤0.05). Comparison between only two pairs of samples (slightly vs. intensely pigmented samples from the same variety) were conducted using the LSD- test (p-value ≤0.05).

To normalize data from volatile compounds, the peak area of each compound was divided by the mean value of all peak areas of that compound from all the evaluated samples. Volatile data were subjected to ANOVA, Principal Component (PCA), Hierarchical Cluster (HCA) and Multifactor Analysis (MFA). ANOVA was performed as described for physico-chemical data. The Pearson correlation coefficient was used as similarity metric and weighted pair-group average linkage as the linkage method for HCA. The MFA was conducted to investigate the relationship between concentration of volatile compounds, sugars and anthocyanins in blood oranges. This involved the constructing a six-row matrix (3 cultivars × 2 pigmentation intensities) with three groups of columns, corresponding to the volatiles, sugars and anthocyanins data.

All these analyses were carried out using XLSTAT software (version 2024, Addinsoft Inc. New York, NY, USA).

3. Results and Discussion

3.1. Relation between physico-chemical parameters and pigmentation

Table 1 shows the mean values of the different physico-chemical characteristics determined per variety and degree of pigmentation. Regarding juice colour, significant differences in the a*/b* values of the blood varieties were found between the Sl-P and the In-P samples. Values ranged between 0.7 and 1.6 for the Sl-P samples and between 2.8 and 3.3 for the In-P ones. For the blond varieties, the a*/b* ratio had negative values that came close to zero.

Table 1. Physico-chemical parameters of the six orange cultivars. Mean values ± standard deviation are presented.

Variety	Degree of pigmentation	Juice colour (a*/b*)	TSS[1] (°Brix)	TA[2] (g citric ac./ 100 mL)	MI[3] (TSS/Ac)	Total anthocyanins (mg/L)
Cadenera		-0.2 ± 0.02[a]	13.6 ± 0.33[e]	1.52 ± 0.08[d]	8.9 ± 0.38[c]	—
Salustiana		-0.1 ± 0.01[a]	11.7 ± 0.19[ab]	1.03 ± 0.07[a]	11.5 ± 0.73[e]	—
Hamlin		-0.2 ± 0.01[a]	12.4 ± 0.53[cd]	1.18 ± 0.08[b]	10.5 ± 0.77[d]	—
Tarocco R.	Slight	0.7 ± 0.15[b]	12.0 ± 0.21[bc]	1.66 ± 0.05[e]	7.2 ± 0.28[a]	14.8 ± 1.3[a]
	Intense	2.8 ± 0.25[d]*	12.8 ± 0.20[d]*	1.63 ± 0.12[de]	7.9 ± 0.66[ab]	45.6 ± 5.1[b]*
Sanguinelli	Slight	1.6 ± 0.22[c]	11.6 ± 0.20[ab]	1.55 ± 0.07[de]	7.4 ± 0.36[a]	17.9 ± 6.0[ab]
	Intense	3.0 ± 0.22[e]*	12.7 ± 0.35[d]*	1.62 ± 0.11[de]	7.8 ± 0.47 [ab]	44.6 ± 14.7[b]*
Moro	Slight	0.7 ± 0.22[b]	11.4 ± 0.57[a]	1.32 ± 0.05[c]	8.6 ± 0.20[bc]	21.6 ± 9.1[b]
	Intense	3.3 ± 0.26[e]*	13.7 ± 0.36[e]*	1.38 ± 0.09[c]	9.9 ± 0.50[d]*	196.5 ± 25[c]*

[1]TSS: total soluble solids, [2]TA: titratable acidity, [3]MI: maturity index. The different letters in the same column indicate a significant difference among cultivars according to the LSD test (p≤0.05). *Indicates significant differences between the slight and intense pigmented samples of the same cultivar for each parameter.

The TSS and TA values were similar between the blood and blond oranges. When focusing on the pigmented varieties, the In-P samples had higher TSS values than the Sl-P samples in the three varieties. This increase in TSS linked with pigmentation intensity was more marked in 'Moro', which was the blood variety with the biggest differences in colour (a*/b*) between the Sl-P and In-P samples. As no differences in TA linked with pigmentation intensity were detected for any of the three blood orange varieties, the higher TSS values in the In-P fruit resulted in a higher MI value, with significant differences in 'Moro'.

The increase in fruit TSS content in conjunction with the degree of pigmentation has already been described for 'Sanguinelli' by Tarancón *et al.* (2022). The fact that this pattern was observed herein in the three evaluated varieties raises the question as to whether a higher TSS content in the In-P samples could be due to the detection of anthocyanins in the TSS measurement, or if it corresponded to a higher sugar concentration. To resolve this doubt, the concentration of individual sugars in Sl-P and In-P samples was analysed by chromatography. Table 2 shows the concentration of the three major sugars, fructose, glucose, and sucrose, for blond and blood orange varieties. For blood oranges, the concentration is separately presented for slightly and intensely pigmented samples.

Sucrose was the most abundant sugar in all the evaluated varieties, whose values ranged between 41 and 63 g/L (Table 2). For the three evaluated blood oranges, sucrose concentrations were significantly higher in the In-P samples than in the Sl-P ones. The fructose and glucose concentrations were similar to one another and were lower (19–35 g/L) than those of sucrose. For 'Tarocco R.' and 'Moro' the glucose concentrations were higher in the In-P samples compared to the Sl-P ones. The biggest differences for sucrose and glucose were detected in the 'Moro' variety as TSS already revealed. In this variety, fructose content also increased in the In-P samples. Therefore, the analysis of individual sugars corroborated that the In-P samples had higher sugar content than the Sl-P ones.

Table 2. Individual sugars (fructose, glucose and sucrose) of the six cultivars. Mean values ± standard deviation are presented.

Variety	Degree of pigmentation	Fructose (g/L)	Glucose (g/L)	Sucrose (g/L)
Cadenera		34.9 ± 2.93^{e}	34.7 ± 3.14^{e}	57.0 ± 1.53^{e}
Salustiana		27.0 ± 0.59^{c}	25.9 ± 0.69^{cd}	52.2 ± 0.88^{c}
Hamlin		28.4 ± 1.18^{cd}	28.8 ± 1.07^{d}	60.4 ± 1.25^{ef}
Tarocco R.	Slight	28.9 ± 0.54^{cd}	23.2 ± 0.85^{bc}	51.4 ± 2.36^{c}
	Intense	29.6 ± 1.04^{cd}	28.9 ± 1.26^{d*}	56.6 ± 2.39^{de*}
Sanguinelli	Slight	19.4 ± 3.14^{a}	19.0 ± 3.21^{a}	41.1 ± 3.96^{a}
	Intense	22.1 ± 1.62^{ab}	22.7 ± 1.77^{ab}	47.3 ± 0.56^{b*}
Moro	Slight	23.4 ± 1,72^{b}	23.7 ± 1.85^{c}	52.7 ± 1.86^{cd}
	Intense	30.7 ± 1.19^{d*}	32.2 ± 1.59^{e*}	62.8 ± 3.62^{f*}

The different letters in the same column indicate significant differences among cultivars according to Duncan's test (p ≤0.05). For a specific blood variety, * in data from the intensely pigmented sample indicates significant differences compared to the slightly pigmented samples of the same variety (LSD test, p ≤0.05) (e.g. sucrose of intensely pigmented 'Moro' samples vs. slightly pigmented 'Moro' samples).

Indirectly, this relation between anthocyanin and sugar accumulation has been previously observed by Muccilli *et al.* (2009). In their proteomic analysis comparing the flesh of 'Moro' variety to that of the blond variety 'Cadenera', the authors reported an overexpression of enzymes involved in sugar metabolism in 'Moro', including pyrophosphate-dependent phosphofructokinase alpha subunit, fructokinase-like protein, glyceraldehyde-3- phosphate dehydrogenase, enolase, phosphoenolpyruvate carboxykinase, sucrose synthase, succinyl CoA ligase beta subunit, and nucleoside diphospahate kinase. These authors suggested that, as previously reported for grape skin (Sarry *et al.*, 2004), these enzymes could play a key role in obtaining the carbon skeletons needed for anthocyanins biosynthesis. In the same line, Carmona *et al.*

(2019) found that storing ‘Moro’ fruit at 9 °C enhanced anthocyanins accumulation and also promoted, among other aspects, the proteins belonging to sugar metabolism.

The difference in colour (a*/b*) observed between In-P and Sl-P samples was explained by the total anthocyanin content (Table 1). All the Sl-P samples presented similar total anthocyanins values, which ranged from 14.8 to 21.6 mg/L. In the In-P samples, ‘Moro’ had the highest anthocyanin concentration with 196.5 mg/L, while both ‘Tarocco Rosso’ and ‘Sanguinelli’ had values of around 45 mg/L. This result falls in line with previous studies in which ‘Moro’ has been highlighted for its high anthocyanin content (Legua *et al.*, 2021; Morales *et al.*, 2021).

In accordance with previous studies, the UPLC analysis revealed cyanidin-3-O-glucoside (Cy-3-glu) and cyanidin-3-(6”malonyl)-O-glucoside (Cy-3-6”mal) as the major anthocyanins in the three blood orange cultivars (Cebadera-Miranda *et al.*, 2019; Carmona *et al.*, 2020; Morales *et al.*, 2021). The presence of more peaks in the chromatograms indicated the existence of a series of minor anthocyanins. Among them, only cyanidin-3-(6“dioxalyl)-O-glucoside (Cy-3-6”diox) and delphinidin-3-O-glucoside (Dp-3-glu) could be unequivocally identified, and therefore they have been included in the results (Figure 2). The anthocyanin profiles of ‘Tarocco Rosso’ and ‘Sanguinelli’ were similar. In both cases, the main differences between In-P and Sl-P samples were detected in the two main anthocyanins, Cy-3-glu and Cy-3-6”mal, whose concentration doubled or tripled in the In-P samples compared to the Sl- P ones. The profile for ‘Moro’ was quite different, especially for the In-P samples, for which the concentration of these two main anthocyanins increased sharply compared to not only ‘Tarocco Rosso’ and ‘Sanguinelli’, but also to their Sl-P counterparts. So the concentration of both Cy-3-glu and Cy-3-6”mal in the In-P samples of ‘Moro’ was about 4-fold higher than in the In-P samples of ‘Tarocco Rosso’ and ‘Sanguinelli’, and was about 10-fold higher than that of the Sl-P counterparts with values over 100 mg/L. A significant increase in the two minority anthocyanins (Dp-3-glu and Cy-3-6”diox) was also observed in all three cultivars under study when comparing In-P and Sl-P samples (Figure 2).

It is worth mentioning that minimal changes in the a*/b* ratio may be linked to marked visual differences in colour and large differences in anthocyanins concentration. This was particularly evident for ’Moro’ and ’Sanguinelli’, whose In-P samples had similar a*/b* values (3.3 and 3.0, respectively), but the anthocyanin content of ’Moro’ was 4-fold higher than that of ’Sanguinelli’, and was easily evidenced by sight (Table 1, Figure 1). This situation has already been observed in other studies (Legua *et al.*, 2021). As the three studied varieties were grown under the same soil and environmental conditions, the differences in anthocyanin concentrations among them confirmed the importance of the genetic factor in anthocyanin accumulation (Cebadera-Miranda *et al.*, 2019; Legua *et al.*, 2021).

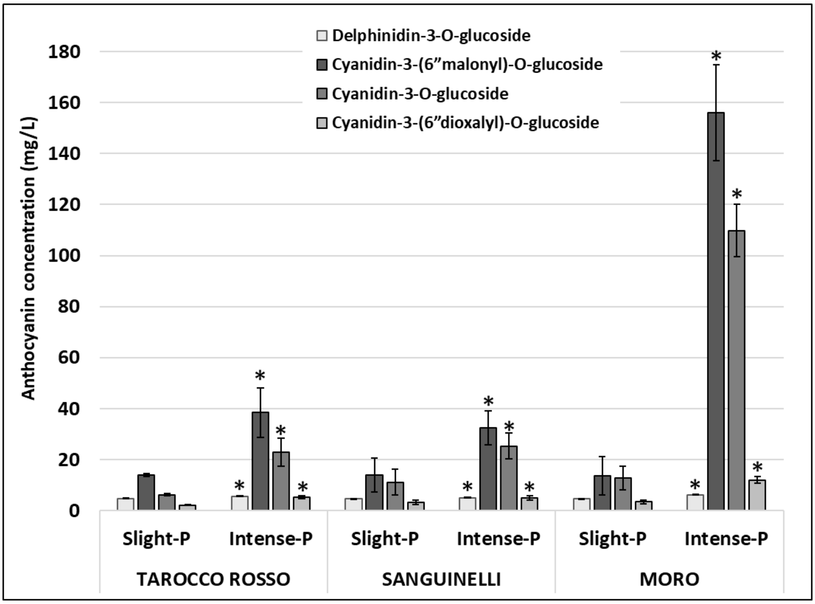

Figure 2. Individual anthocyanins (delphinidin 3-glucoside, cyanidin 3-glucoside, cyanidin 3-(6”malonyl)-glucoide and cyanidin 3-(6”-dioxalyl)-glucoside) of the slightly and intensely pigmented samples of the evaluated varieties ‘Tarocco Rosso’, ‘Moro’ and ‘Sanguinelli’. Vertical bars represent standard deviations. For each compound, * in the intensely pigmented samples indicates significant differences respect to the slightly pigmented samples of the same cultivar (LSD test, $p \leq 0.05$).

3.2. Volatile Composition of Fruit

The two main aroma-related objectives of this study were to investigate to what extent the volatile profile of blood oranges differed from that of blond varieties, and to determine if the volatile composition of fruit depends on their pigmentation intensity for a specific blood variety. To answer these two questions in an orderly manner, this section was divided into two subsections.

3.2.1. Volatile profile of blood vs. blond orange varieties

Applying SPME–GC–MS to analyse the volatile profile of three blond and blood orange varieties allowed 101 compounds to be identified: 26 esters, 23 monoterpenes, 20 aldehydes, 16 sesquiterpenes, 11 alcohols, 1 ketone, 1 alkaloid, 1 alkatetraene, 1 unknown compound and carbon dioxide (Supplementary Table S1). A Principal Component Analysis (PCA) was performed to visualise the main differences and similarities in the varieties' volatile compounds profile (Figure 3). The PCA figure (Figure 3A) shows the distribution of samples, while the loading plot (Figure 3B) depicts the influence of the 101 compounds identified in the distribution of the samples. We indicate only those compounds with the strongest impact on the differentiation of samples.

The 'Moro' variety presented a characteristic profile that positioned it to the left of the space (Figure 3A). Two compounds were key for this separation because they were specific of this variety profile: (Z)-4-decenal and (E)-methylgeranate (Figure 3B, Supplementary Table S2). Compound (Z)-4-decenal has been described as having mainly citrus-related aromatic notes such as 'citrusy', 'juicy' or 'orange-like' (Hognadottir and Rouseff, 2003, Kiefl *et al.*, 2017). It has also been described as 'spicy' and 'waxy' in essential peel oils of Makino (*Citrus tachibana*) (Miyazato, 2018). To the best of our knowledge, in orange juice it has only been detected in the blond variety 'Pera Rio' from Brazil and described as having 'citrus-like' aroma (Seideneck and Schieberle, 2011). (E)-methylgeranate was first detected in citrus fruit by Miyazaki *et al.* (2010) in tangerine hybrids, but its aroma was not described. In other fruit like feijoa, it has been reported to confer 'green', 'flower' and 'fruit' aromas (Song *et al.*, 2023). 'Moro' fruit are also characterised by having high concentrations of several aldehydes, such as octanal, neral, decanal, undecanal, dodecanal and citral, which have been described to confer citrus fruit a ‘green’ and ‘fatty’ aroma (Miyazaki *et al.*, 2011). Besides, fruit from this variety showed increased levels of β-famesene, which has been reported to have ‘wood’, ‘citrus’ and ‘sweet’ aroma (Ye *et al.*, 2024), and octyl acetate with ‘soapy’ and ‘floral’ notes (Plotto *et al.*, 2008).

Although the behaviour related to the above-mentioned compounds was common to the Sl-P and In-P samples of ‘Moro’, both sample types were clearly differentiated in the PCA space, with the In-P samples showing positive values for the second component and the Sl-P samples with negative values. The compounds implied in such a separation between In-P and Sl-P samples are later discussed.

On the opposite side of the graph, to the right, blood orange variety ‘Tarocco Rosso’ and blond varieties ‘Cadenera’ and ‘Hamlin’ appear. These cultivars shared one aspect: they had high concentrations of aldehydes (E)-2-pentenal and (E,E)-2,4-hexadienal, 3-hexenal and (E)-2-octenal, and of esters ethyl hexanoate, methyl hexanoate and ethyl 3-hydroxyhexanoate. The major difference between ‘Cadenera’ and ‘Hamlin’ vs. ‘Tarocco Rosso’ was that the two blond varieties had relatively high levels of (E)-2-hexen-1-ol acetate and (Z)-3-hexenyl hexanoate, but these two compounds were not detected in the ‘Tarocco’ variety (Figure 3B, Supplementary Table S2). As observed in ‘Moro’, the In-P samples also differed from the Sl-P samples in ‘Tarocco Rosso’. In fact, the Sl-P samples were closer in the PCA space to the blond variety ‘Hamlin’ than to their In-P counterparts.

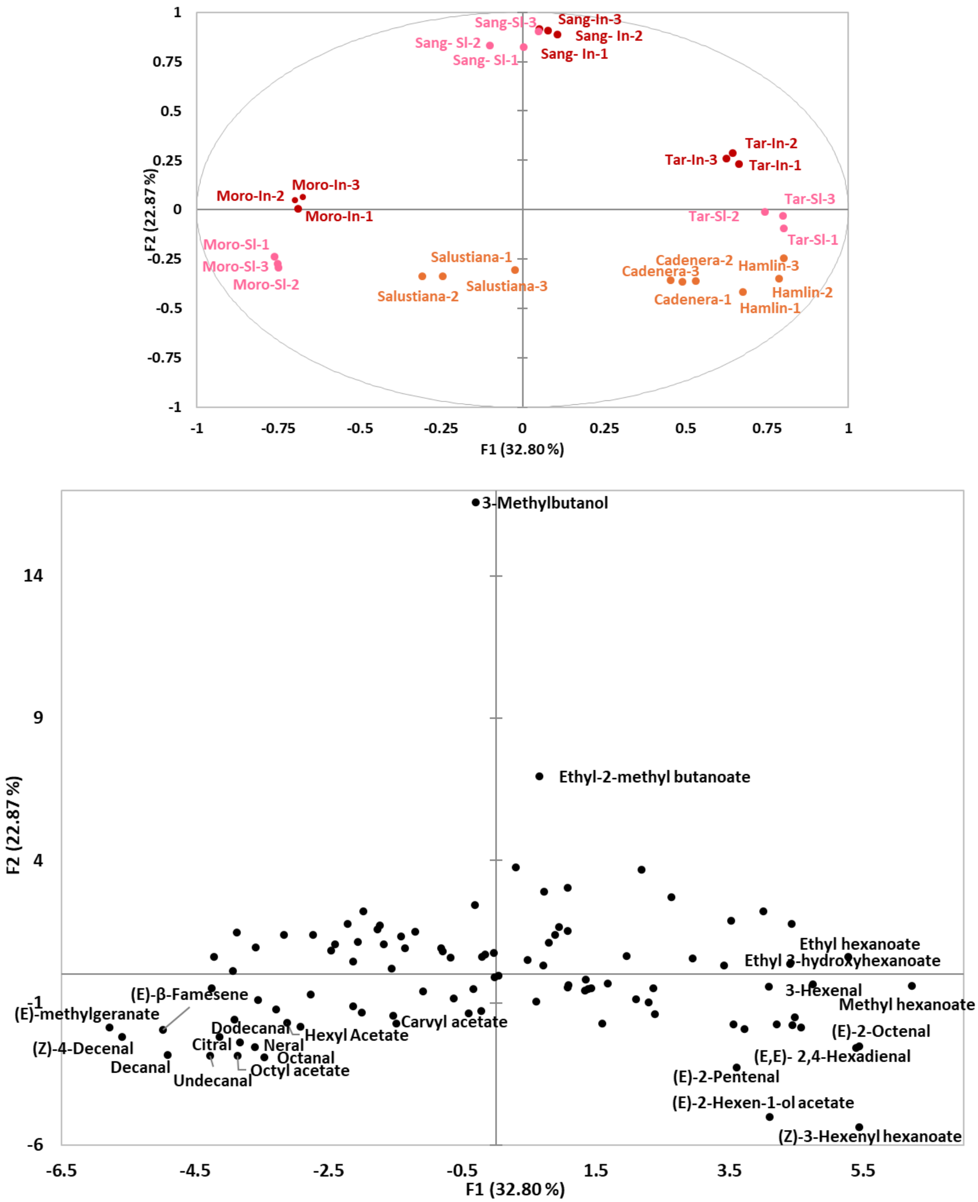

Figure 3. Principal Component Analysis (PCA) based on volatile compound composition. The score and loading plots for the first and second principal components are shown. Three replicates were analysed per cultivar and pigmentation intensity (Sl-Slightly pigmented, In-Intensely pigmented).

'Sanguinelli' was allocated between 'Moro' and 'Tarocco Rosso' along the PCA first component but was clearly separated from them along the second component as it was allocated to the upper part of the space. Of the main reasons for this, we found a high 3-methylbutanol concentration in 'Sanguinelli', but this compound was not detected in any of the other blood or blond orange varieties (Figure 3B, Supplementary Table S2). This compound, also known as isoamyl alcohol, has been previously described as the major alcohol compound in 'Moro' and 'Sanguinello' cultivated in Turkey (Selli and Kelebek, 2011). However, in the present study it was detected only in 'Sanguinelli'. Another relevant fact about this variety is that it was the only one in which compounds neral and hexyl acetate were not detected (Figure 3B, Supplementary Table S2). Neral has been reported to contribute to the main citrus odour notes (Pérez-Cacho and Rouseff, 2008), while hexyl acetate has been previously described to confer 'sweet', 'floral' and 'apple' aromas in limes and lemons (Cano-Lamadrid *et al.*, 2018). The Sl-P and In-P samples of 'Sanguinelli' were close to one another in the space, which *a priori* indicates that their volatile profile does not depend on pigmentation. However, an independent PCA performed only with the 'Sanguinelli' samples showed a separation among them, as later discussed.

Finally, the blond variety 'Salustiana' was allocated close to the origin of the coordinates along the first component, which indicates that it shares characteristics with most of the evaluated varieties. It differed from 'Cadenera' and 'Hamlin' because, among other aspects, it was the only blond variety in which (Z)-3-hexenyl hexanoate was not detected (Figure 3B, Supplementary Table S2), and also because it had higher concentrations of other compounds, such as carvyl acetate. This compound has been detected in Malaysian pomelo peel and has been described as 'herbaceous' and 'spicy' (Cheong *et al.*, 2011). It is worth noting that the three blond varieties were allocated further down in the PCA space than the three blood varieties. This was mainly because of compound ethyl-2-methyl butanoate, which was not detected in the blond varieties in this study but was present at different levels in the blood ones. Moreover, compounds (E)-2-hexen-1-ol acetate and (Z)-3-hexenyl hexanoate were not detected in any of the blood varieties but were present in the blond ones. To further investigate the similarity/dissimilarity between the volatile profiles of the different varieties, a hierarchical cluster analysis (HCA) was carried out (Figure 4). According to their profile, the six evaluated varieties were organised into two clusters (A and B), and each one was, in turn, divided into two subclusters (A1, A2, B1 and B2). The HCA confirmed the greater similarity of 'Tarocco Rosso' to the blond varieties 'Cadenera' and 'Hamlin' (Cluster A) than to the other blood varieties (Cluster B); subcluster A1 included variety 'Cadenera', while subcluster A2 consisted of 'Hamlin' and 'Tarocco Rosso'. Cluster B included two blood varieties, 'Moro' and 'Sanguinelli', and blond variety 'Salustiana'. Blood variety 'Moro' was more similar to blond variety 'Salustiana' (subcluster B2) than to 'Sanguinelli' (subcluster B1).

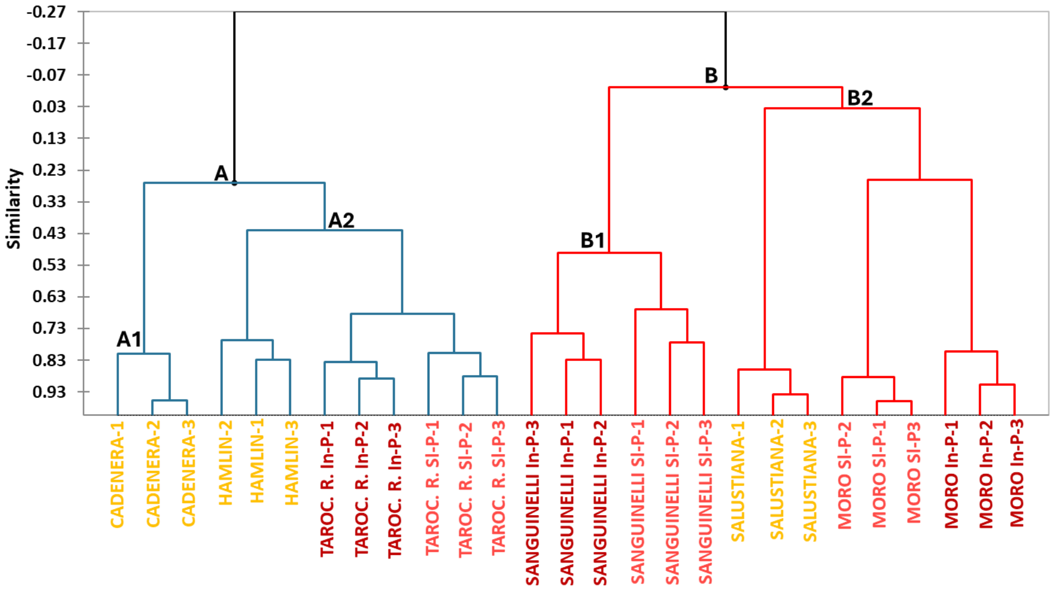

Figure 4. Hierarchical Cluster Analysis (HCA) of the evaluated samples based on their volatile compound profile.

Our results show, therefore, that by taking into account the complete volatile compound profile, the three evaluated blood varieties showed no greater similarities to one another than to the blond varieties. In 2006, Arena *et al.* came to a different conclusion, which was based on the fact that, in their study, methyl butanoate and ethyl octanoate were present at olfactory active levels in blood varieties ‘Moro’ and ‘Tarocco’ but not in the blond varieties they evaluated (‘Washington Navel’ and ‘Valencia Late’). In the present study, we evaluated three blond varieties different from those in Arena *et al.* (2006), and in all three (‘Cadenera’, ‘Salustina’ and ‘Hamlin’) methyl butanoate and ethyl octanoate compounds were detected; in fact, methyl butanoate was noted at higher levels in ‘Hamlin’ than in ‘Moro’ and ethyl octanoate was at higher concentrations in ‘Hamlin’ than in ‘Sanguinelli’ (Figure 5). In the same line, ethyl octanoate has been previously detected in blond variety ‘Navelina’ by Sdiri *et al.* (2017) and methyl butanoate in ‘Valencia’ orange by Plotto *et al.* (2008).

At this point it is important to mention that ethyl-2-methyl butanoate was the only compound in the present study to be detected only in the blood varieties (Figure 6, Supplementary Table S2). Based only on such a result, we could conclude that it is specific of blood varieties. However, this would be a false conclusion because this compound has also been found in blond varieties ‘Washington Navel’ and ‘Valencia Late’ by Arena *et al.* (2006). Therefore, if we consider the results of both studies, that of Arena *et al.* (2006) and the present one, there is no compound that is specific to blood oranges vs. blond oranges.

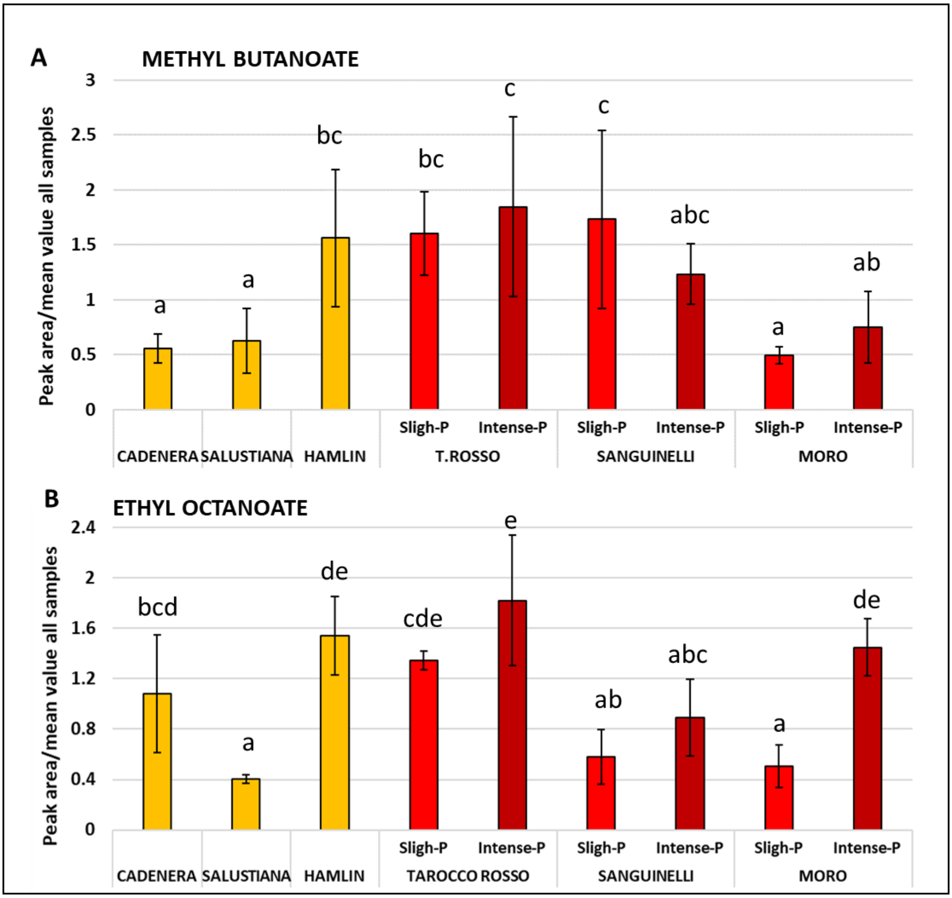

Figure 5. Normalised peak areas (peak area/mean value of all the evaluated samples) of the compounds methyl butanoate (A) and ethyl octanoate (B) in each of the orange varieties evaluated. Vertical bars represent standard deviations. Different letters indicate significant differences among varieties according to Duncan's test ($p \leq 0.05$).

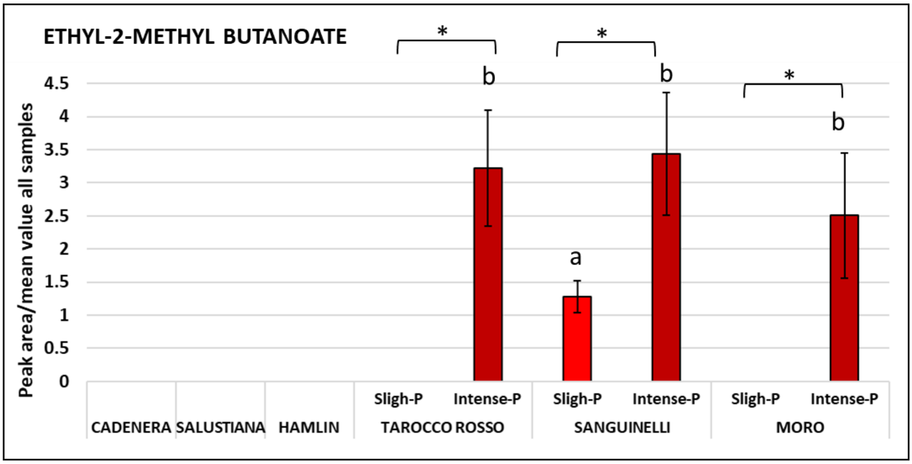

Figure 6. Normalised peak areas (peak area/mean value of all the evaluated samples) of the compound ethyl-2-methyl butanoate in each of the orange varieties evaluated. For each type of samples, vertical bars represent standard deviations. * Indicates significant differences between the slightly and intensely pigmented samples of a specific variety (LSD test, $p \leq 0.05$).

3.2.2. Blood oranges. Volatile Profile of the Slightly vs. Intensely Pigmented Fruit

In order to gain a deeper understanding of the aroma profiles of the In-P samples compared to the Sl-P ones, a PCA was carried out individually for each blood variety (Figure 7). A common pattern was detected in the three of them, with the In-P samples characterised by increased levels of some volatile compounds compared to the Sl-P ones. The compounds found at higher concentrations in the In-P samples were mainly esters. Three of them, ethyl acetate, ethyl-2-methyl butanoate and ethyl-2-butenoate are highlighted for being identified at high levels in the In-P samples of the three evaluated varieties. This pattern was also followed by the ethanol compound, which appeared at higher levels in the In-P samples for the three evaluated varieties (Figures 6, 7 and 8).

In a study performed by Plotto *et al.* (2008) to determine the odour activity values for flavour volatiles in an orange juice matrix, ethyl-2-

methyl butanoate was described to confer 'sweet' and 'fruity' notes, and ethyl acetate was described as 'fruity', 'sweet', 'tangerine', 'estery' and 'solvent'. Such odour descriptions were corroborated in a study performed on blackberry fruit (Wang *et al.*, 2005), which also described ethyl-2-butenoate as having a 'fruity', 'green' odour. Ethyl-2-butenoate was described as a potent aroma-active compound in the peel of quince, in which it was found to confer 'sweet' and 'fruity' aroma notes (Choi *et al.*, 2018). A priori, such odour descriptions suggests that these compounds may play an important role in the aroma of the In-P fruit, perhaps by making them more aromatic than the Sl-P ones. However, Tietel *et al.* (2011) performed an experiment with 'Mor' mandarins and linked the accumulation of ethyl acetate, ethyl-2-butenoate and ethyl-2-methylbutanoate among other esters to the ethanol fermentation process under anaerobic conditions. According to the authors, ethanol would serve as substrate for downstream esterification reactions. These authors linked the accumulation of ethanol and ethanol-derived esters to the development of off-flavours under anaerobiosis. Although the fruit was not subjected to anaerobic conditions in this study, it is necessary to carry out sensory studies to clarify whether the changes in volatiles associated with anthocyanin accumulation described herein have a positive or negative influence on fruit flavour.

In the same line, in 'Moro' and 'Tarocco Rosso', the increment in these esters in the In-P samples was accompanied by lower levels of aldehydes (Z)-4-decenal, decanal, octanal, nonanal, undecanal, citral, dodecanal, (E,E)-2,4-hexadienal, (E)-2-octenal, heptanal, (E)-2-nonenal, (E)-2-heptenal and (E)-2-pentenal compared to the Sl-P samples. For 'Sanguinelli' and 'Moro', the In-P samples had a lower content of some monoterpenes, such as β-thujene, γ-terpinene α-terpinene, and sabinene. These compounds belong to the monoterpene chemical family and have been previously described as important contributors to citrus-related aromatic characteristics (Cano-Lamadrid *et al.*, 2018). From these results, we can predict that fruit aroma will depend on pigmentation intensity, but we cannot infer what these changes mean at the organoleptic level, i.e. which fruit will be more aromatic or have a more appealing odour.

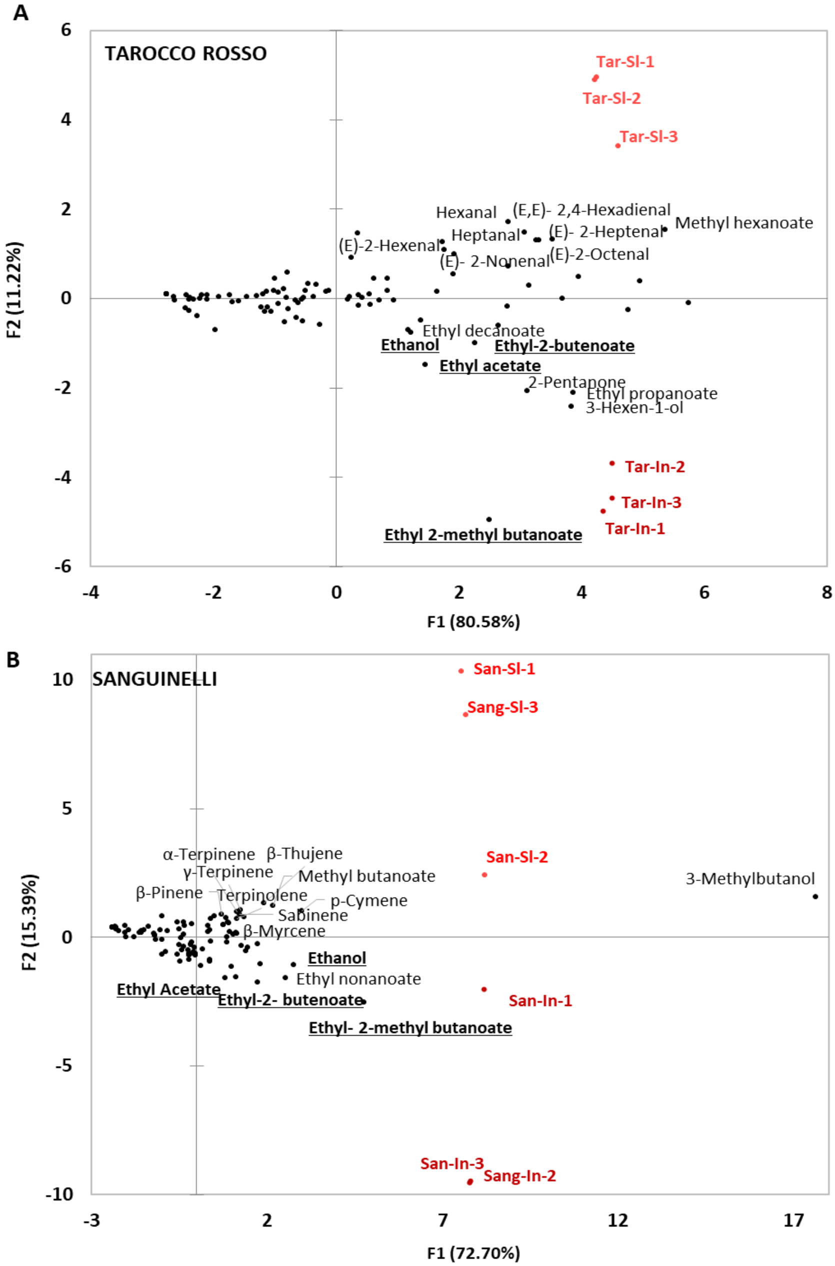

Figure 7. Principal component analysis (PCA) of the slightly and intensely pigmented samples of varieties ‘Tarocco Rosso’ (A), ‘Sanguinelli’ (B) and ‘Moro’ (C) based on their volatile compound profile (continued on the following page).

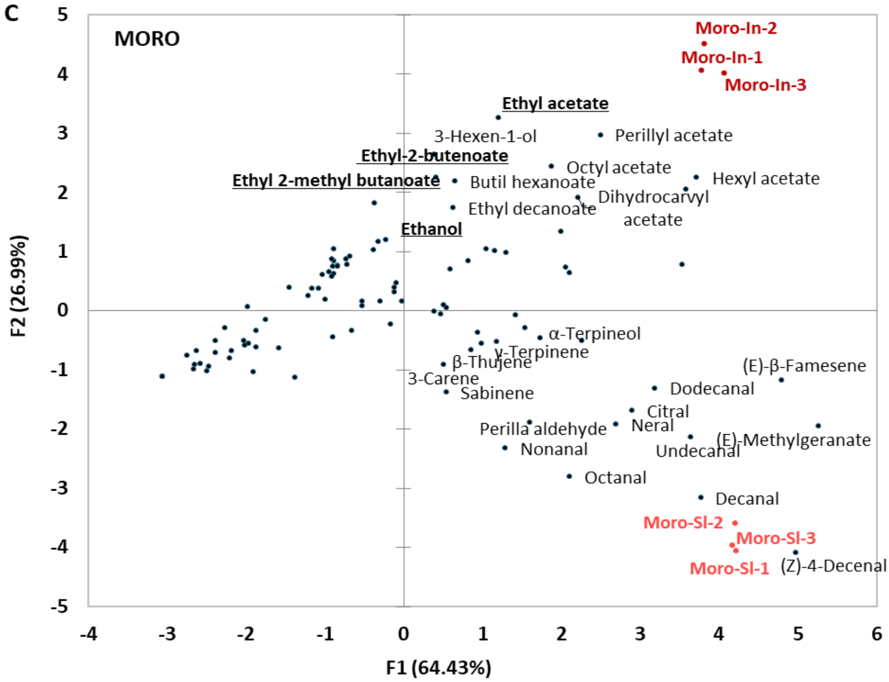

Figure 7. Principal component analysis (PCA) of the slightly and intensely pigmented samples of varieties 'Tarocco Rosso' (A), 'Sanguinelli' (B) and 'Moro' (C) based on their volatile compound profile.

The results obtained from the present study open the door to further investigate the relation between anthocyanins and sugar accumulation, and changes in volatile composition in blood oranges. It has been demonstrated that applying ethanol at low concentrations can enhance anthocyanin accumulation in grapes (Canals *et al.*, 2005; Miyazaki *et al.*, 2010; Margherita *et al.*, 2024). Therefore, it is possible that the herein observed increased ethanol levels in the In-P samples may play a role in anthocyanin synthesis. In addition, Margherita *et al.* (2024) reported an increase in sugar content as a result of treating grapes with ethanol. In the same line, recent studies in plants, specifically in fern, have begun investigating possible links between the biosynthetic pathways of anthocyanins and volatile compounds (Zhang *et al.*, 2022). Furthermore, in tomato fruit, it has been demonstrated that the overexpression of a single gene leads to multiple changes, including an increment in anthocyanin content, soluble solids, and changes in volatile composition, among others. Therefore, a hypothesis worth mentioning is about the possible link between the higher concentration of sugars found in the In-P fruit compared to the Sl-P ones, and the increase in the concentration of ethanol and certain esters (ethyl acetate, ethyl-2-methyl butanoate and ethyl-2-butenoate) linked with pigmentation intensity.

To investigate this possibility in more detail, an MFA was conducted, including data on volatiles, anthocyanins, and sugars from slightly and intensely pigmented blood orange samples. The results of this analysis are shown in Figure 9. It can be observed that sucrose was the sugar most correlated with anthocyanins. This finding is consistent with Abdullah *et al.* (2018), whose review highlights sucrose as the most significant sugar involved in the regulation of the anthocyanin biosynthesis pathway. These authors indicate that the exact molecular mechanism of sugar sensing and signalling are not yet clear, and some confusion still remains, necessitating further studies.

Additionally, the MFA confirmed the higher accumulation of the four previously mentioned compounds (ethyl acetate, ethyl-2-methyl butanoate, ethyl-2-butenoate, and ethanol) in the more intensely pigmented samples, which consequently had higher concentrations of anthocyanins and sugars. Moreover, it revealed other volatile compounds that correlated with anthocyanin accumulation. Notable among these were the esters butyl hexanoate and ethyl decanoate, the terpenes β-guaiene, selina-3,7(11)-diene, and the alcohol neointermedeol. While in this study the two esters were unequivocally identified using standards, β-guaiene, selina-3,7(11)-diene, and neointermedeol were identified tentatively, necessitating further studies to corroborate these findings.

There are many unknowns still to be resolved, but our results corroborate the link between anthocyanin and sugar accumulation at harvest time that has been previously reported by Tarancón *et al.* (2022). They also reveal, for the first time, changes in the volatile composition associated with anthocyanin accumulation in blood oranges, thus opening the door for further investigation into the relationship among anthocyanins, sugars, and volatile composition in blood oranges.

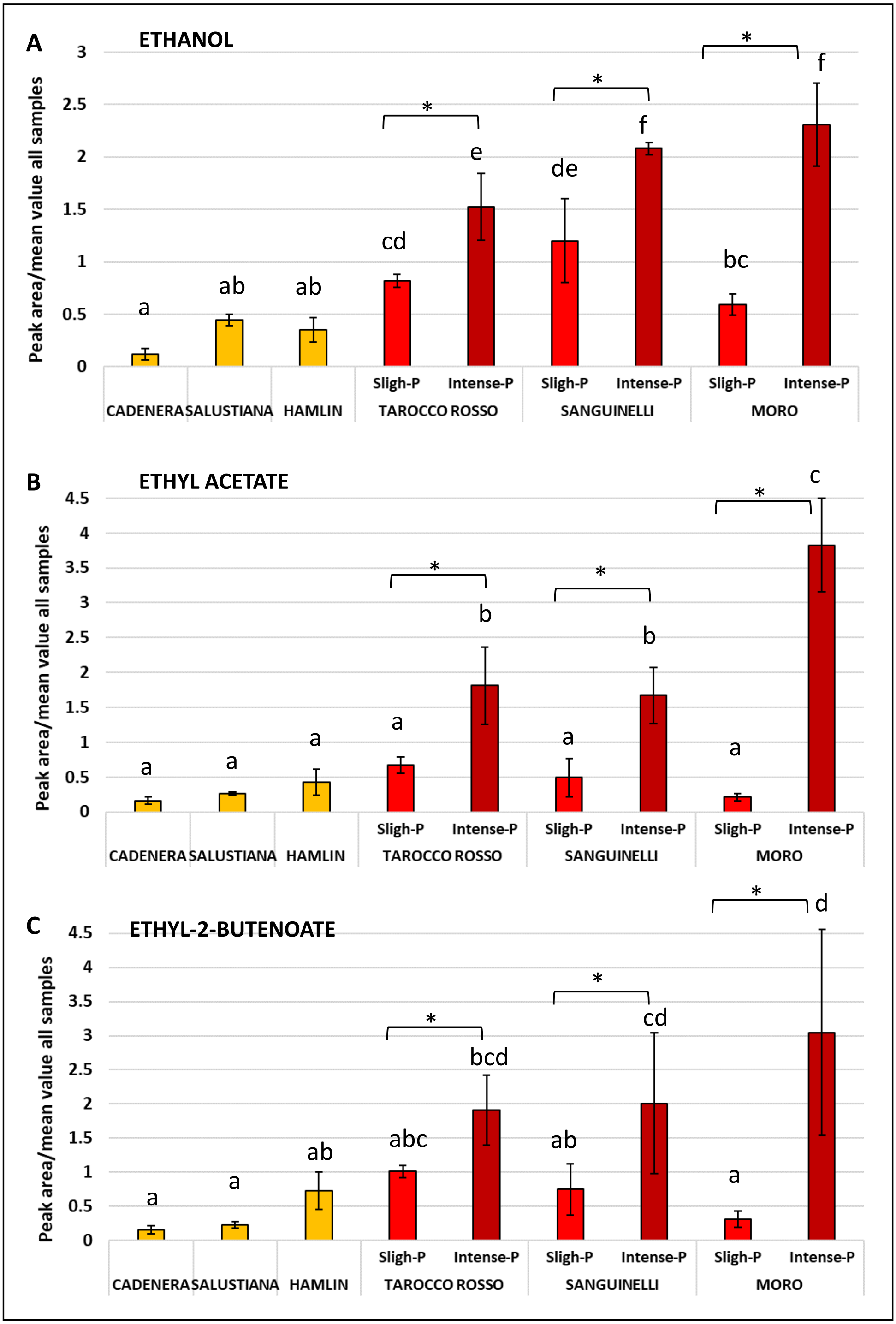

Figure 8. Description on the following page.

Figure 8. Normalised peak area (peak are/mean value of all the evaluated samples) for compounds ethanol (A), ethyl acetate (B) and ethyl-2-butenoate (C). For each type of samples, vertical bars represent standard deviations. *Indicates significant differences between the slightly and intensely pigmented samples of a specific variety (LSD test, $p \leq 0.05$).

4. Conclusion

The set of analytical determinations and statistical analyses performed allowed us to achieve the two main objectives of this study. On one hand, blood oranges were shown not to have a unique volatile compounds profile that makes them more similar to each other than to blond varieties. On the other hand, the comparative analysis of the slightly and intensely pigmented samples from blood orange varieties revealed that the concentration of sugars and the composition of volatile compounds depend on the intensity of pulp pigmentation. Greater anthocyanin accumulation in intensely pigmented fruits is associated with higher sugar concentrations, mainly sucrose, and changes in the aroma profile. Future studies should aim to elucidate the metabolic connection between the accumulation of sugars and anthocyanins and the observed changes in the volatile profile in blood oranges

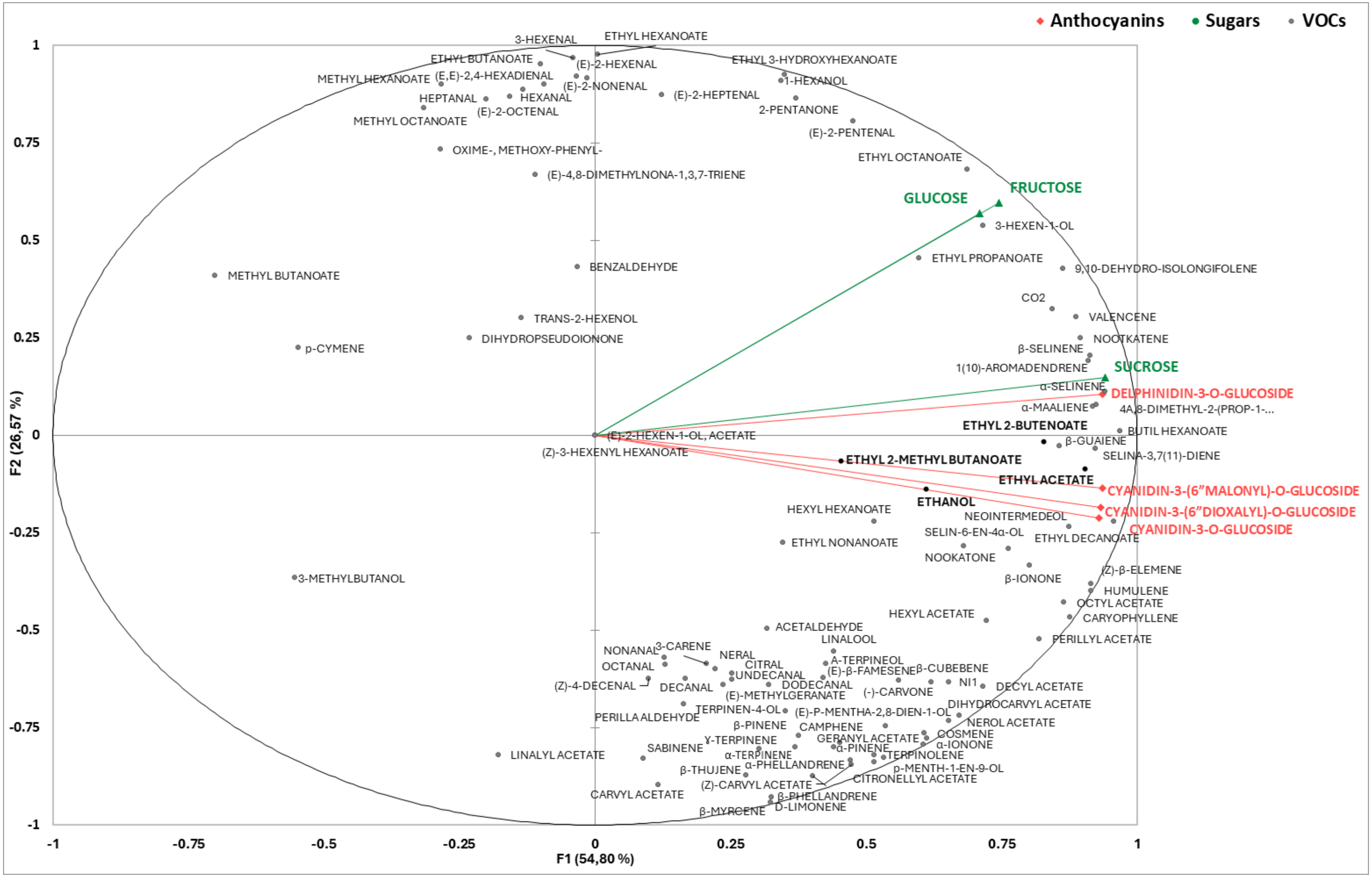

Figure 9. Description on the following page

Figure 9. Multifactor analysis (MFA) considering mean values of volatile compounds from each type of blood orange samples (3 varieties x 2 pigmentation intensities) as active dataset and sugars and anthocyanin concentrations as supplementary dataset.

Acknowledgements

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Supplementary Material

Table S1. Identification of volatile compounds in the orange varieties 'Tarocco Rosso', 'Sanguinelli', 'Moro', 'Cadenera', 'Salustiana' and 'Hamlin'. For each compound, the retention time (min) and the reverse match of the mass spectra against NIST library (1000 = 100% identical to the NIST library entry) are shown; (+) after the NIST reverse match value indicates that identification was confirmed by an authentic chemical standard injection (continued on the following page)**.**

Code	Volatile Compound	Type	Retention Time (min)	NIST Reverse Match
1	CO_2	miscellaneous compound	3.55	999
2	Acetaldehyde	aldehyde	4.27	846 (+)
3	Ethanol	alcohol	4.97	973 (+)
4	Ethyl acetate	ester	8.69	933 (+)
5	2-Pentanone	ketone	11.3	928 (+)
6	Ethyl propanoate	ester	12.15	927 (+)
7	Methyl butanoate	ester	12.64	930 (+)
8	3-Methylbutanol	alcohol	13.13	894
9	(E)-2-Pentenal	aldehyde	14.05	896 (+)
10	Ethyl butanoate	ester	15.69	954 (+)
11	3-Hexenal	aldehyde	15.71	871
12	Hexanal	aldehyde	15.83	969 (+)
13	Ethyl 2-butenoate	ester	17.49	923
14	Ethyl 2-Methyl Butanoate	ester	17.66	922 (+)
15	3-Hexen-1-ol	alcohol	17.74	899 (+)
16	(E)-2-Hexenal	aldehyde	17.99	952 (+)
17	trans-2-Hexenol	alcohol	18.28	911
18	1-Hexanol	alcohol	18.39	879 (+)
19	Oxime-, methoxy-phenyl-	alkaloid	18.69	832
20	Heptanal	aldehyde	19.82	869 (+)
21	(E,E)- 2,4-Hexadienal	aldehyde	20.28	891
22	Methyl hexanoate	ester	20.51	902 (+)
23	β-Thujene	monoterpene	21.06	950
24	α-Pinene	monoterpene	21.52	944 (+)

Table S1. Identification of volatile compounds in the orange varieties 'Tarocco Rosso', 'Sanguinelli', 'Moro', 'Cadenera', 'Salustiana' and 'Hamlin'. For each compound, the retention time (min) and the reverse match of the mass spectra against NIST library (1000 = 100% identical to the NIST library entry) are shown; (+) after the NIST reverse match value indicates that identification was confirmed by an authentic chemical standard injection (continued on the following page)**.**

Code	Volatile Compound	Type	Retention Time (min)	NIST Reverse Match
25	(E)-2-Heptenal	aldehyde	21.98	930 (+)
26	Camphene	monoterpene	22.3	916 (+)
27	Benzaldehyde	aldehyde	22.59	907 (+)
28	Sabinene	monoterpene	22.92	966
29	β-Myrcene	monoterpene	23.13	975 (+)
30	Ethyl hexanoate	ester	23.24	929 (+)
31	β-Pinene	monoterpene	23.32	940 (+)
32	Octanal	aldehyde	23.62	965 (+)
33	Hexyl acetate	ester	23.72	916 (+)
34	(E)-2-Hexen-1-ol, acetate	ester	23.78	915
35	α-Phellandrene	monoterpene	24.15	922 (+)
36	Cosmene	alkatetraene	24.08	922
37	3-Carene	monoterpene	24.29	915 (+)
38	α-Terpinene	monoterpene	24.51	961 (+)
39	p-Cymene	monoterpene	24.82	847 (+)
40	D-Limonene	monoterpene	25.09	924 (+)
41	β-Phellandrene	monoterpene	25.24	948
42	(E)-2-Octenal	aldehyde	25.64	889 (+)
43	ɣ-Terpinene	monoterpene	25.89	946 (+)
44	Terpinolene	monoterpene	26.91	964 (+)
45	Linalool	alcohol	27.017	928 (+)
46	Nonanal	aldehyde	27.16	891 (+)
47	(E)-4,8-Dimethylnona-1,3,7-triene	monoterpene	27.44	921
48	Methyl octanoate	ester	27.6	869 (+)
49	Ethyl 3-hydroxyhexanoate	ester	27.86	844 (+)
50	(E)-p-Mentha-2,8-dien-1-ol	alcohol	28.19	828

Table S1. Identification of volatile compounds in the orange varieties 'Tarocco Rosso', 'Sanguinelli', 'Moro', 'Cadenera', 'Salustiana' and 'Hamlin'. For each compound, the retention time (min) and the reverse match of the mass spectra against NIST library (1000 = 100% identical to the NIST library entry) are shown; (+) after the NIST reverse match value indicates that identification was confirmed by an authentic chemical standard injection (continued on the following page)**.**

Code	Volatile Compound	Type	Retention Time (min)	NIST Reverse Match
51	(E)-2-Nonenal	aldehyde	29.07	911 (+)
52	Butil hexanoate	ester	29.77	903
53	Ethyl octanoate	ester	29.9	929 (+)
54	(Z)-4-Decenal	aldehyde	30.12	915 (+)
55	Terpinen-4-ol	alcohol	30.23	956 (+)
56	Octyl acetate	ester	30.33	892 (+)
57	Decanal	aldehyde	30.45	899 (+)
58	α-terpineol	alcohol	30.59	909 (+)
59	Neral	aldehyde	31.64	900
60	Linalyl acetate	ester	31.68	869 (+)
61	(-)-Carvone	monoterpene	32.16	948 (+)
62	Citral	aldehyde	32.45	908 (+)
63	Ethyl nonanoate	ester	32.93	935 (+)
64	Perilla aldehyde	aldehyde	33.27	957
65	Undecanal	aldehyde	33.53	930 (+)
66	(E)-Methylgeranate	monoterpene	33.88	889
67	Carvyl acetate	monoterpene	34.17	835 (+)
68	Dihydrocarvyl acetate	ester	34.43	882
69	Citronellyl acetate	ester	34.54	873 (+)
70	Nerol acetate	ester	34.83	901
71	(Z)-Carvyl acetate	ester	35.16	898 (+)
72	Geranyl acetate	ester	35.36	920 (+)
73	(Z)-3-Hexenyl hexanoate	ester	35.47	930
74	Hexyl hexanoate	ester	35.57	903 (+)
75	Ethyl decanoate	ester	35.78	842 (+)
76	Decyl acetate	ester	36.15	959 (+)
77	Dodecanal	aldehyde	36.41	975 (+)

Table S1. Identification of volatile compounds in the orange varieties 'Tarocco Rosso', 'Sanguinelli', 'Moro', 'Cadenera', 'Salustiana' and 'Hamlin'. For each compound, the retention time (min) and the reverse match of the mass spectra against NIST library (1000 = 100% identical to the NIST library entry) are shown; (+) after the NIST reverse match value indicates that identification was confirmed by an authentic chemical standard injection**.**

Code	Volatile Compound	Type	Retention Time (min)	NIST Reverse Match
78	(Z)-β-Elemene	sesquiterpene	36.46	937
79	Non identified		36.6	
80	p-Menth-1-en-9-ol	monoterpene	36.93	849 (+)
81	α-Ionone	monoterpene	37.22	886
82	Perillyl acetate	monoterpene	37.31	924 (+)
83	Dihydropseudoionone	monoterpene	37.49	921
84	(E)-β-Famesene	sesquiterpene	37.6	960 (+)
85	Caryophyllene	sesquiterpene	37.73	951
86	β-Cubebene	sesquiterpene	37.9	931
87	Aromadendrene	sesquiterpene	38.3	909
88	Humulene	sesquiterpene	38.7	948 (+)
89	β-Ionone	monoterpene	38.77	905 (+)
90	9,10-Dehydro-Isolongifolene	sesquiterpene	39.075	875
91	1(10)-Aromadendrene	sesquiterpene	39.16	902
92	β-Guaiene	sesquiterpene	39.26	871
93	4a,8-Dimethyl-2-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,7-octahydronaphtalene	sesquiterpene	39.37	940
94	Valencene	sesquiterpene	39.57	937 (+)
95	α-Selinene	sesquiterpene	39.71	928 (+)
96	Nootkatene	sesquiterpene	40.19	949
97	α-Maaliene	sesquiterpene	40.39	927
98	Selina-3,7(11)-diene	sesquiterpene	40.9	934
99	Neointermedeol	alcohol	43.84	864
100	Selin-6-en-4α-ol	alcohol	44.12	859
101	Nookatone	sesquiterpene	47.71	944

Table S2. Volatile compounds that were specific for some of the evaluated varieties. Normalised area is shown (continued on the following page).

Compound	**Cadenera**	**Salustiana**	**Hamlin**	**Tarocco R.**		**Sanguinelli**		**Moro**	
				Slight	**Intense**	**Slight**	**Intense**	**Slight**	**Intense**
2-Pentanone	-	1.675	0.763	1.039	2.449	0.596	0.781	0.166	1.421
Ethyl propanoate	-	0.209	0.379	1.151	2.704	0.816	1.938	0.156	1.941
3-Methylbutanol	-	-	-	-	-	6.506	6.578	-	-
Ethyl 2-Methyl butanoate	-	-	-	-	3.216	1.281	3.437	-	2.506
3-Hexen-1-ol	-	-	-	1.063	2.642	0.169	0.746	-	3.283
1-Hexanol	3.125	0.502	2.082	1.364	1.298	0.223	0.729	-	1.014
(E)-2-Heptenal	0.642	0.807	1.795	1.950	1.577	-	0.237	0.117	0.559
Camphene	-	1.127	-	-	-	0.987	0.823	3.047	3.098
(E)-2-Hexen-1-ol. acetate	9.979	0.623	1.415	-	-	-	-	-	-
(E)-2-Octenal	0.305	1.114	2.436	1.963	1.648	-	-	-	-
Linalool	-	0.961	0.276	0.508	0.518	-	0.062	4.060	2.613
(E)-p-Mentha-2.8-dien-1-ol	-	1.111	0.904	-	-	0.483	0.753	2.982	2.471
(E)-2-Nonenal	0.440	1.213	1.879	1.505	1.245	0.134	0.199	-	0.274
(Z)-4-Decenal	-	-	-	-	-	-	-	9.469	2.067
Decanal	0.023	1.519	0.103	0.142	0.195	0.036	-	7.520	1.997

Table S2. Volatile compounds that were specific for some of the evaluated varieties. Normalised area is shown.

Compound	Cadenera	Salustiana	Hamlin	Tarocco R.		Sanguinelli		Moro	
				Slight	Intense	Slight	Intense	Slight	Intense
Neral	0.031	1.406	0.314	0.347	0.434	-	-	5.638	2.120
Linalyl acetate	-	0.716	0.087	0.058	0.141	0.964	1.124	0.485	0.518
Undecanal	-	1.553	0.385	0.232	0.328	-	-	6.578	2.444
(E)-Methylgeranate	-	-	-	-	-	-	-	7.771	3.231
Carvyl acetate	-	2.185	0.590	-	-	0.255	0.654	0.935	0.546
Dihydrocarvyl acetate	-	0.934	0.234	-	-	0.949	1.410	2.135	3.738
Citronellyl ecetate	0.240	0.727	0.737	-	0.293	1.103	1.536	1.832	2.826
(Z)-Carvyl acetate	-	0.965	0.438	-	-	0.954	1.268	2.915	3.174
Geranyl acetate	0.130	0.565	0.500	-	0.215	0.805	0.943	1.795	2.639
(Z)-3-Hexenyl hexanoate	8.793	-	3.200	-	-	-	-	-	-
Decyl acetate	0.113	1.832	0.599	-	0.174	0.527	0.693	2.419	3.348
Perillyl acetate	0.222	1.061	0.338	-	-	0.541	0.873	1.568	4.335
(E)-β-Famesene	-	0.224	0.280	0.076	0.159	-	-	6.687	3.441
Neointermedeol	1.830	2.046	1.006	-	0.464	0.163	1.071	0.320	1.884

CHAPTER VI

Volatile Organic Compounds Profile of 'Tarocco Ippolito' Blood Orange: Changes Associated with the Final Phases of Ripening

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Abstract

The ‘Tarocco Ippolito’ blood orange has gained popularity in recent years due to its intensely red-coloured flesh and highly valued organoleptic properties. It has been demonstrated that flavour liking is the main reason why consumers choose blood oranges, with aroma playing a key role in flavour perception. The aim of this study was to determine the volatile composition of ‘Tarocco Ippolito’ blood oranges and describe the changes occurring from the stage when anthocyanins begin to accumulate in the flesh to full ripening at commercial harvest. Using the SPME-GC-MS technique, 81 volatile compounds were identified, mainly including terpenes, esters, aldehydes and alcohols. Three distinct patterns of volatile compound evolution were detected during the final stages of ripening: 1) compounds whose relative concentrations decrease, notably aldehydes, whose decrease likely contributes to the reduction of unripe fruit aromatic notes; 2) compounds whose relative concentrations remain stable, including key contributors to citrus aroma such as valencene and D-limonene; and 3) compounds whose relative concentrations increase, with monoterpenes and esters being predominant. Our findings reveal dynamic changes in volatile compounds during the ripening of ‘Tarocco Ippolito’ oranges and highlight the importance of monoterpenes and esters in defining the characteristic aroma of this variety.

Keywords: anthocyanins; blood oranges; maturation; aroma

1. Introduction

Blood oranges (*Citrus Sinensis* (L.) Osbeck) are characterized by the accumulation of anthocyanins, which are bioactive compounds that give the fruit its red colouration (Lo Piero, 2015; Fallico *et al.*, 2017). These varieties have traditionally been grown in Sicily, where most of them originate and where the environmental conditions are favourable for the red colouring of the fruit. Over the last decade, the popularity of blood oranges has increased significantly worldwide, and the scientific community has sought to understand not only the molecular basis of anthocyanin accumulation but also consumer preferences (Huang *et al.*, 2019; Catalano *et al.*, 2020; Giménez-Sanchis *et al.*, 2022).

Their attractive appearance and health benefits associated with anthocyanin accumulation are the main commercial claims for blood oranges. However, a recent study involving consumers from four different countries reported that flavour liking is the main reason for them to choose these red coloured oranges (Giménez-Sanchis *et al.*, 2022). Although both taste and aroma are well integrated in their contribution to the overall flavour of citrus fruits, aroma is often considered dominant (Goff and Klee, 2006), being a crucial factor in consumer preference (Giménez-Sanchis *et al.*, 2021).

A recent study performed by our group showed that, at commercial maturity stage, the volatile organic compounds (VOCs) profile of blood oranges differs between fruits with intense pigmentation and those with slight pigmentation, revealing changes in volatile compounds associated with the accumulation of anthocyanins (Giménez-Sanchis *et al.*, 2024). Given this result, it would be very interesting obtain more information about the changes in VOCs associated with the maturation of blood oranges, about which there is only information for 'Tarocco' variety (Liu *et al.*, 2023).

Among blood orange varieties, 'Tarocco Ippolito' has recently attracted market interest due to its intensely red-coloured flesh and highly appreciated organoleptic characteristics. Consequently, it has begun to be cultivated in several countries worldwide, including Spain, Iran, and

Australia, among others (Jafari *et al.*, 2021; Vanella *et al.*, 2023; Vithana *et al.*, 2024).

The regulation for the commercialization of oranges in the EU establishes minimum internal quality requirements, which, for blood oranges, include a maturity index (MI) of 6.5 and a juice yield of 30% (UNECE, 2023). Since the main characteristic of blood oranges is their reddish coloration, it is desirable that anthocyanin synthesis has at least begun before commercialization; otherwise, they would not be distinguishable from traditional blond varieties. However, in commercial practice, the 'Tarocco Ippolito' variety is typically harvested with a MI much higher than the minimum requirement. This is not only to achieve a more intense coloration but also to allow the fruit to fully develop its organoleptic characteristics.

Considering the growing commercial importance of 'Tarocco Ippolito', the lack of information on its volatile compound profile, and the limited data on VOC changes associated with blood orange ripening, we decided to investigate the main changes in VOC composition during the final ripening stages that contribute to the development of its characteristic flavour. To this end, we selected two maturity stages: the onset of anthocyanin synthesis, which is the main commercial stage when the blood orange character becomes apparent, and a more advanced maturity stage, when the fruit has fully developed its organoleptic characteristics.

2. Materials and Methods

2.1. Plant Material and Experimental Design

In the 2023 season, 'Tarocco Ippolito' oranges (*Citrus sinensis* (L.) Osbeck) were collected at the beginning of anthocyanin synthesis in the flesh (9 December 2023) when red coloration of flesh was visually detectable, and again at the stage when the fruit had fully developed it organoleptic characteristics, when most of the fruit is typically harvested (15 January 2024). These oranges were harvested from 6-year-old trees grafted onto 'Carrizo' citrange (*Citrus sinensis* Osb. × *Poncirus trifoliata* Raf.) rootstock (Figure 1). The orchard was situated in Pedralba, in the centre of the province of Valencia, Spain (39° 36′ 00″ N, 0° 43′ 12.0″ W, at an elevation of 301 m.a.m.s.l). A total of eighteen fruits were harvested at each maturity stage. After assessing the skin colour, juice was extracted from three sets of six fruits, which were designated as A1, A2 for fruits at the stage when anthocyanin synthesis began, and as H1, H2 and H3 for the second stage of maturity, when the bulk of harvest is typically carried out. The following parameters were analysed: juice colour, titratable acidity, total soluble solids, juice yield and maturity index. Additionally, juice samples were preserved at -80°C for further analysis of total anthocyanin content and the VOC profile.

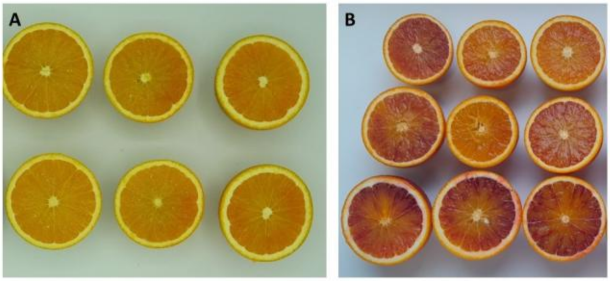

Figure 1. Fruit samples of 'Tarocco Ippolito' at the time of the onset of anthocyanin synthesis (A) and at the time of harvest (B).

2.2. Analytical Determinations

2.2.1. Quality Parameters

The skin and juice colouration were assessed using a Minolta colorimeter CR-400 (Minolta Co. Ltd., Osaka, Japan), calculating the a*/b* ratio (CIELAB), which is a standard colour index for blood oranges (Buccheri *et al.*, 2015; Tarancón *et al.*,2022). Skin colouration was evaluated by taking three measurements around the equatorial region of each fruit.

The maturity index (MI) was determined as the ratio of total soluble solids to titratable acidity. Total soluble solids were measured with a digital refractometer model PR-32α (ATAGO CO., LTD., Fukuya, Japan) and were expressed as percentage. Titratable acidity was assessed using a potentiometric titrator model HI 901 (Hanna Instruments S.L., Eibar, Spain), which is based on an acid-base titration whit NaOH (0.01N), and it was expressed as g citric acid. 100 mL^{-1}.

Total anthocyanin content was quantified using the pH-differential method as described by Rapisarda *et al.* (2000) with slight modifications. Briefly, aliquots (1 mL) of orange juice were frozen at -80 °C. For analysis, the samples were thawed at room temperature, centrifuged at 6,000g for 20 min, and the supernatant was collected. Anthocyanin content was analyzed by dissolving 200 μL aliquots in 2.3 mL of buffer 1 (12.5 mL KCl 0.2M + 37.5 mL HCl 0.2M, pH 1.0) and buffer 2 (150 mL CH_3COONa + 53 mL HCl 1M, pH 4.5). After incubating the samples in the dark for one hour, absorbance was measured at 510 nm. Anthocyanin content was calculated based on the difference in absorbance at both pH levels. A calibration curve was established using cyanidin-3-glucoside, and results were expressed as cyanidin-3-glucoside equivalents (mg/L).

2.2.2. Volatile Organic Compounds Profile

The analysis of VOCs was performed in the facilities of the Institute of Molecular and Cellular Biology of Plants (Valencia, Spain) following the methodology described by González-Mas *et al.* (2011). Two mL of juice sample, previously thawed at 27.5 °C for 15 minutes, were subjected to Headspace Solid Phase Microextraction (HS-SPME) in a 5 mL crimp-cap headspace vial. After preincubation at 70 °C for 10 minutes, the extraction was performed over a 40-minute period with a 65-μm PDMS/DVB fiber (Supelco, PA, USA).

Volatile organic compounds trapped on the fibre were analysed by GC–MS using an autosampler COMBI PAL CTC Analytics (Zwingen, Switzerland), a 6890N Gas Chromatograph (Agilent Technologies, CA, USA) and a 5975B Inert XL Mass Selective Detector (Agilent), equipped with an Agilent J&W Scientific DB-5 ms fused silica capillary column (5 %-phenyl-95 %-dimethylpolysiloxane as stationary phase, 60 m length, 0.25 mm i.d., and 1 μm thickness film). Splitless mode desorption was set to 250 °C for 1 minute. Oven temperature conditions were 40 °C for 2 min, 5 °C/min ramp until 250 °C and then held isothermally at 250 °C for 5 min. The carrier gas was helium 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 35–220; seven scans per second). Chromatograms and spectra were recorded and processed using the Enhanced ChemStation software for GC-MS (Agilent).

The NIST version 2.3 mass spectra library was used for compound identification by comparing the mass spectra of each potential compound. Additionally, a custom library created from analytical grade commercial compounds (Sigma-Aldrich Química, Madrid, Spain) allowed us to unequivocally confirm the identification of certain compounds, by matching the GC retention times and mass spectra information against the standards in the library.

2.3. Data Processing and Statistical Analysis

VOCs data were expressed as the normalized peak area. It was obtained by dividing the peak area of each compound by the mean value of all peak areas of that compound from all the evaluated samples. After normalization, the data were subjected to Principal Component Analysis (PCA) and a heat map was generated. These analyses were performed using XLSTAT software (version 2024, Addinsoft Inc. New York, NY, USA).

3. Results and Discussion

At the stage when the onset of anthocyanin accumulation took place in the flesh, the fruit had a maturity index value of 8.7 and a juice yield of 40.6 %, thus meeting the standard requirements (UNECE, 2023). The skin and juice colour showed a*/b* values of 0.21 and -0.10, respectively, with red pigmentation becoming visibly in the flesh for the first time, which was associated with an anthocyanin accumulation of 2.6 mg/L. However, the bulk of the commercial harvest was not carried out until one month later. At that point, not only had the red colouration drastically increased in flesh (a*/b* values of 0.55) and peel (0.96), but the fruit maturity index and juice yield had also risen to 10.8 and 42.0 % respectively. The anthocyanin concentration in the flesh had risen to 37.2 mg/L which led to an intense red coloration (Figure 1).

The main objective of this study was to determine whether, beyond the changes in the maturity index associated with ripening, there are changes in the volatile profile that contribute to sensory differences between the early commercial stages, i.e., when the fruit meets the required standards and red pigmentation begins to appear, and the maturity stage considered commercially optimal by traders, at which the bulk of the harvest is typically carried out.

The SPME-GC-MS technique allowed the identification of 81 VOCs: 21 monoterpenes, 19 esters, 17 sesquiterpenes, 15 aldehydes, 5 alcohols, 1 alkatetraene, 1 ketone, 1 epoxide, and carbon dioxide (Table S1). The PCA analysis (Figure 2) provided a global overview of the main differences in volatile composition between fruit at the two studied maturity stages. It clearly distinguished the juice samples from oranges where anthocyanin accumulation had just begun (samples A) from those at the commercial maturity stage (samples H). The volatile profile of samples A was characterized by high levels of aldehydes, while samples H were associated with elevated levels of terpenes and esters, with the compounds CO_2 and ethanol also present.

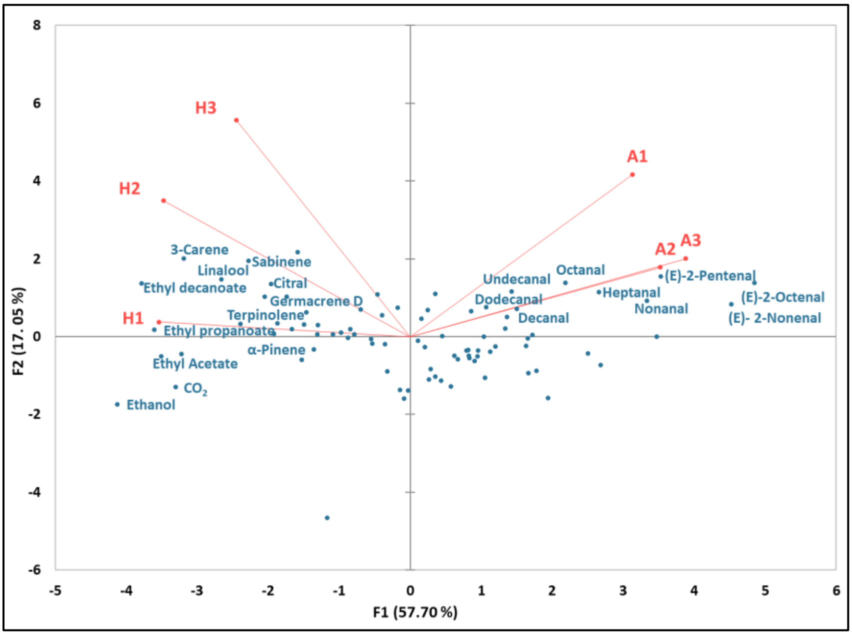

Figure 2. Principal component analysis performed with the normalised peak area (peak area/mean value of all the evaluated samples) of each compound. Samples of fruit at the onset of anthocyanin accumulation in the flesh (A) and at the time of commercial harvest (H) are shown.

To evaluate in more detail the evolution of the different family of compounds with the maturation, a heat map was performed (Figure 3). This analysis grouped the compounds into two clusters (cluster A and cluster B). Cluster A was subdivided in cluster A1 and A2. These three clusters (A1, A2 and B) reflected three different patterns of evolution during ripening: Cluster A1 - volatile compounds whose relative concentration increases with maturation; Cluster A2 - volatile compounds whose relative concentration keeps quite stable during maturation; Cluster B - volatile compounds whose relative concentration decreases with maturation (Figure 3).

The 81 VOCs identified were present in greater or lesser concentrations in both groups of samples. Among the compounds whose concentration decrease with ripening (cluster B), a group of aldehydes stands out by their high concentration at the stage of anthocyanins began to accumulate: heptanal, octanal, nonanal, decanal, undecanal, dodecanal, (Z)-2-heptenal, (E)-2-pentenal, (E)-2-nonenal, (E)-2-octenal and benzaldehyde. Many of these aldehydes have been associated with odour notes linked to the unripe stage of different fruits (El Hadi *et al.*, 2013; Arias-Pérez *et al.*, 2021). In citrus fruit, the straight-chain aldehydes such as heptanal, octanal, nonanal or decanal have been associated with citrus notes but also as contributors of green and fatty odour notes (Miyazawa *et al.*, 2010; Deterre *et al.*, 2016; Porat *et al.*, 2016). Specifically in blood oranges, nonanal has been described as apporting melon and floral odour notes in varieties 'Moro' and 'Tarocco' (Arena *et al.*, 2006) and decanal as a citrus-like odour contributor in 'Moro' and 'Sanguinello' (Selli and Kelebek, 2011). The aldehydes with more complex structures such as (E)-2-pentenal, (E)-2-octenal or (E)-2-nonenal, which have an alkene group in their structure, have been respectively described as contributors of fruity odour notes in tangerines (Leclair *et al.*, 2012) and green and fatty odour notes in sweet oranges and blood oranges (Näf *et al.*, 1996; Deterre *et al.*, 2016). Despite the decrease in aldehyde concentration with maturation, this family of compounds still plays a role in the final VOC profile of 'Tarocco Ippolito', representing 15% of the aromatic compounds (Figure 4).

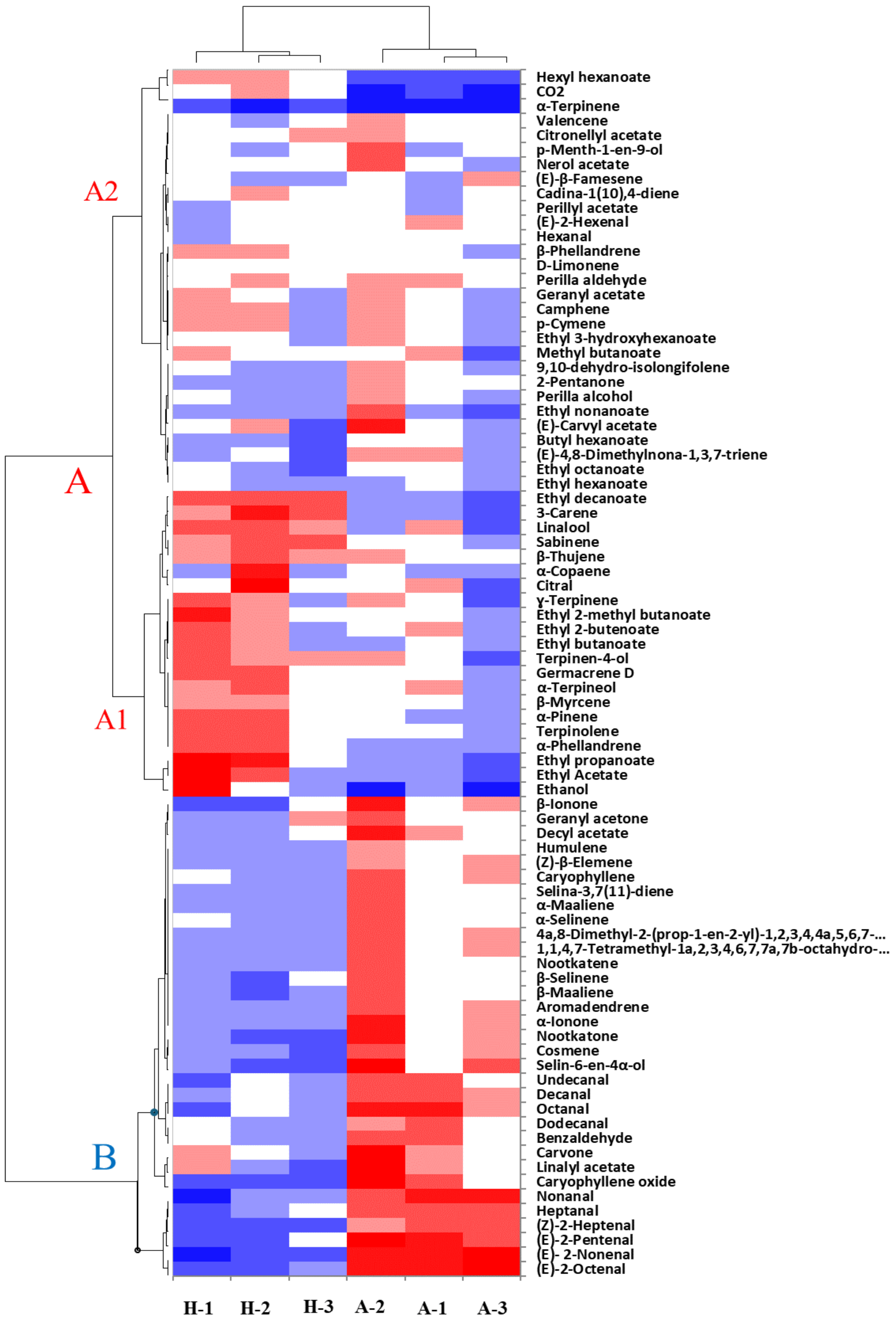

Figure 3. Description on the following page.

Figure 3. Heat map performed with the normalised peak area (peak area/mean value of all the evaluated samples) of each compound identified in the samples collected at the onset of anthocyanin accumulation in the flesh (A) and at the time of commercial harvest (H). Cluster A1 – volatile compounds whose relative concentration increases with ripening; cluster A2 – volatile compounds whose relative concentration keeps quite stable during ripening; cluster B – volatile compounds whose relative concentration decreases with ripening.

Among the VOCs that decrease with ripening were also found compounds belonging to other families of compounds, although their initial concentration was lower than that of aldehydes. Among them, there were a wide group of compounds belonging to the sesquiterpene family (humulene, (Z)-β-elemene, caryophyllene, selina-3,7(11)-diene, α-maaliene, α-selinene, etc.) and monoterpene family (α-ionone, β-ionone, geranyl acetone, etc.). Most of them have been previously reported in different citrus species (Sharon-Asa *et al.*, 2003; Chisholm *et al.*, 2003; Deterre *et al.*, 2011; Chaudhary *et al.*, 2017), and some of them, such as caryophyllene or β-ionone, have been detected in blood orange varieties (Moufida and Marzouk, 2003; Habibi *et al.*, 2020).

The group of compounds whose concentration increased with maturity (cluster A1) were mainly monoterpenes (3-carene, sabinene, β-thujene, ɣ-terpinene, β-myrcene, α-pinene, terpinolene and α-phellandrene), and esters (ethyl decanoate, ethyl 2-methyl butanoate, ethyl 2-butenoate, ethyl butanoate, ethyl propanoate and ethyl acetate). Most of the terpenes herein detected have previously been reported to be part of the volatile profile of different citrus fruits, included blood oranges (Arena *et al.*, 2006). Compounds sabinene, terpinolene and α-pinene have been linked to citrus, herbaceous, sweet and woody odour notes in limes and lemons (Cano-Lamadrid *et al.*, 2018), while compound 3-carene was described to contribute with sweet notes to mandarin aroma (Tietel *et al.*, 2010). Among the esters, ethyl butanoate, ethyl 2-methyl butanoate, ethyl 2-butenoate and ethyl acetate have been repeatedly reported in blood oranges (Arena *et al.*, 2006; Yu *et al.*, 2017; Habibi *et al.*, 2020), with the aroma of ethyl butanoate linked to pineapple odour notes and ethyl 2-methyl butanoate described as fruity (Arena *et al.*, 2006). Furthermore, ethyl 2-methyl butanoate, ethyl 2-butenoate and ethyl acetate have recently been found to be at higher concentration in blood orange fruits with intense red pigmentation compared to those with slight pigmentation. This fact was observed by Giménez-Sanchis *et al.* (2024) when comparing fruits with different pigmentation intensities that had been harvested at commercial maturity stage from the same tree, and the authors reported this pattern to be consistent across three different blood orange varieties. Herein we observed that fruit at commercial ripe stage, with an elevated concentration of anthocyanins, presented higher levels of these three esters than the fruit in which the anthocyanin biosynthesis had only recently begun. Therefore, our results are in line with those of Giménez-Sanchis *et al.* (2024), as higher anthocyanin concentrations were associated with higher levels of these three esters.

Among the compounds increasing with maturation were also the aldehyde citral and a group of alcohols (linalool, terpinen-4-ol, α-terpineol and ethanol). Most of these compounds have been previously reported in blood oranges. Linalool has been described to apport floral, green and spicy odour notes (Arena *et al.*, 2006; Selli and Kelebek, 2011), α-terpineol contributes with green and violet/lilac notes (Selli and Kelebek, 2011; Cano-Lamadrid *et al.*, 2018) and terpinen-4-ol with floral and fresh odour notes (Selli and Kelebek, 2011).

Finally, the levels of a large group of volatile compounds remained stable during the evaluated period of ripening (cluster A2). Of them, esters represent the largest group of compounds (hexyl hexanoate, citronellyl acetate, nerol acetate, methyl butanoate, geranyl acetate, ethyl 3-hydroxyhexanoate, ethyl nonanoate, (E)-carvyl acetate, butyl hexanoate, ethyl octanoate and ethyl hexanoate). Some of these compounds have been described to apport citrus-like, fruity, floral and herbaceous odour notes to citrus fruit (Elsharif and Buettner, 2017; Sdiri *et al.*, 2017). Methyl butanoate has been described to impart strawberry odour notes in two blood orange varieties (Arena *et al.*, 2006) and fruity odour notes in Chinese sweet orange (Shui *et al.*, 2019). Within the group of compounds that did not experience significant changes in the last stages of ripening, the presence of monoterpenes and sesquiterpenes was also relevant.

Among them, we can find D-limonene and valencene, two compounds that are well-known for playing a key role in citrus fruit aroma (Sharon-Asa *et al.*, 2003; Kvittingen *et al.*, 2021). Other terpenoids are also present in this group, such as p-cymene or α-terpinene, which are important contributors to citrus aroma (Tietel *et al.*, 2010; Cano-Lamadrid *et al.*, 2018) and have been found in the blood orange variety 'Moro' (Habibi *et al.*, 2020).

Taken together, our data suggest that at the maturity stage that the onset of anthocyanin accumulation in the flesh occurs, the fruits contain significant levels of numerous compounds responsible for citrus aroma, although some aldehydes may be associated with green notes characteristic of unripe stages. It is in the final stages of ripening that the aroma prolife of the variety reach its final composition. The ripening of the 'Tarocco Ippolito' variety being characterized by an increase in the level of esters and monoterpenes and significant decrease in the concentration of certain aldehydes (Figure 4).

An increase in relevant terpenes (α-pinene, β-myrcene) and esters (ethyl butanoate, ethyl hexanoate) in the last phases of ripening has been also reported in navel oranges by Obenland *et al.* (2009). Other common aspects of navel maturation in line with our findings are that limonene concentrations were high during the maturation period and that levels of aldehydes such as hexanal, pentanal, octanal and (E)-2-nonenal decreased in the final stages of ripening. Contrarily, Liu *et al.* (2023) reported for 'Tarocco' that not the total concentration of aldehydes neither that of terpenes were modified during the last phases of ripening. Moreover, they reported a decrease of esters during this period. As these authors discussed in their study the changes of VOC profile in citrus fruit during maturation is determined by the variety.

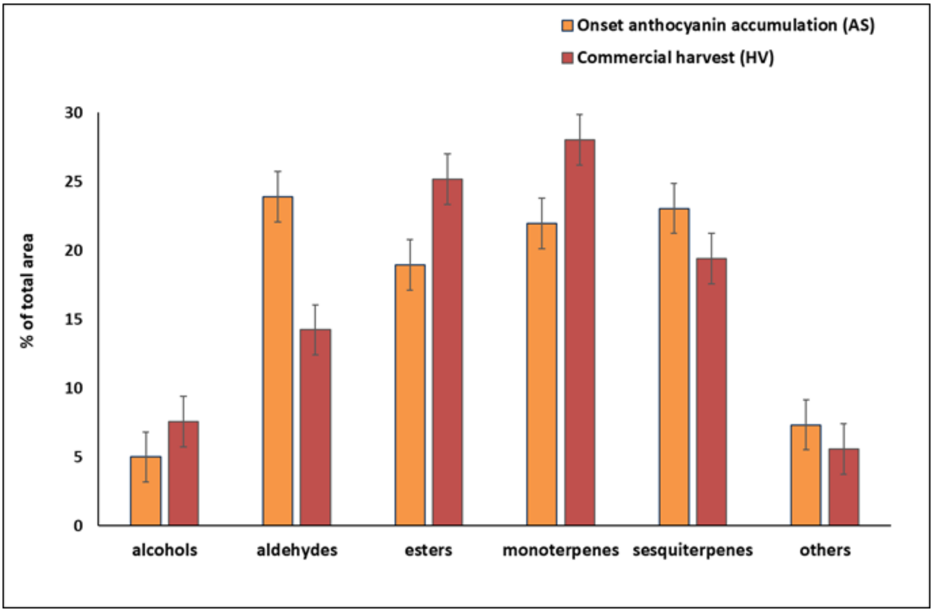

Figure 4. Percentages of each family of volatile compounds, obtained from the normalised peak area (peak area/mean value of all the evaluated samples) of each compound identified in the samples collected at the start of anthocyanin synthesis (AS) and at the time of commercial harvest (HV).

4. Conclusions

A total of 81 VOCs were identified in blood oranges 'Tarocco Ippolito'. Relevant changes in the volatile composition were detected in the last stages of maturation, from the onset of anthocyanin accumulation in the flesh to the full ripen at commercial harvest. The heat map analysis revealed three distinct patterns of volatile compound evolution during this period: compounds whose relative concentration decreases, compounds whose relative concentration remains fairly stable and compounds whose relative concentration increases. Aldehydes were the main group of compounds that decreased during the last phases of ripening, what probably contributed to the decrease in aromatic notes associated with unripe fruit. A significant number of volatile compounds remained stable throughout the ripening process, among which key citrus odour compounds such as valencene and D-limonene can be found. Of the compounds that increased throughout maturation, monoterpenes and esters were predominant, which may contribute to the characteristic aroma of this variety. These included compounds such as ethyl 2-methyl butanoate and 3-carene, which are known for providing fruity and sweet notes respectively to citrus fruit. Our findings show dynamic changes of the VOCs during the ripening of 'Tarocco Ippolito' oranges, revealing the importance of monoterpenes and esters in defining the characteristic aroma of the fruit.

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Supplementary Material

Table S1. Identification of volatile compounds in the orange variety 'Tarocco Ippolito'. For each compound, the retention time (min) and the reverse match of the mass spectra against NIST library (1000 = 100% identical to the NIST library entry) are shown; (+) after the NIST reverse match value indicates that identification was confirmed by an authentic chemical standard injection (continued on the following page).

Code	Volatile Compound	Type	Retention Time (min)	NIST Reverse Match
1	CO_2	miscellaneous compound	3.55	999
2	Ethanol	alcohol	4.88	937 (+)
3	Ethyl acetate	ester	8.54	935 (+)
4	2-Pentanone	ketone	11.15	961 (+)
5	Ethyl propanoate	ester	12.0	834 (+)
6	Methyl butanoate	ester	12.5	877 (+)
7	(E)-2-Pentenal	aldehyde	13.89	826 (+)
8	Ethyl butanoate	ester	15.55	903 (+)
9	Hexanal	aldehyde	15.65	971 (+)
10	Ethyl 2-butenoate	ester	17.33	877
11	Ethyl 2-Methyl Butanoate	ester	17.48	866 (+)
12	(E)-2-Hexenal	aldehyde	17.82	950 (+)
13	Heptanal	aldehyde	19.65	899 (+)
14	β-Thujene	monoterpene	20.87	906
15	α-Pinene	monoterpene	21.3	912 (+)
16	(Z)-2-Heptenal	aldehyde	21.8	890
17	Camphene	monoterpene	22.1	860 (+)
18	Benzaldehyde	aldehyde	22.4	880 (+)
19	Sabinene	monoterpene	22.75	959
20	β-Myrcene	monoterpene	22.95	968 (+)
21	Ethyl hexanoate	ester	23.05	895 (+)
22	Octanal	aldehyde	23.45	955 (+)

Table S1. Identification of volatile compounds in the orange variety 'Tarocco Ippolito'. For each compound, the retention time (min) and the reverse match of the mass spectra against NIST library (1000 = 100% identical to the NIST library entry) are shown; (+) after the NIST reverse match value indicates that identification was confirmed by an authentic chemical standard injection (continued on the following page).

Code	Volatile Compound	Type	Retention Time (min)	NIST Reverse Match
23	α-Phellandrene	monoterpene	24.15	922 (+)
24	3-Carene	monoterpene	24.09	935 (+)
25	α-Terpinene	monoterpene	24.32	934 (+)
26	p-Cymene	monoterpene	24.6	870 (+)
27	D-Limonene	monoterpene	24.85	936 (+)
28	β-Phellandrene	monoterpene	25.02	943
29	(E)-2-Octenal	aldehyde	25.45	870 (+)
30	ɣ-Terpinene	monoterpene	25.7	929 (+)
31	Terpinolene	monoterpene	26.72	923 (+)
32	Linalool	alcohol	26.82	918 (+)
33	Nonanal	aldehyde	26.97	875 (+)
34	(E)-4,8-Dimethylnona-1,3,7-triene	monoterpene	27.24	913
35	Ethyl 3-hydroxyhexanoate	ester	27.68	898 (+)
36	(E)-2-Nonenal	aldehyde	28.88	914 (+)
37	Butyl hexanoate	ester	29.58	879
38	Ethyl octanoate	ester	29.72	934 (+)
39	Terpinen-4-ol	alcohol	30.04	933 (+)
40	Decanal	aldehyde	30.27	924 (+)
41	α-terpineol	alcohol	30.41	921 (+)
42	Linalyl acetate	ester	31.49	877 (+)
43	Carvone	monoterpene	31.96	920 (+)
44	Citral	aldehyde	32.27	923 (+)
45	Ethyl nonanoate	ester	32.74	896 (+)
46	Perilla aldehyde	aldehyde	33.08	940
47	Undecanal	aldehyde	33.34	924 (+)
48	Carvyl acetate	monoterpene	33.98	820 (+)

Table S1. Identification of volatile compounds in the orange variety 'Tarocco Ippolito'. For each compound, the retention time (min) and the reverse match of the mass spectra against NIST library (1000 = 100% identical to the NIST library entry) are shown; (+) after the NIST reverse match value indicates that identification was confirmed by an authentic chemical standard injection (continued on the following page).

Code	Volatile Compound	Type	Retention Time (min)	NIST Reverse Match
49	Cosmene	alkatetraene	34.17	924
50	Citronellyl acetate	ester	34.35	918 (+)
51	Nerol acetate	ester	34.64	885
52	Perilla alcohol	ester	34.77	841 (+)
53	Geranyl acetate	ester	35.17	898 (+)
54	Hexyl hexanoate	ester	35.37	814 (+)
55	Ethyl decanoate	ester	35.58	844 (+)
56	Decyl acetate	ester	35.96	868 (+)
57	Dodecanal	aldehyde	36.22	955 (+)
58	(Z)-β-Elemene	sesquiterpene	36.26	855
59	Perillyl acetate	monoterpene	36.42	849 (+)
60	p-Menth-1-en-9-ol	monoterpene	36.75	856 (+)
61	α-Ionone	monoterpene	37.02	877
62	Geranyl acetone	monoterpene	37.29	913 (+)
63	(E)-β-Famesene	sesquiterpene	37.4	916 (+)
64	Caryophyllene	sesquiterpene	37.52	955
65	Germacrene D	sesquiterpene	37.69	805
66	Aromadendrene	sesquiterpene	38.09	880
67	Humulene	sesquiterpene	38.49	929 (+)
68	β-Ionone	monoterpene	38.57	893 (+)
69	β-Maaliene	sesquiterpene	38.73	876
70	9,10-Dehydro-Isolongifolene	sesquiterpene	38.87	851
71	1,1,4,7-Tetramethyl-1a,2,3,4,6,7,7a,7b-octahydro-1H-cyclopropa[e]azulene	sesquiterpene	38.95	907

Table S1. Identification of volatile compounds in the orange variety 'Tarocco Ippolito'. For each compound, the retention time (min) and the reverse match of the mass spectra against NIST library (1000 = 100% identical to the NIST library entry) are shown; (+) after the NIST reverse match value indicates that identification was confirmed by an authentic chemical standard injection.

Code	Volatile Compound	Type	Retention Time (min)	NIST Reverse Match
72	4a,8-Dimethyl-2-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,7-octahydronaphtalene	sesquiterpene	39.16	954
73	Valencene	sesquiterpene	39.39	954 (+)
74	α-Selinene	sesquiterpene	39.51	929
75	β-Selinene	sesquiterpene	39.63	920
76	Cadina-1(10),4-diene	sesquiterpene	39.71	916
77	Nootkatene	sesquiterpene	39.98	938
78	α-Maaliene	sesquiterpene	40.18	919
79	Caryophyllene oxide	epoxide	41.89	871
80	Selin-6-en-4α-ol	alcohol	44.12	859
81	Nookatone	sesquiterpene	47.71	944

CHAPTER VII

Determination of Chilling Requirements for Anthocyanin Accumulation in Blood Orange 'Tarocco Ippolito'

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Abstract

This study investigates the influence of solar radiation, thermal amplitude, and temperature on anthocyanin accumulation in 'Tarocco Ippolito' blood oranges. Over three consecutive seasons, environmental factors and major physicochemical changes during fruit maturation were monitored in parallel, including the timing of anthocyanin accumulation in both the pulp and peel of the fruit. The results show that anthocyanin biosynthesis is not directly linked to fruit maturation, with temperature emerging as the key environmental factor driving this process. For the first time, the Chilling Hours, Utah, Positive Utah, and Dynamic models were evaluated to determine the chilling requirements necessary to trigger anthocyanin biosynthesis in the fruit.

Our findings confirm that anthocyanin biosynthesis in the flesh and peel of blood oranges occurs as independent processes. The onset of anthocyanin accumulation in the flesh was associated with exposure to low temperatures, corresponding to a total cold accumulation of 4-5 chill portions according to the Dynamic model and ≈128 chilling P-units according to the Positive Utah model. A greater cold requirement was observed for anthocyanin accumulation in the peel, with a delay of 8 to 15 days compared to the flesh. The red colour associated with anthocyanin accumulation in the peel was detected after meeting a cold accumulation requirement of ≈10 chilling portions (Dynamic model) or ≈240 chilling P-units (Positive Utah model). The Chilling Hours and the original Utah model were found to be unsuitable for this purpose.

Keywords: anthocyanins; Dynamic model; Utah model, chilling requirements; solar radiation; thermal amplitude; blood oranges

1. Introduction

Blood oranges (*Citrus sinensis* (L.) Osbeck) are a group of varieties that differ from blond oranges by their high content of anthocyanins, bioactive compounds that give the fruit its characteristic red colour (Lo Piero, 2015). Traditionally, these varieties have been grown mainly in Sicily, where most of the commercial varieties originate and where the environmental conditions favour their red colouring. Nowadays, the popularity of these oranges is growing worldwide, and commercial orchards have been established in countries such as Spain, China, South Africa, Brazil, Australia and the USA (Magalhães *et al.*, 2019; Seminara et al., 2023). For a long time, the most important varieties grown outside Italy were 'Tarocco Rosso', 'Moro' and 'Sanguinelli' (Magalhães *et al.*, 2019; Simons *et al.*, 2019; Morales *et al.*, 2021; Tarancón *et al.*, 2022). In recent years, the cultivar 'Tarocco Ippolito' has also gained popularity due to its organoleptic characteristics and has started to be cultivated in several countries around the world including Spain, Iran, and Australia, among others (Jafari *et al.*, 2021; Forner-Giner *et al.*, 2023; Vithana *et al.*, 2024).

Anthocyanins are water-soluble pigments in the flavonoid family that give red, purple, and blue colour to various fruits and vegetables (Khoo *et al.*, 2017). These compounds have been extensively studied, primarily for their health-promoting properties, which are mainly related to their antioxidant properties. The process of anthocyanin accumulation during the ripening of blood oranges was described for the 'Moro' cultivar grown in Italy by Cotroneo *et al.*, (2006) and for 'Sanguinelli' cultivar grown in Spain by Martínez-Nicolás *et al.* (2016). In both cases, the accumulation of anthocyanins in the flesh began in November, with higher concentrations of anthocyanins at harvest time in 'Moro' compared to 'Sanguinelli'.

To date, three environmental factors have been suggested as key in the biosynthesis of anthocyanins in blood oranges:

1) Thermal amplitude. As pointed out by Caruso *et al.* (2016), it is generally assumed that anthocyanin pigmentation in blood oranges on

the tree is influenced by the differences in temperature between day and night. However, these assumptions are not based on research studies because they are somewhat difficult to measure.

2) Chilling temperatures. It has been proved that the Ruby gen is involved in the activation and deactivation of anthocyanin biosynthesis (Huang et al., 2018; Catalano et al., 2020), and its activation, which is necessary for pigmentation, is cold dependent (Butelli et al., 2012). The key role of cold in the biosynthesis of anthocyanins observed at the molecular level has been corroborated by postharvest studies, which show that storing blood oranges at low temperatures promotes the accumulation of these compounds, thereby enhancing red colouration (Lo Piero et al., 2005; Carmona et al., 2017; Habibi et al., 2020, 2021). As a result, cold stress is currently considered the key factor in anthocyanin biosynthesis in blood oranges.

3) Sun radiation. Huang *et al.* (2019) observed that bagging blood oranges while on the tree inhibited the biosynthesis of anthocyanins in the fruit peel, whereas the process of anthocyanin accumulation in the flesh remained unaffected. According to results of these authors the presence of sun light seems to be a prerequisite for the colouration of the blood orange peel, but not for the flesh. By combining in-field and postharvest studies, they concluded that the accumulation of anthocyanins in the peel of blood oranges is regulated by one gene activated by light and another gene dependent on cold, whereas in the flesh, only the cold-dependent gene is involved.

Understanding the influence of environmental conditions on anthocyanin accumulation in blood oranges is crucial, as they are a significant factor limiting their global expansion. Indeed, although blood oranges are grown in countries other than Italy, in some seasons entire crops may be unusable due to sub-optimal climatic conditions during fruit ripening, resulting in absent or insufficient red colouration in places such as Brazil or Florida (Butelli *et al.*, 2012). Obtaining information on the influence of thermal amplitude, chilling temperatures and sun radiation on the accumulation of anthocyanins in blood oranges is essential for a successful introduction in regions where they have not been traditionally grown.

Chilling requirements of fruit trees have primarily been studied in relation to their role in breaking bud dormancy. Several models have been proposed for this purpose, with the most important being the Chilling Hours model, the Utah model, and the Dynamic model (Rodríguez *et al.*, 2019, Fernández *et al.*, 2020). The Chilling Hours model is the oldest and simplest one, measuring chilling exposure by counting the number of hours when temperatures fall between 0 and 7.2°C (Bennett, 1949; Weinberger, 1950). The Utah model (Richardson *et al.*, 1974) employs chilling units and acknowledges that different temperature ranges elicit varying responses, with temperatures above a certain threshold negatively impacting chilling accumulation. This model was adapted in various ways to account for different climates (Norvell and Moore, 1982; Shaltout and Unrath, 1983). The adaptation for warm and subtropical climates is the Positive Utah Model, in which warm temperatures do not negate chilling as they do in the original Utah model (Linsley-Noakes and Allan, 1994). Finally, chilling portions are the units of the Dynamic model (Fishman *et al.*, 1987a, 1987b), which takes into account the temporal sequence of cool and warm temperature periods in the accumulation of chilling.

The aim of this study was to evaluate the impact of thermal amplitude, chilling temperatures, and sun radiation on the accumulation of anthocyanins in the blood orange ‘Tarocco Ippolito’. Conducted over three consecutive seasons, the study describes the main physicochemical changes occurring during the growth and ripening of this cultivar under Mediterranean conditions, with particular focus on the onset of anthocyanin accumulation in both the flesh and the peel. To assess the role of chilling temperatures in anthocyanin biosynthesis, we employed a novel approach by evaluating, for the first time, the utility of the Chilling Hours, Utah, Positive Utah, and Dynamic models in determining the chilling requirements associated with anthocyanin accumulation in fruit, specifically in blood oranges.

2. Materials and Methods

2.1. Plant Material and Experimental Design

The study was carried out during three seasons (2021, 2022 and 2023) in a commercial citrus orchard located in Pedralba in the centre of the province of Valencia, Spain (39° 36′ 00″ N, 0° 43′ 12.0″ W, 301 m.a.m.s.l). The experiment was performed on young 'Tarocco Ippolito' blood orange (*Citrus sinensis* (L.) Osbeck) trees grafted onto 'Carrizo' citrange (*Citrus sinensis* Osb. × *Poncirus trifoliata* Raf.) rootstock, with a tree spacing of 6 m×4 m. The trees were 4-year-old in 2021. Soil textural class, according to the USDA classification, is loam to sandy clay loam with percentages of clay ranging from 22 to 34 % within the orchard. Soil organic matter was on average 1.3 %.

A scheme of the study design is shown in Figure 1. For this study, 8 trees were selected. At the end of September, when fruit was completely green, fruit was picked to determinate the stage of the fruit at the beginning of the study. In addition, 10 fruits per tree were marked around the canopy and used to evaluate colour and diameter evolution until the commercial harvest in January of the next year.

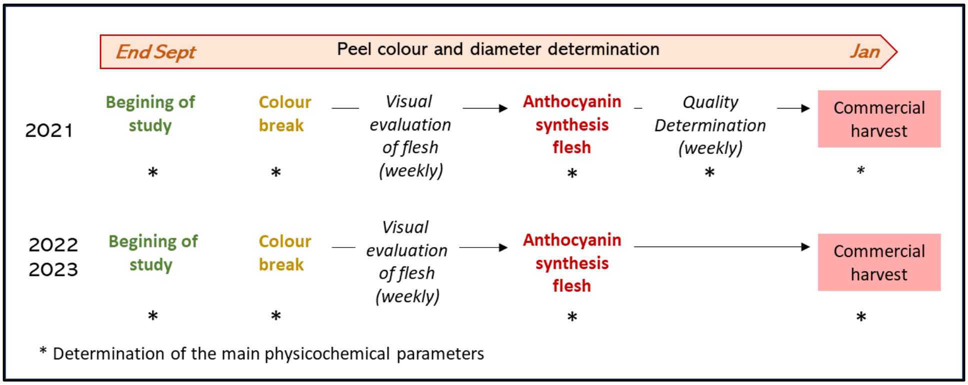

Figure 1. Scheme of the study.

During the season 2021 a detailed evaluation of the fruit ripening was carried out. For this purpose, the colour and diameter of the 10 marked fruits of each tree were measured weekly. When the colour break from green to yellow tones took place, we harvested fruit to analyse its internal quality. From this point on, weekly visits to the field included not only colour and diameter measurements, but also a visual assessment of 16 fruits at the internal level. To do this, two fruits per tree, other than those monitored for colour and diameter, were cut in half and the flesh colouration was visually evaluated in order to determine the onset of anthocyanin biosynthesis in the flesh. Anthocyanins were first observed in the flesh during the second half of November, and from this moment until the commercial harvest, 16 fruits (2 fruits per tree) were harvested weekly for the determination of the main physico-chemical parameters in the laboratory: external colour, juice colour, juice yield, titratable acidity, total soluble solids, and maturity index. Additionally, juice samples were stored at -80°C to determine total and individual anthocyanins.

In 2022 and 2023 we focused on the key points of the ripening: colour break from green to yellowish tones, onset of anthocyanin biosynthesis in the flesh and peel, and commercial harvest. As described for the first season, we evaluated the stage of the fruit at the beginning of the study, and 10 fruits were marked for periodical determination of external colour and diameter. When the colour changed from green to yellow tones, fruit was harvested again for evaluation. From this point on, the external and internal aspect of 16 fruits was weekly evaluated to detect the onset of anthocyanin biosynthesis in both tissues. At the moment, when anthocyanins were firstly observed in the flesh fruit was harvested for the determination of the main physicochemical parameters: external colour, juice colour, juice yield, titratable acidity, total soluble solids, maturity index, total anthocyanin content. These parameters were also evaluated at commercial harvest.

It is worth noting that monitoring the external colour allowed us to determine the onset of anthocyanin biosynthesis in the fruit peel during each season. In the first season, the internal quality of the fruit at this stage was assessed, but it was not evaluated in the second and third seasons. In all three seasons, the commercial harvest date was determined by the citrus cooperative that owns the orchard. The criteria for their decision on the harvest date will be discussed later.

2.2. Monitoring of Ambient Conditions and Chilling Requirements Models

Air temperature, relative humidity, solar radiation, wind and speed direction and rainfall were monitored every 15 minutes with an automatic weather station (Vantage Pro2, Davis Instruments Corporation, CA, USA) located 50 m from the orchard under study. For the objective of this study only temperature and solar irradiation data have been analysed.

Solar radiation was calculated as the daily mean radiation and was expressed in Wm^{-2}.

The thermal amplitude was calculated as the difference between the daily maximum and minimum temperatures and was expressed in °C.

The foundations of the three models of chilling requirements that were evaluated were the following:

1. The Chilling Hour Model sums the number of hours with temperatures between 0 and 7.2 °C (Bennett, 1949; Weinberger, 1950).

2. The Utah Model, a weighted Chilling Hour Model with high temperatures having a negative effect on chilling accumulation, was developed in the 1970s (Richardson *et al.*, 1974). This model defines chilling units as the hours of exposure to temperatures considered optimal for chilling accumulation (2.5-12.5°C). Temperatures below 1.4°C do not contribute to chilling accumulation and are assigned a value of 0; temperatures between 1.5 and 2.4°C contribute 0.5 chilling units; temperatures between 12.5 and 15.9°C also do not contribute to chilling accumulation, while temperatures between 16 and 18°C contribute negatively with -0.5 chilling units; and temperatures above 18°C are assigned a value of -1 chilling units, meaning they subtract from the chilling units accumulated at temperatures in the 2.5 to 12.5°C range.

3. The Positive Utah Model is derived from the Utah Model and is a specific adaptation for warm and subtropical climates. In it, higher

temperatures do not have the effect of subtracting from the accumulated cold (Linsley-Noakes and Allan, 1994). In this study, the chilling units calculated under this model will be named 'chilling P-units' to differentiate them from the 'chilling units' derived from the Utah model.

4. The Dynamic Model (Fishman *et al.*, 1987a, 1987b) was developed in the 1980s. Chilling is assumed to accumulate in a two-step process. Cold temperatures initially result in the formation of an intermediate chilling product; high temperatures can destroy this product. Once a critical amount of this chilling product has accumulated, it converts to a chill portion, which cannot be destroyed. The equations used to calculate chill portions are more complex than the other models. The Dynamic Model takes into account the temperatures that precede and follow a particular time as well as those occurring at that particular time. In this model, ''the period of chilling duration and the frequency of high temperature infusion during the chilling period are of prime importance" (Erez *et al.*, 1979).

2.3. Analytical Determinations

2.3.1. Quality Parameters

Peel and juice colour were measured with a Minolta colourimeter model CR- 400 (Minolta Co. Ltd., Osaka, Japan) by calculating the a*/b* ratio (CIELAB), which is the colour index usually used for blood oranges (Buccheri *et al.*, 2015; Tarancón *et al.*, 2022). Peel colour was determined by taking three measurements around the equatorial area of each fruit. The maximum diameter of the equatorial section of each fruit was measured using a digital calliper (Mitutoyo, Guipúzcoa, Spain). For internal quality evaluation, four juices were prepared using four fruits each. Total soluble solids (TSS) were determined by using a digital palette refractometer model PR-32α (ATAGO CO., LTD., Fukuya, Japan) and the titratable acidity (TA) was measured using a potentiometric titrator model HI 901 (Hanna Instruments S.L., Eibar, Spain). TSS were expressed as percentage and TA as g citric acid. 100mL^{-1}. The maturity index (MI) was calculated as TSS/TA. Juice yield (JY) was measured and expressed as a percentage, calculated by dividing the volume of juice by the total fruit weight. The total anthocyanin content was determined by the pH-differential method according to Rapisarda *et al.* (2000), which is based on the differences in absorbance at two different pH values (1.0 and 4.5) at 510 nm.

2.3.2. Anthocyanin Profile

A sample of 1 mL of filtered juice was placed in an Eppendorf tube, vortexed for 15 seconds (LBX instruments V05 series vortex homogeniser, VORX-005-001), and centrifuged at 12000 rpm for 20 minutes (Eppendorf centrifuge 5425, Eppendorf Hamburg, Germany). The precipitate was discarded, and the supernatant was filtered through a 0.45 μm nylon membrane, collected in a vial, and stored at -20°C until chromatographic analysis on an HPLC-DAD system equipped with a ZQ2000 mass detector (Waters, Barcelona, Spain), using a C18 Tracer Excel 5 μm 120 OSDB (250 mm x 4.6 mm) reversed-phase column (Teknokroma, Barcelona, Spain). The compounds were eluted using a gradient phase composed consisting of acetonitrile (phase A) and 0.2% formic acid (phase B). The conditions were as follows: start with 10% A for 2 min; 75% A for the following 28 min; and return to the initial conditions maintaining 10% A for 5 min, with a total experimental time of 35 min at a flow rate of 1 mL/min and an injection volume of 10 μL. The column temperature was maintained at 35 °C and the autosampler at 4 °C. Chromatograms were recorded at an absorbance of at 520 nm, and HPLC-MS was performed under positive electrospray conditions from 400 to 650 m/z.

Compounds were identified by comparison of their retention times, UV-Vis spectra and mass spectral data with authentic standards and literature data. Concentrations were determined using an external calibration curve with delphinidin-3-O-glucoside (Dp-3-glu, RT = 10.5 min, [MH]+ 465 m/z) and cyanidin-3-O-glucoside (Cy-3-glu, RT = 12.5 min, [MH]+ 449 m/z). Due to the unavailability of cyanidin-3-(6"-malonyl)-O-glucoside (Cy-3-6"mal, RT = 18.0 min [MH]+ 535 m/z)) and cyanidin-3-(6"-dioxalyl)-O-glucoside (Cy-3-6"diox, RT = 21.5 min [MH]+ 593 m/z), their calibration curves were constructed using Cy-3-glu as an external standard and quantified as Cy-3-O-glu equivalents. Dp-3-O-glu and Cy-3-O-glu standards were purchased from Cymit Química S.L. (Barcelona, Spain), and all solvents used were HPLC mass spectrometry grade and purchased from Scharlab S.L. (Barcelona, Spain). Three replicates were analysed for each sample. The concentrations obtained were expressed as mg L^{-1} juice. Empower 2 software (Waters, Barcelona, Spain) was used for data processing.

2.4. Statistical Analysis

ANOVA was used to evaluate the differences in the main physicochemical parameters throughout the ripening period. Multiple comparisons between means were performed using the LSD test (p-value ≤ 0.05). This analysis was performed using the Statgraphics Centurion XVI software (Statgraphics Technologies, Inc. The Plains, Virginia, USA).

3. Results and Discussion

3.1. Fruit Maturation. Identification of Key Points of Anthocyanin Accumulation

As explained above, fruit development was closely monitored during the 2021 season in order to describe in detail the ripening process of the cv. Tarocco Ippolito. At the end of September, the fruit had an average size of 67 mm and an intense green peel colour, reflected in a*/b* values of -0.6 (Table 1). The colour break from green to yellow tones started in early November when the fruit had a diameter of 72.8 mm (Table 1, Figure 2).

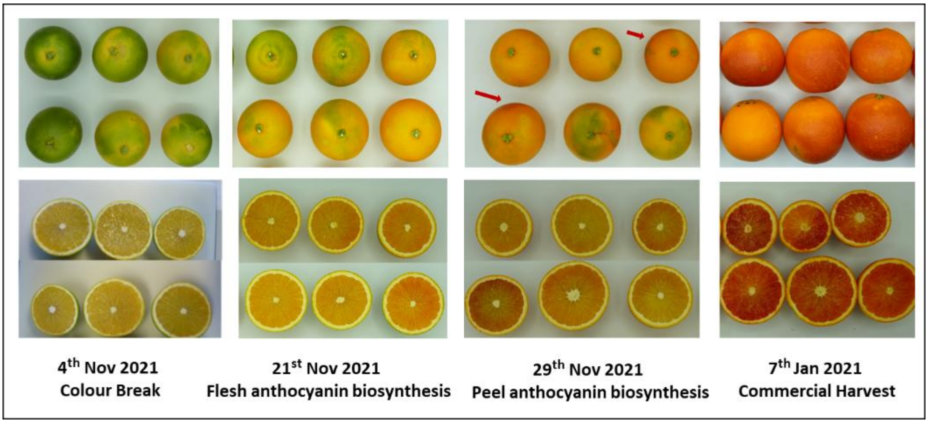

Figure 2. Internal and external appearance of the fruit at key stages of the maturation process. Red arrows indicate red colouration in the peel.

The degradation of chlorophylls and the biosynthesis of carotenoids continue throughout November, resulting in a predominantly orange colouration by the 21st, when the a*/b* index showed positive values (Table 1, Figure 2). Fruit size remained relatively stable after colour break, with measurements around 73 mm.

In November, a significant change in flesh colour also occurred. Juice colour values shifting from -30 at the beginning of the month to 0.06 by 29th November. Notably, anthocyanin biosynthesis in the fruit flesh became evident during this period, with the first detection occurring on 21st November (Table 1, Figure 2). This observation aligns with previous studies on other varieties. It is worthy to comment that at the moment when anthocyanins were first detected in the flesh, the peel of the fruit was mostly orange with some green remnants, with no distinguishable reddish colouration (Figure 2 and Supplementary Figure S1). At this time, when the accumulation of anthocyanins in the flesh became evident, the fruit had an MI of 6.7 and a juice content of 35.6% (Table 1). The shift in peel colour from orange to red tones was detected one week later than in the flesh, on 29th November, when the a*/b* index reach values of 0.37.

During December, the increase in red colouration was notable both in the peel and the flesh, associated with an accumulation of anthocyanins, as reflected in the data of total anthocyanin concentration in the flesh (Table 1). Concurrently, there was a decrease in acidity and an increase in total soluble solids, resulting in a MI of 10 by the end of the year (Table 1). Juicy yield experienced a slight increase during the first weeks of December, stabilizing and even slightly decreasing in early January when the commercial harvest took place. At this time, the fruit had an external colour of 0.60 and a flesh colour of 0.70, corresponding to a total anthocyanin concentration of 30.4 mg L^{-1} in juice. The diameter at harvest was 73.6 mm. The MI was 10, with an TSS of 10.5 and a TA of 1.1 mg citric acid 100 mL^{-1}.

Table 1. Main physicochemical parameters during maturation of blood oranges 'T. Ippolito'. Different letter in the same column and season indicates significant differences (LSD test, p-value=0.05). 'CB'- colour break, 'Fl-An'-onset of anthocyanins synthesis in the flesh, 'Sk-An'-onset of anthocyanins synthesis in the peel. *The Sk-An were detected on 14th December in 2022 (a*/b*=0.31), and 19th December in 2023 (a*/b*=0.35) (continued on the following page).

Dates	Diameter (mm)	Rind colour (a*/b*)	Juice colour (a*/b*)	TSS (%)	TA (g citric ac. 100 mL^{-1})	MI (TSS/Ac.)	JY (%)	Flesh anthocyanin content (mg L^{-1})
2021								
28th Sep	67.2 ± 3.9 a	-0.61 ± 0.05 a	-0.51 ± 0.02 a	7.9 ± 0.2 a	2.2 ± 0.09 f	3.7 ± 0.11 a	28.6 ± 3.8 a	-
4th Nov (CB)	72.8 ± 4.2 ab	-0.16 ± 0.15 ab	-0.30 ± 0.01 b	9.5 ± 0.1 b	1.6 ± 0.03 e	5.8 ± 0.12 b	30.8 ± 1.3 ab	-
21st Nov (Fl-An)	72.7 ± 4.4 b	0.18 ± 0.14 c	-0.08 ± 0.13 c	9.5 ± 0.3 b	1.4 ± 0.06 d	6.7 ± 0.45 c	35.6 ± 1.2 c	1.6 ± 3.8 a
29th Nov (Sk-An)	73.4 ± 4.4 b	0.37 ± 0.10 d	0.06 ± 0.09 cd	10.0 ± 0.1 c	1.4 ± 0.02 d	7.1 ± 0.04 cd	35.2 ± 0.5 bc	2.9 ± 3.0 ab
9th Dec	72.8 ± 4.1 b	0.39 ± 0.09 d	0.34 ± 0.14 de	10.1 ± 0.3 cde	1.3 ± 0.05 cd	7.6 ± 0.50 de	39.1 ± 2.6 cd	12.2 ± 4.2 bc
16th Dec	72.9 ± 4.4 b	0.42 ± 0.08 d	0.48 ± 0.25 ef	10.0 ± 0.2 cd	1.3 ± 0.08 bc	7.9 ± 0.53 e	41.2 ± 4.4 cd	18.2 ± 7.6 c
22nd Dec	74.5 ± 4.6 c	0.54 ± 0.11 e	0.57 ± 0.41 ef	10.4 ± 0.1 ef	1.2 ± 0.12 b	9.0 ± 0.87 f	42.3 ± 5.5 d	19.3 ± 14.8 cd
29th Dec	74.5 ± 4.6 c	0.54 ± 0.14 e	0.74 ± 0.39 f	10.3 ± 0.3 def	1.0 ± 0.08 a	10.0 ± 0.72 g	42.6 ± 2.5 d	29.1 ± 14.5 de
7th Jan (HV)	73.6 ± 4.2 bc	0.60 ± 0.16 f	0.70 ± 0.27 f	10.5 ± 0.2 f	1.0 ± 0.05 a	10.0 ± 0.33 g	39.7 ± 1.8 d	30.4 ± 10.5 e

Table 1. Main physicochemical parameters during maturation of blood oranges 'T. Ippolito'. Different letter in the same column and season indicates significant differences (LSD test, p-value=0.05). 'CB'- colour break, 'Fl-An'-onset of anthocyanins synthesis in the flesh, 'Sk-An'-onset of anthocyanins synthesis in the peel. *The Sk-An were detected on 14th December in 2022 (a*/b*=0.31), and 19th December in 2023 (a*/b*=0.35).

Dates	Diameter (mm)	Rind colour (a*/b*)	Juice colour (a*/b*)	TSS (%)	TA (g citric ac. 100 mL^{-1})	MI (TSS/Ac.)	JY (%)	Flesh anthocyanin content (mg L^{-1})
2022*								
21st Sep	60.4 ± 3.9 a	-0.54 ± 0.04 a	-0.53 ± 0.06 a	10.5 ± 1.4 a	3.0 ± 0.3 c	3.5 ± 0.24 a	34.1 ± 3.9 a	-
3rd Nov (CB)	68.6 ± 4.4 b	-0.36 ± 0.10 b	-0.30 ± 0.01 a	11.1 ± 0.8 a	1.5 ± 0.2 b	7.3 ± 0.51 b	42.2 ± 4.5 b	-
1st Dec (Fl-An)	70.6 ± 4.3 c	0.15 ± 0.11 c	-0.17 ± 0.04 a	12.3 ± 0.9 ab	1.4 ± 0.1 ab	8.7 ± 0.77 b	40.9 ± 1.1 b	1.2 ± 0.8 a
19th Jan (HV)	71.7 ± 4.6 c	0.46 ± 0.10 d	1.57 ± 0.48 b	13.4 ± 1.0 b	1.2 ± 0.2 a	11.1 ± 1.40 c	42.7 ± 2.3 b	61.2 ± 26.6 b
2023*								
27th Sep	65.0 ± 5.5 a	-0.44 ± 0.05 a	-0.44 ± 0.05 a	9.5 ± 0.2 a	2.1 ± 0.2 c	4.5 ± 0.4 a	35.6 ± 1.2 a	-
9th Nov (CB)	68.4 ± 5.8 b	-0.02 ± 0.15 b	-0.22 ± 0.00 b	11.4 ± 0.2 b	1.3 ± 0.1 b	8.7 ± 0.5 b	36.5 ± 2.0 a	-
4th Dec (Fl-An)	70.0 ± 5.5 b	0.21 ± 0.08 c	-0.10 ± 0.02 b	11.6 ± 0.4 cd	1.2 ± 0.0 ab	9.7 ± 0.1 b	42.0 ± 0.5 b	4.3 ± 3.6 a
15th Jan (HV)	70.3 ± 5.1 b	0.55 ± 0.16 d	0.98 ± 0.38 c	12.1 ± 0.4 d	1.1 ± 0.1 a	10.8 ± 1.0 c	41.3 ± 3.0 b	40.1 ± 17.7 b

In addition to the total anthocyanin content in the juice, individual anthocyanins were analysed by HPLC (Figure 3). The concentration of individual anthocyanins was not high enough to detect them by chromatography until 29th November, when cyanidin-3-O-glucoside (c-3-glu), cyanidin-3-(6”malonyl)-O-glucoside (c-3-6”mal-glu) and cyanidin-3-(6”dioxalyl)-O-glucoside (c-3-6”dio-glu) were identified. At all stages of the ripening, the major anthocyanins in the juice of ‘Tarocco Ippolito’ were c-3-glu and c-3-6”mal-glu, increasing exponentially from 2.5 and 3.6 mg L^{-1} on 29th November to 12.5 and 18.8 mg L^{-1} respectively at commercial harvest. c-3-6”dio-glu showed lower levels throughout all the maturation period, reaching values around 5 mg L^{-1} at harvest. Delphinidin-3-O-glucoside (d-3-glu) was not detected analytically until mid-December and its concentration remained stable at around 5 mg L^{-1} throughout maturation. The importance of these four anthocyanins, particularly c-3-glu and c-3-6”mal-glu, is consistent with that reported for other blood orange varieties such as ‘Moro’, ‘Tarocco’ and ‘Sanguinelli’ (Fabroni *et al.*, 2016; Cebadera-Miranda *et al.* 2019; Morales *et al.* 2021).

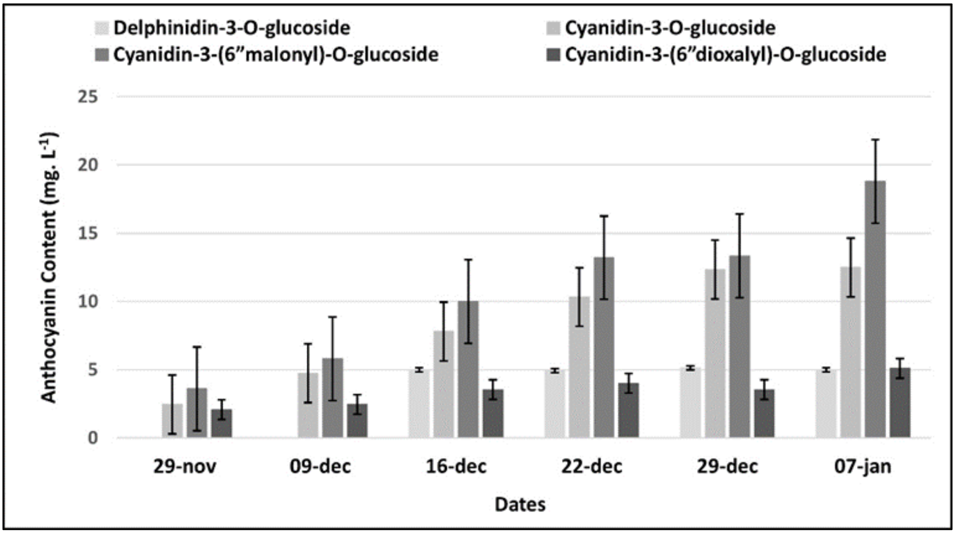

Figure 3. Concentration of individual anthocyanins during the maturation of ‘Tarocco Ippolito’ oranges. Error bars represent the LSD intervals (p-value=0.05) from an ANOVA performed to evaluate the effect of date on the concentration of each individual anthocyanin.

As in the first season, in the second and third seasons the colour break from green to yellow tones occurred in early November. However, there was more variability between seasons in the onset of anthocyanins accumulation in the flesh and the peel. As explained above, in the first season anthocyanin biosynthesis in the flesh was detected on 21st November and in the peel eight days later. In the second season, there was a delay of 10 days in the accumulation of anthocyanins respect to the first season, being firstly detected on 1st December, while in the third season, it was delayed significantly more, with the reddish colouration in the flesh not becoming evident until 4th December (Table 1). With regard to the detection of red colouration in the peel, in the second and third seasons it was firstly detected on 14th December (a*/b*= 0.31) and 19th December respectively (a*/b*= 0.35). Therefore, a common pattern observed across all three harvests was that anthocyanin biosynthesis in the peel was delayed compared to the flesh, with red colouration in the peel appearing 8 to 15 days later than in the flesh. Previous studies have suggested that anthocyanin accumulation in citrus fruits exhibits tissue dependent (Cebadera-Miranda *et al.*, 2019; Huang *et al.*, 2019), and this result support this finding.

Regarding the other physicochemical parameters, if we focus on the MI values at the onset of anthocyanin accumulation in the flesh, there is significant variability between seasons, with values of 6.7, 8.7, and 9.7 for the first, second, and third seasons of the study, respectively (Table 1). Therefore, it is evident that although the accumulation of anthocyanins in the fruit occurs alongside its ripening, the onset of this process is not associated with a specific stage of maturity.

There were also significant differences in the MI at the time of commercial harvest. In the first season, the MI was 10, associated with TSS values of 10.5 % and TA of 1.1 mg 100 mL^{-1}. In the second and third seasons, although acidity values were similar to those in the first season, the fruit accumulated a higher sugar content, resulting in MI values around 11. It is important to note that the final harvest date was decided by the citrus cooperative managing the plot. The fact that the harvest in the second and third seasons was carried out 12 and 8 days later than in the first season was due to the interest in allowing the fruit to achieve optimal external red colouration. While it is commercially desirable to market fruit as soon as possible for better pricing, it is also important to allow the fruit to develop the intense red colour characteristic of blood oranges. During the three seasons covered by this study, flesh colouration was not a limiting factor, with the flesh developing intense red tones. However, peel colouration was limiting in the second and third seasons, as the peel did not achieve intense red tones at the beginning of January. Consequently, a compromise was reached by harvesting the fruit as early as possible while ensuring that the characteristic red colour had developed. In the first season, the fruit was harvested on 7th January with external colour values of a*/b*=0.60. In contrast, in the second and third seasons peel colour values were lower, a*/b*=0.46 and 0.55 respectively, despite the harvest being postponed to mid-January or even a few days later (Table 1).

3.2. Relation between Anthocyanins Accumulation and Environmental Factors

In this section of the study, after having confirmed that the onset of anthocyanin accumulation is not directly related to a specific stage of maturation, we evaluate each of the three main factors that have been associated with the biosynthesis and accumulation of anthocyanins in blood oranges: thermal amplitude, cold temperatures and sun radiation. The aim is to elucidate the extent to which each of them is involved in the initiation of anthocyanin biosynthesis in 'Tarocco Ippolito' oranges. We also investigate whether, beyond their involvement in the triggering of the biosynthesis, any of these factors is correlated with the total accumulation of anthocyanins at the time of commercial harvest.

3.2.1. Thermal Amplitude

The daily thermal amplitude, i.e. the difference between the maximum and minimum temperature in a day is shown in Figure 4.

In the season 2021, the thermal amplitude ranged from 10 to 15°C during the first weeks of November. In the week immediately preceding the onset of anthocyanin accumulation in the flesh, the thermal amplitude showed values close to 10°C to then decreased markedly, falling below

5°C in the three days immediately preceding anthocyanin observation. During the week between the detection of anthocyanins in the flesh and peel, the thermal amplitude remained relatively constant, averaging 7°C.

In the season 2022, a completely different pattern was observed. During the first weeks of November, the thermal amplitude fell below 5 degrees on several occasions. The thermal amplitude during the week immediately before anthocyanin accumulation in the flesh was higher than 5°C, reaching values close to 15°C on some days. In the following days, until anthocyanins were detected in the peel, the average thermal amplitude was 7 °C but with a considerable variability, with values ranging from 2.3 °C to over 12 °C.

Finally, in 2023, the thermal amplitude during the month of November reached higher values, with entire weeks showing values close to or exceeding 15 degrees. In the days preceding anthocyanin biosynthesis in the flesh, the thermal amplitude showed high variability, with some days below 5°C, while the period immediately before anthocyanin observation reached values around 10°C. In the subsequent days, until the biosynthesis in the peel occurred, the average thermal amplitude was 11.5 °C with values in this case between 5.2°C to over 16°C.

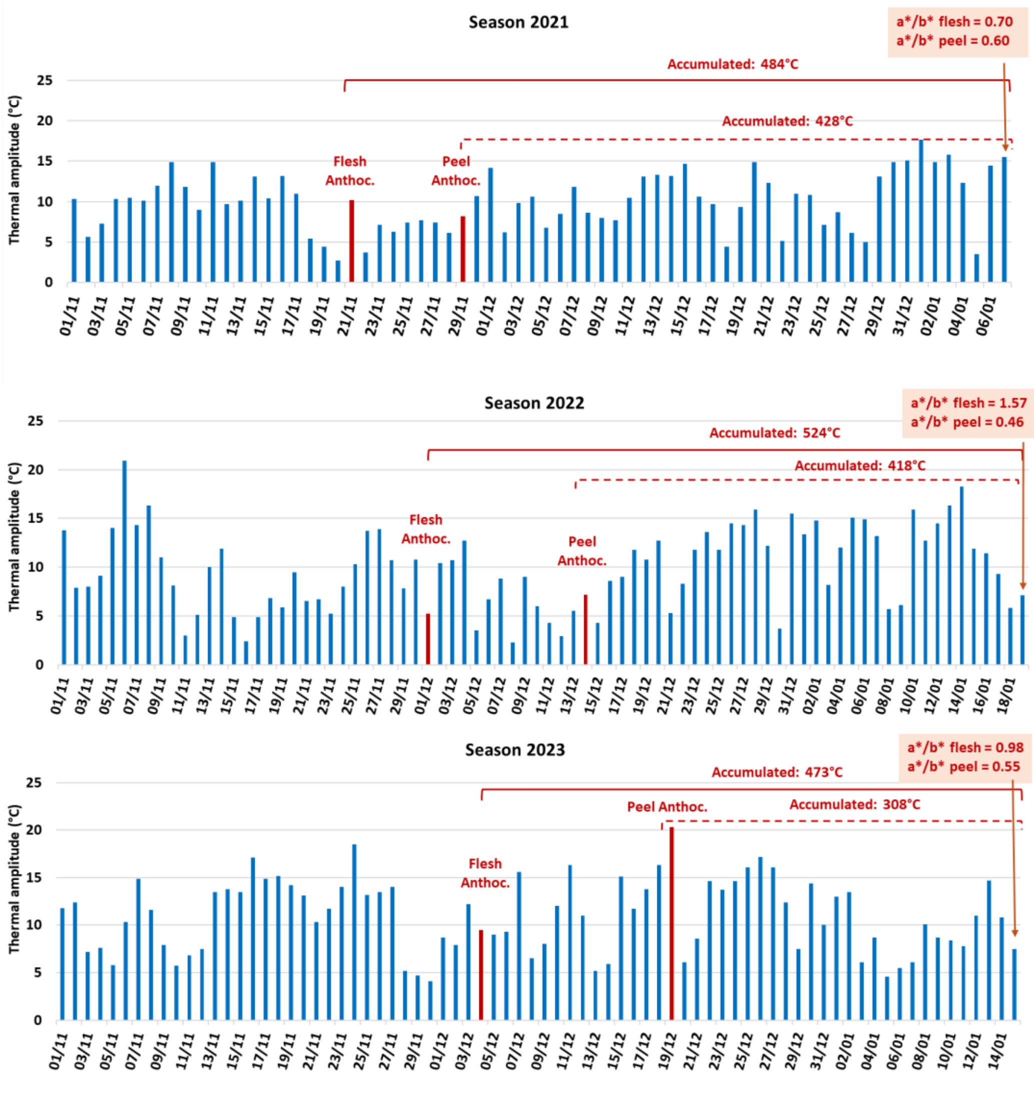

Figure 4. Daily thermal amplitude and accumulated amplitude from the date on which the accumulation of anthocyanins in the flesh and peel was first detected until harvest.

Therefore, across the three evaluated seasons, distinct patterns of thermal amplitude were observed in the weeks and days preceding the onset of anthocyanin accumulation in both the flesh and peel. This suggests that, at least in the case of 'Tarocco Ippolito' oranges, there is no consistent relationship between the thermal amplitude and the activation of the anthocyanin biosynthesis process.

Beyond the onset of anthocyanin accumulation, as indicated by Butelli *et al.* (2012) and Caruso *et al.* (2016), the general belief, despite the lack of scientific proof, is that significant temperature changes between day and night are necessary for intense colour formation in fruit flesh. In other words, a large thermal amplitude is associated with more intensely coloured fruit. When examining the period between the start of biosynthesis in the flesh and harvest time, and calculating the accumulated thermal amplitude for each season, the order from greatest to least is: season 2022 > season 2021 > season 2023, with accumulated thermal amplitudes of 524 °C, 484 °C, and 473 °C, respectively (Figure 4). However, at harvest, the fruit with the most intense flesh colouration was that collected in 2022, followed by 2023 and 2021 (Figure 4). Therefore, in the last two seasons of this study, the relationship between thermal amplitude and internal colouration intensity did not hold. Similarly, it was not possible to establish a relationship between the peel colouration at harvest and the thermal amplitude accumulated during the period between the dates when red colouration was first detected in the peel and the harvest dates (Figure 4).

This result suggests that the commonly held belief—that strong day-night thermal differences are directly responsible for blood oranges to achieve intense flesh colouration—is not accurate, at least not for the cv. Tarocco Ippolito under the evaluated conditions.

3.2.2. Chilling Temperatures. The Importance of Chilling Quality

To the best of our knowledge, this is the first time that the chilling requirements for triggering anthocyanin accumulation in fruit have been investigated. To achieve this, we applied the main models available, Chilling Hours, Utah, Positive Utah and Dynamic model. Since the weather station was located very close to the plot from which the fruit was harvested, we can be confident that our data accurately reflect the temperatures to which the fruit was exposed.

Figure S2 shows the daily evolution of chilling hours, chilling portions, chilling units and chilling positive units from October 1st, when temperatures were still mild, until the date when peel red colouration was first detected in each of the seasons.

The first notable observation is the significant difference between the models. The Chilling Hours model, which calculates the number of hours per day within a temperature range of 0-7.2 °C, has a maximum possible value of 24 (Figure S2). During the three seasons under study, the highest value recorded by this model was 17 at mid-December of 2023. The Dynamic model yields considerably lower values, with no negative values and the highest detected value being 1.47 at the beginning of December 2022. The Utah model exhibits the greatest variability between days, with values ranging from -25 to +20 chilling units. The Utah Positive model resulted in values between 0 to +19 chilling P-units.

The graph shows that, for all three seasons, chilling temperatures do not begin until November. In fact, during October, minimum temperatures did not drop below 8°C in any of the three seasons under study (data not shown).

The main working hypothesis of this research was that like what occurs with the breaking of dormancy in fruit trees, a certain level of chilling exposure is required for the process of anthocyanin synthesis in blood oranges to begin. This hypothesis led us to evaluate the main existing chilling accumulation models, which were developed for dormancy breaking. Consequently, we calculated the accumulated chilling according to the different models at the time anthocyanins were first detected in the pulp and the peel of the oranges, using 1st November as a starting date (Figure 5). Let's examine each model to understand the events related to anthocyanin biosynthesis in both the flesh and peel:

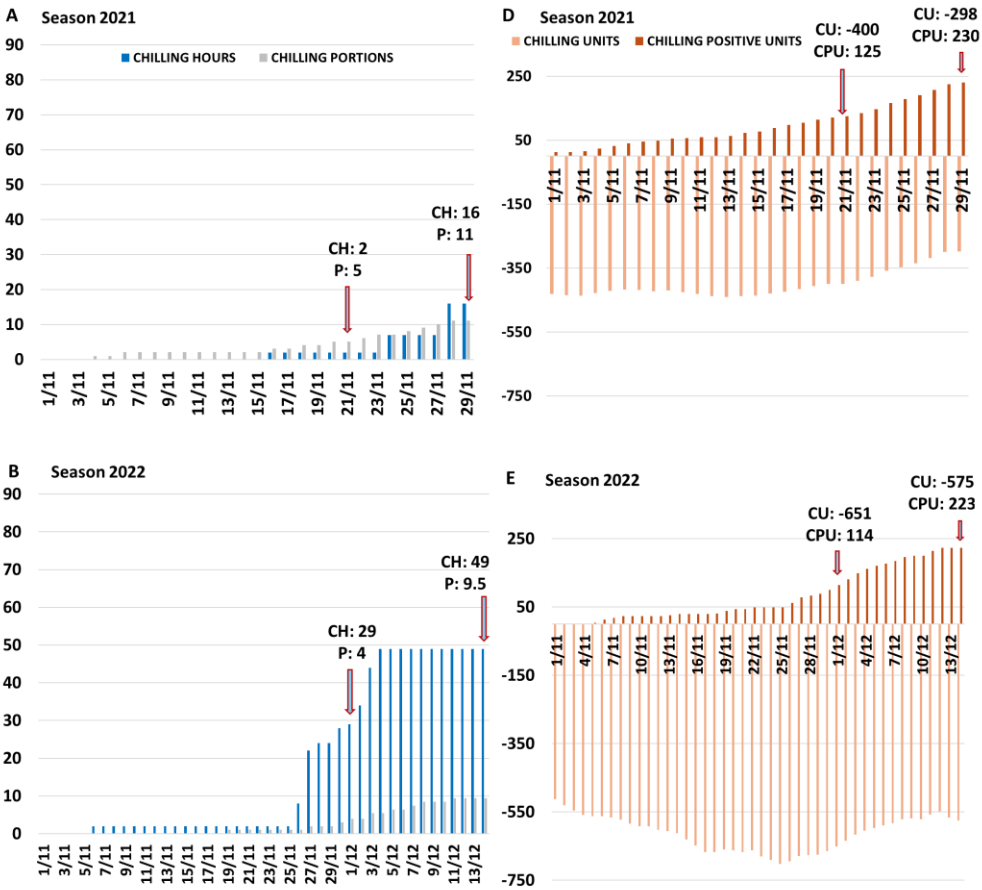

Figure 5. Accumulated Chilling Hours, Chilling Portions (A, B and C), Chilling Units and Chilling Positive Units (D, E, F) from November 1st to the date of first detection of anthocyanins in the peel. For each season, the dates of the first detection of anthocyanins in the flesh and peel are indicated with arrows, and the accumulated chilling is specified (continued on the following page).

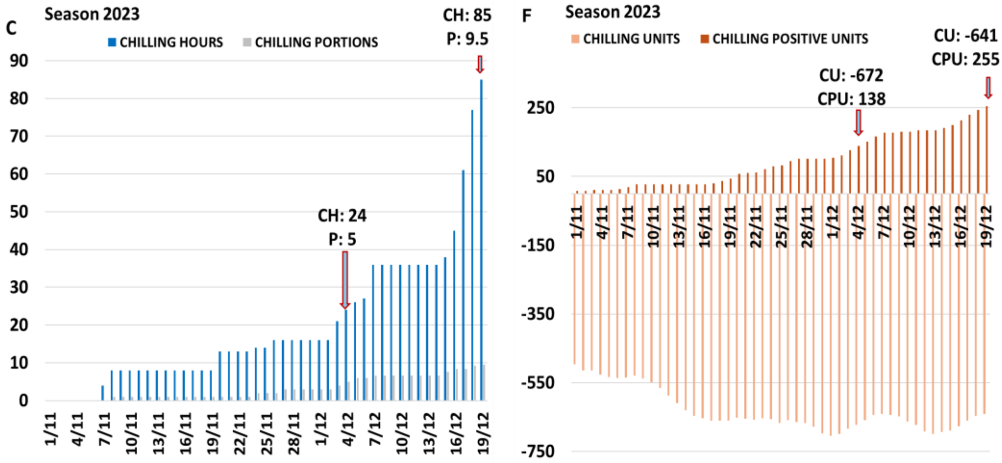

Figure 5. Accumulated Chilling Hours, Chilling Portions (A, B and C), Chilling Units and Chilling Positive Units (D, E, F) from November 1st to the date of first detection of anthocyanins in the peel. For each season, the dates of the first detection of anthocyanins in the flesh and peel are indicated with arrows, and the accumulated chilling is specified.

1. The chilling hours accumulated before the appearance of red colouration in the flesh varied widely from values of 2 chilling hours in the 2021 season to 29 chilling hours in the 2022 season. Similarly, there was considerable variability in the chilling accumulated before anthocyanins accumulation in the peel, ranging from 16 chilling hours in the 2021 season to 85 hours in the 2023 season (Figure 5A, 5B and 5C). Therefore, the chilling hours model did not reveal a pattern for the chilling requirements in either case.

2. Upon analysing the dynamic model, a pattern of behaviour appears to emerge. The number of chilling portions accumulated before anthocyanin were first detected in the flesh ranged from 4 in season 2022 to 5 in seasons 2021 and 2023. If we take the midpoint between these two values, we can establish values ≈ 4.5 as the chilling requirement. This results in a variability range of 11 %, which seems sufficiently small to assume the existence of a pattern across the three sessions studied (Figure 5A, 5B and 5C). Moreover, we were pleasantly surprised by the

results concerning the chilling portions accumulated before the development of red colouration in the peel. In all three seasons under study, the accumulated chilling portions were ≈ 10.2, with values ranging from 9.5 in the 2022 and 2023 seasons to 11.1 in the 2021 season. In this case, the variability is even less than 10 %.

3. The chilling units of the Utah model showed negative values when accumulated. Similar to the chilling hours model, there was considerable variability among seasons in the chilling units accumulated before the onset of anthocyanin biosynthesis in both the flesh and peel. Values ranged from -400 for the flesh and -298 for the peel in the 2021 season to -672 and -641 for flesh and peel respectively in the 2023 season (Figure 5D, 5E and 5F). As a result, no clear pattern could be established based on the accumulated values of this model.

4. The Positive Utah model revealed more interesting results than the original Utah model. The onset of anthocyanins in the flesh was linked to ≈ 128 chilling P-units (125 in season 2021, 114 in season 2022, and 138 in season 2023) (Figure 5D, 5E and 5F). The chilling P-units required for anthocyanins accumulation to begin in the peel was 230 in season 2021, 223 in season 2022, and 255 in season 2023. Therefore, we can stablish a value of ≈ 240 chilling P-units as the chilling requirement for red colouration to initiate in this tissue.

Therefore, our results show that the onset of anthocyanin synthesis in both the pulp and peel is indeed linked to specific chilling requirements, which are higher in the peel than in the pulp. These requirements were revealed by the Dynamic Model and the Positive Utah Model, while the Chilling Hours Model and the Utah Model were not suitable for their calculation. The requirements of chilling for anthocyanins synthesis to begin in the flesh was stablish in 4-5 chilling portions (Dynamic Model), which was equivalent to ≈ 128 Utah P-units. The requirements for the process to initiate in the peel were estimated to be ≈ 10 chilling portions and ≈ 240 chilling P-units.

So far, we have discussed the onset of anthocyanin accumulation, which was the main objective of this study. However, in addition to determining the chilling requirements for this process to begin, it is also valuable to explore the correlation between cold exposure and the fruit's final colouration at harvest. To this end and based on the previously described results regarding the onset of anthocyanin synthesis, we focused on the Dynamic and Positive Utah models. Supplementary Figure S3 presents the accumulated chilling values from November 1st until the commercial harvest.

Focusing on the dynamic model, we observe that in the first season, fruit was harvested on 7th January with a peel colour value of 0.60 (Table 1), at which point the accumulated chilling portions were 27.1 (Figure S3). However, in the second and third seasons, the accumulated chilling portions on 7th January were 10 portions less. This may explain why the harvest had to be delayed in these two seasons to allow the fruit to develop a more attractive colour. In fact, if we rank the seasons by the intensity of peel colouration at harvest time, the order is: season 2021 (a^*/b^* = 0.60) > season 2023 (a^*/b^* = 0.55) > season 2022 (a^*/b^* = 0.46) (Table 1). The same order is observed when focusing on the accumulated chilling portions at the time of harvest: season 2021 (27.1) > season 2023 (22.4) > season 2022 (21.3) (Figure S3). This relationship was confirmed by performing a correlation analysis between the chilling portions accumulated each season by the time of harvest and the peel colour index. As displayed in Figure S4, a correlation coefficient of $r = 0.76$ was obtained. Therefore, according to our data, the higher the accumulated chilling portions, the more intense the external red colouration at harvest.

A parallel pattern was detected when applying the Positive Utah Model, with a correlation as higher as $r = 0.88$ between the peel colour and the Utah P-units accumulated at harvest time (Figure S5).

A different pattern was observed for internal colouration. The most intense flesh colouration was achieved in the 2022 season (a^*/b^* = 1.57), followed by the 2023 season (a^*/b^* = 0.98). The least intense red colouration was seen in the fruit from the 2021 season (a^*/b^* = 0.70) (Table 1). It was not possible to explain the intensity of internal colouration based on the accumulated values of chilling P-units, or chilling portions. However, it is noteworthy that the later the fruit was harvested in the season, the more intense the red colouration became. This suggests that once the biosynthesis of internal anthocyanins begins, it progresses gradually over time, and the longer the fruit remains on the tree, the more intense its internal colour will be.

As previously suggested by other researchers (Butelli *et al.*, 2012; Carmona *et al.*, 2017; Huang *et al.*, 2019), several findings from the present study support the notion that anthocyanin biosynthesis in blood oranges is a tissue-dependent process that is not synchronized between the flesh and peel (Huang *et al.*, 2019): 1) The onset of anthocyanin accumulation was consistently delayed in the peel compared to the flesh across all three seasons; 2) The chilling requirements for initiation of anthocyanin biosynthesis in the peel were nearly double those whose required to trigger anthocyanin biosynthesis in the flesh; 3) The accumulation of anthocyanins in the peel at harvest was related to the total accumulated chilling portions and chilling P-units, while anthocyanin accumulation in the flesh increased the later the harvest occurred in the season.

A specificity in anthocyanin accumulation across tissues has been previously reported in other fruits. In grapevine varieties known as *teinturier* varieties, anthocyanins are synthesized in both the peel and flesh, and it has been observed in several of these varieties that the transcriptional regulation of anthocyanin biosynthesis is tissue-specific, with anthocyanins beginning to accumulate in the flesh before they accumulate in the peel (Castellarin *et al.*, 2011; Xie *et al.*, 2015). In the same vein, it has been reported in red-fleshed apples that the gene responsible for red flesh is not responsible for peel colour (Umemura *et al.*, 2013). Therefore, several studies, including the present one, suggest that different mechanisms regulate the anthocyanin biosynthetic pathway in different fruit tissues.

Let's now discuss why, among the four models studied, the Positive Utah and the Dynamic Model yielded chilling requirements for anthocyanin synthesis, while the Chilling Hours and the Utah Model did not.

The first point is that, as mentioned in the introduction, the Positive Utah Model was developed to adapt the Utah Model to warm temperatures and subtropical zones (Linsley-Noakes and Allan, 1994). This explains why, between these two models, the Positive Utah Model is better suited to the conditions of southeastern Spain, a region characterized by a Mediterranean climate and, therefore, classified as a warm temperature zone.

The second point to considerer is the advantages of the Positive Utah and Dynamic Models over the Chilling Hours Model. The main difference between them is that the Positive Utah and Dynamic Models place significant emphasis on the quality of chilling, rather than merely its quantity.

As explained in the Material and Methods section, the Chilling Hours Model is the simplest, based on counting the number of hours when temperatures fall between 0 and 7.2°C.

The Positive Utah model defines chilling units as the exposure to temperatures considered optimal for chilling accumulation (2.5-12.5 °C). Temperatures lower than 1.4 °C and between 12.5 and 15.9 °C do not contribute to chilling accumulation, while temperatures between 1.5 and 2.4 °C contribute to chilling units (+0.5 unit).

Explaining the equations of the dynamic model in detail is quite complex, but the key idea to take away is that it is more demanding in terms of the quality of chilling compared to the Chilling Hours and even the Utah model. The dynamic model not only considers the amount of time spent at chilling temperatures (1.6 to 12.7 °C), but also takes into account the sequence and quality of these temperatures, making it a more refined and stringent approach for assessing chilling accumulation.

Habibi *et al.* (2020, 2021) found in postharvest studies on the varieties 'Moro', 'Tarocco', 'Sanguinello', and 'Sanguine' that anthocyanin accumulation was higher in both the flesh and peel when fruit was stored at 5 °C compared to 2 °C. Previously, Carmona *et al.* (2017) had reported greater anthocyanin accumulation in 'Moro' cultivar at 9 °C than at 5 °C. Furthermore, Hamedani *et al.* (2012) observed the greatest increase in anthocyanins in 'Tarocco' oranges stored at 8 °C, compared to 15 °C and 22 °C. Therefore, as far as current knowledge suggests, temperatures around 8–9 °C are the most effective for stimulating anthocyanin biosynthesis in the blood oranges under postharvest conditions. These temperatures fall within the range that the Positive Utah and Dynamic models consider optimal for chilling accumulation, suggesting that this may partly explain why these two models are well-suited for explaining the triggering of anthocyanin biosynthesis. However, we must be cautious with this hypothesis, as current knowledge does not allow us to assume that the effect of temperature on anthocyanin accumulation is the same after harvest as it is while the fruit is still on the tree.

Another aspect worth commenting on is that our data showed that the chilling requirements for anthocyanin biosynthesis in the peel are higher than those for the flesh, a conclusion further supported by the observed delay of 8 to 15 days in the onset of anthocyanin accumulation in the peel compared to the flesh.

Although the chilling requirements for anthocyanin biosynthesis have not been studied in other fruits, it has been reported that low temperatures play a role in anthocyanin accumulation in the peel of grapes. Research on grapes has focused on nighttime temperatures, and several studies have shown that exposing vines to cool nights results in greater total anthocyanin accumulation compared to warm nights (Mori *et al.*, 2005; Gaiotti *et al.*, 2018). Considering the principles of the Dynamic and Positive Utah models, it is logical to infer that nighttime temperatures, being lower than daytime temperatures, play a crucial role in the accumulation of chilling portions and chilling P-units. Therefore, the effect of low temperatures on anthocyanin accumulation in grapes and blood oranges appears to be similar, with more pronounced accumulation occurring when the fruit is exposed to quality cold.

3.2.3. Sun Radiation

Since Huang *et al.* (2019) demonstrated that sunlight exposure was necessary for peel colouration but not for flesh colouration of 'Tarocco' oranges, we focused on exploring the possibility of a relationship between anthocyanin accumulation in the peel and irradiation data. Figure 6 shows the average daily radiation and the accumulated radiation at the time the onset of anthocyanins accumulation was detected in the peel and at the harvest time.

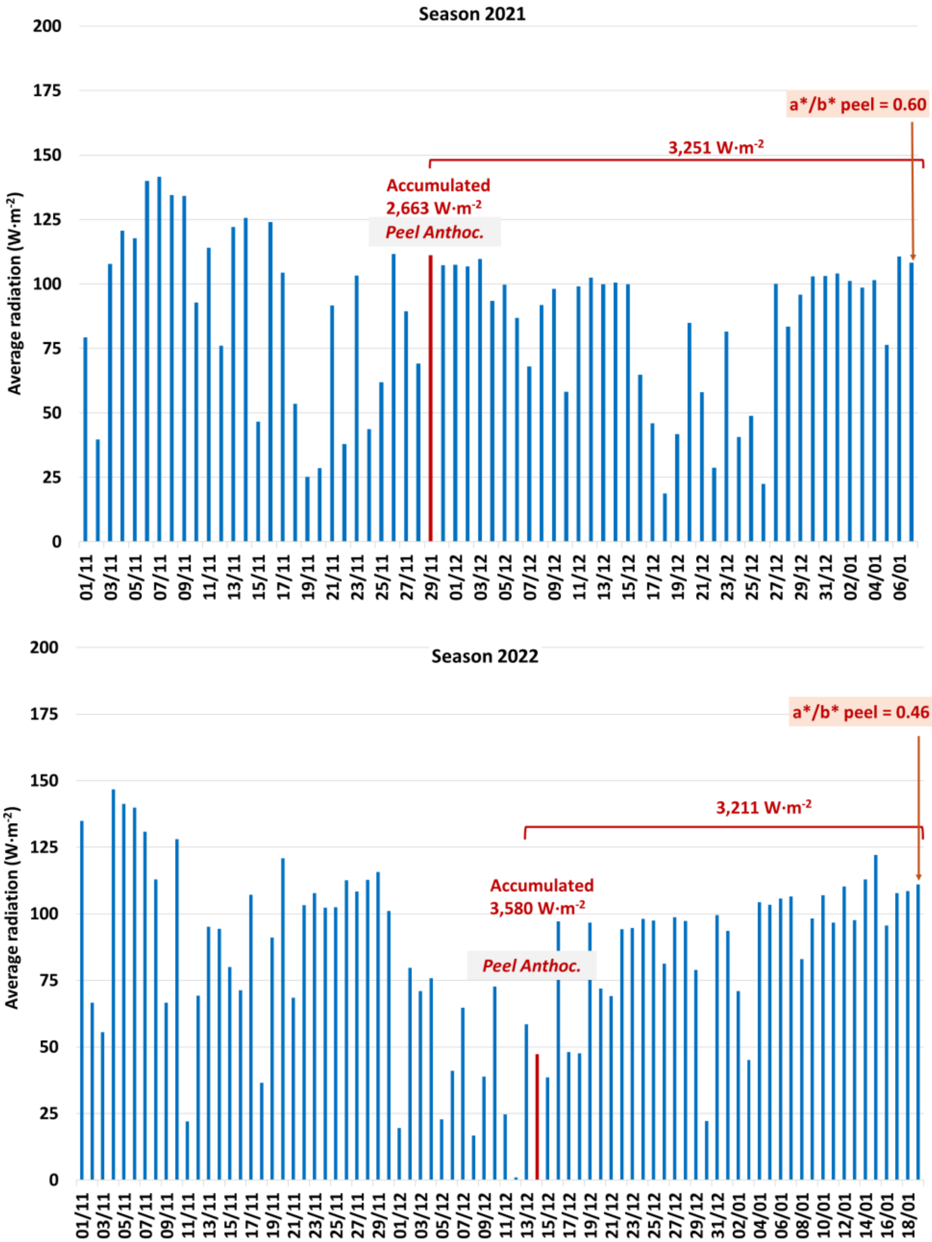

Figure 6. Average values of daily radiation (W·m^{-2}). For each season, the date of first detection of peel anthocyanins, and the peel colour at harvest time are indicated (continued on the following page).

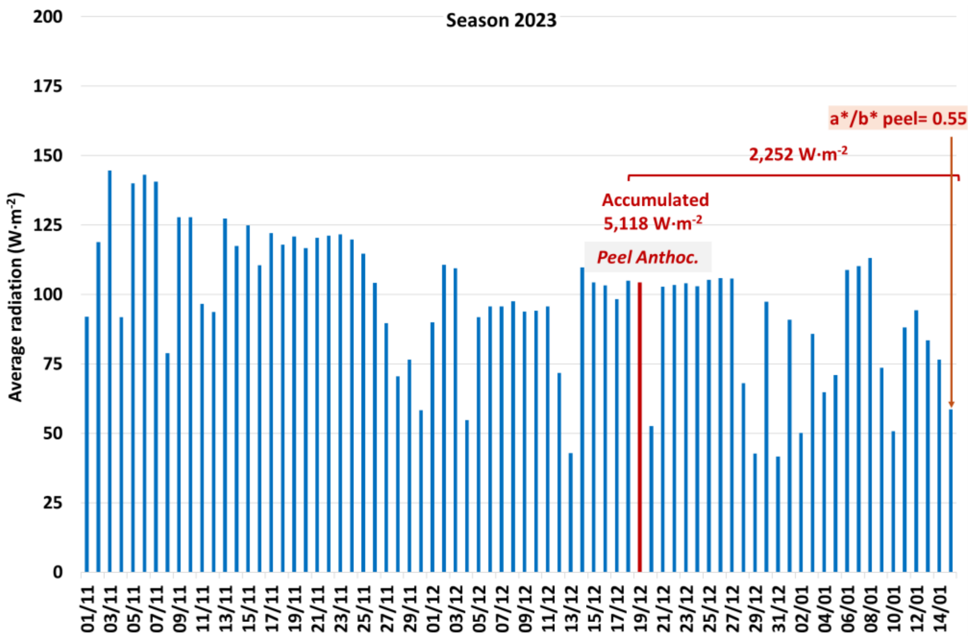

Figure 6. Average values of daily radiation (W·m^{-2}). For each season, the date of first detection of peel anthocyanins, and the peel colour at harvest time are indicated.

We did not identify a specific pattern of requirements, neither when analysing the daily data nor when evaluating the accumulated values.

The accumulated radiation from November 1st until the beginning of anthocyanin synthesis in the peel ranged from 2,663 W·m^{-2} in the 2021 season to 5,118 W·m^{-2} in the 2023 season. Therefore, it is not possible to determine specific requirements in this regard.

If we focus only on the days immediately preceding the detection of red colouration in the peel, radiation levels also varied significantly between seasons. During the 2021 season, radiation was mostly below 50 W·m^{-2}, with occasional peaks near 100 W·m^{-2} (Figure 6). In the 2022 season, values ranged between 25 and 75 W·m^{-2}. In contrast, during the 2023 season, most days immediately preceding the detection of red colouration in the fruit peel exhibited radiation levels of 100 W·m^{-2} or higher.

We also could not find a correlation between the radiation accumulated from the start of anthocyanin biosynthesis in the peel and the fruit colouration at harvest (Figure 6). The accumulated radiation during this period was very similar in the 2021 and 2022 seasons, but colouration was significantly more intense in 2021 ($a^*/b^*= 0.60$) than in 2022 ($a^*/b^*=0.46$), allowing for an earlier harvest. Additionally, in the 2023 season, the accumulated radiation during this period was lower than in 2022, yet the final external colouration of the fruit was redder ($a^*/b^*=0.55$) (Table 1, Supplementary Figure S6).

Huang *et al.* (2019) found that two different genes are involved in anthocyanin biosynthesis in the peel 'Tarocco' oranges, one responsive to cold and the other to light. It is therefore possible that while light activation may be necessary, the cold and its quality could be the determining factors in anthocyanin accumulation, or at least this is what our data suggest for the cv. Tarocco Ippolito. It is also possible that below a certain threshold of radiation, its intensity plays a role in colouration intensity, but beyond this point, the amount and quality of chilling become the determining factors. More studies are necessary to elucidate the role of sun radiation in anthocyanin accumulation in the peel of blood oranges.

4. Study Limitations and Strengths

This study was conducted on the blood oranges cv. Tarocco Ippolito, and initially, these results are exclusively applicable to this cultivar. It is not advisable to extrapolate them to other cultivars without conducting specific studies for that purpose. In fact, various studies conducted with a range of blood orange cultivars grown in the same plot, primarily in collections or germplasm banks, have demonstrated that some varieties accumulate more pigmentation in the peel than in the flesh, while in other cases, the opposite occurs (Caruso *et al.*, 2016; Cebadera-Miranda *et al.*, 2019). Therefore, the cultivar factor is a crucial consideration that must be taken into account. In the same vein, various studies have demonstrated that the effect of temperature and solar radiation on anthocyanin accumulation in grapes and apples depends on the cultivar (Gouws *et al.*, 2014; Fernades de Oliveira and Nieddu, 2016).

Beyond its limitations, this study has significant strengths. Not only is this the first time that chilling requirements for anthocyanin accumulation in blood oranges have been calculated, but to the best of our knowledge, it is also the first time that the Chilling Hours, Utah, Positive Utah and Dynamic models have been applied to determine the chilling requirements for triggering anthocyanin biosynthesis in fruit. Our positive results open the door for applying these models to other fruits where the accumulation of anthocyanins is critical, such as grapes, apples, and others.

Another strength of this study is that the data were collected from actual orchard environments rather than controlled indoor conditions. This means that the calculated chilling requirements have precedence over the potential influence of other factors, such as radiation intensity or day-night temperature differences, as previously discussed. In other words, if the chilling needs remain consistent while other environmental conditions vary, it suggests that the model could be applicable over a larger geographic area.

5. Conclusion

The evaluation of the influence of solar radiation, thermal amplitude, and cold exposure on anthocyanin accumulation in 'Tarocco Ippolito' blood oranges over three seasons showed that temperature is the determining factor in the accumulation of these compounds.

Anthocyanin synthesis in the flesh and peel of blood oranges are processes that do not occur in parallel. The onset of anthocyanin synthesis in the flesh took place between late November and early December and was associated with exposure to a total cold accumulation of 4-5 chill portions according to the Dynamic model and ≈ 128 chilling P-units according to the Positive Utah model. For anthocyanin accumulation to begin in the peel of 'Tarocco Ippolito' oranges, more cold is required than for accumulation in the flesh, with a delay of 8 to 15 days observed for the initiation of this process in the peel relative to the flesh. The cold requirements for anthocyanin accumulation in the peel, were identified to be ≈ 10 chilling portions (dynamic model) or ≈ 240 chilling P-units (positive Utah model). The Chilling Hours and Utah model should be discarded for this purpose.

These results open the door to evaluating the Positive Utah and Dynamic models for explaining anthocyanin synthesis in other blood orange varieties, and even in other anthocyanin-accumulating fruits, such as grapes.

Acknowledgements

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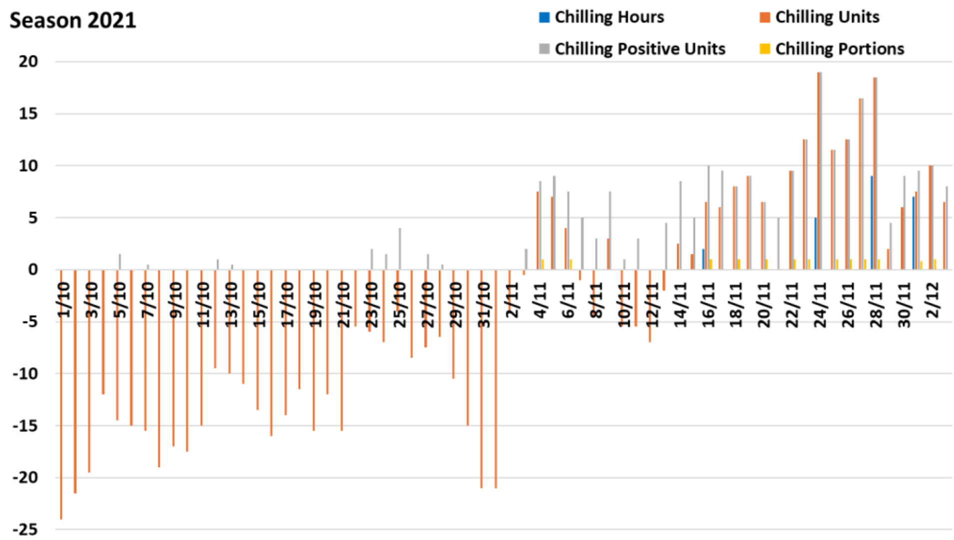
Supplementary Material

Figure S1. External and internal appearance of the fruit at the time when anthocyanin accumulation in the flesh was first detected.



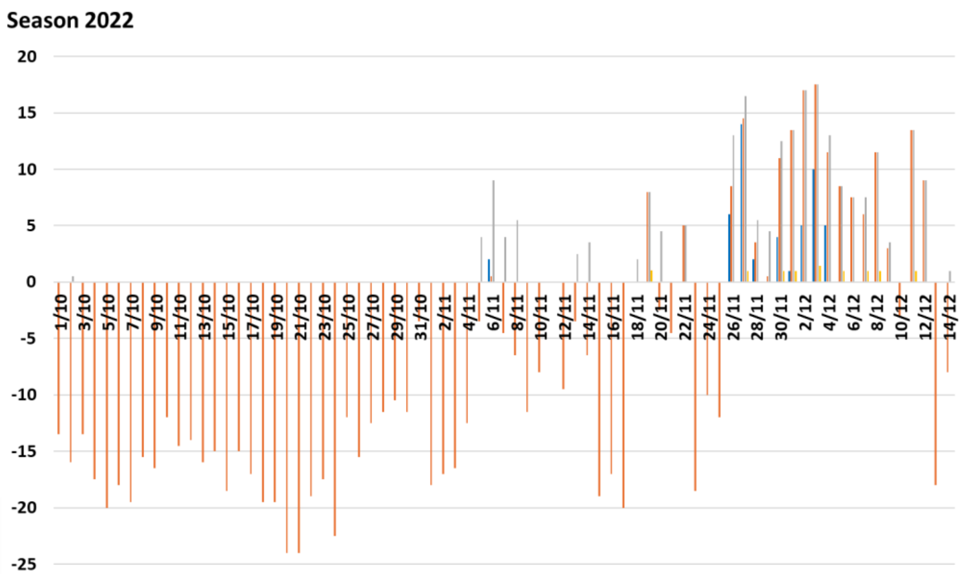
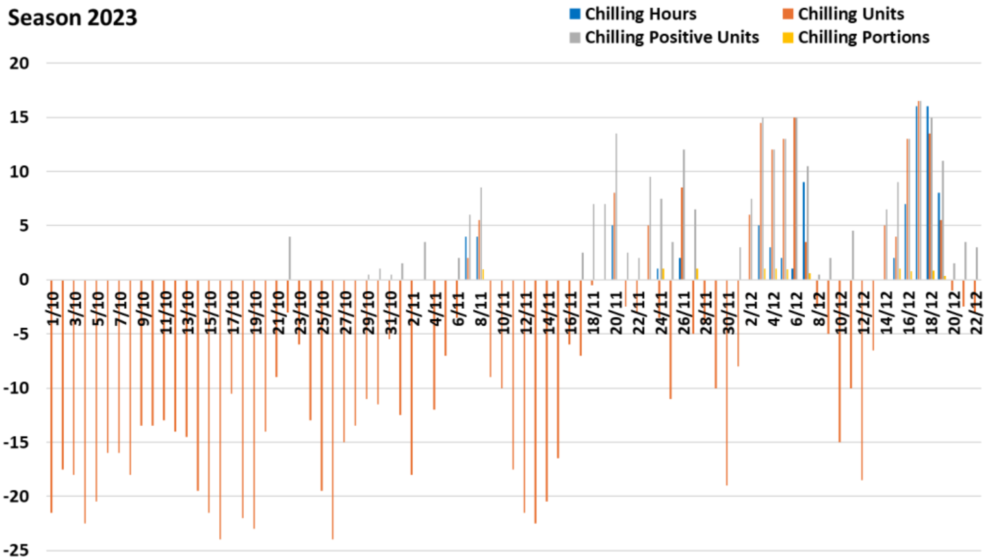


Figure S2. Daily values of chilling hours (Chilling Hours model), chilling units (Utah model), chilling P-units (Utah Positive model) and chilling portions (Dynamic model) from October 1st to the date when the accumulation of anthocyanins in fruit peel was first detected (Peel Anthoc.) (continued on the following page).



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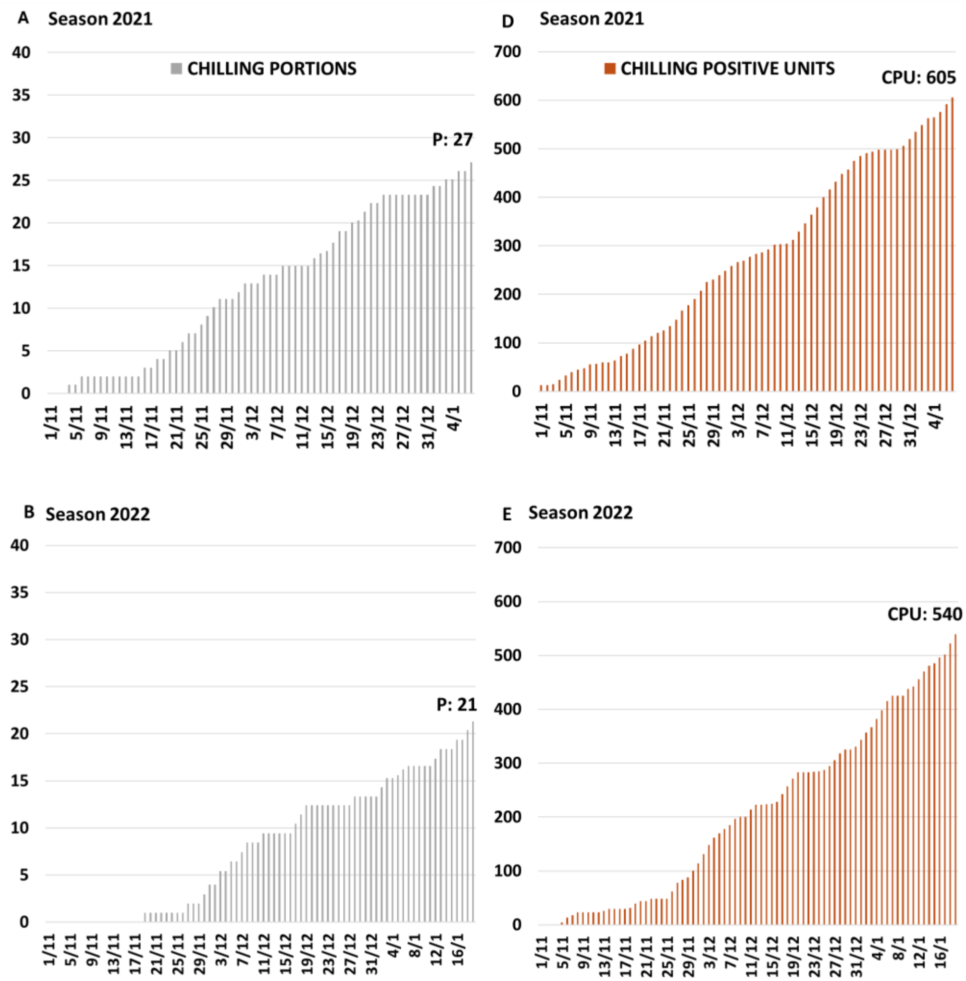

Figure S3. Accumulated Chilling Portions (A, B and C) and Chilling Positive Units (D, E, F) from November 1st to harvest time (continued on the following page).

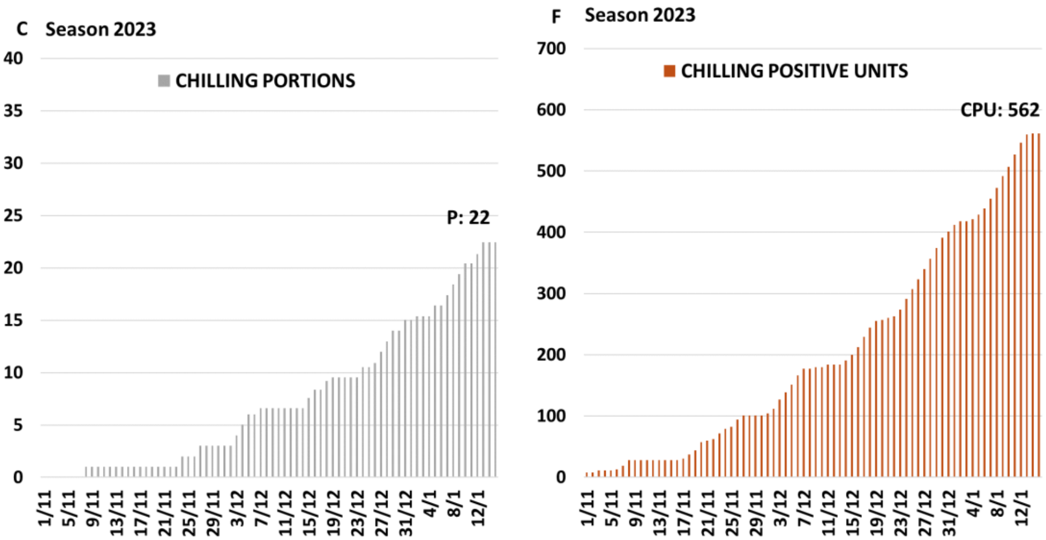

Figure S3. Accumulated Chilling Portions (A, B and C) and Chilling Positive Units (D, E, F) from November 1st to harvest time.

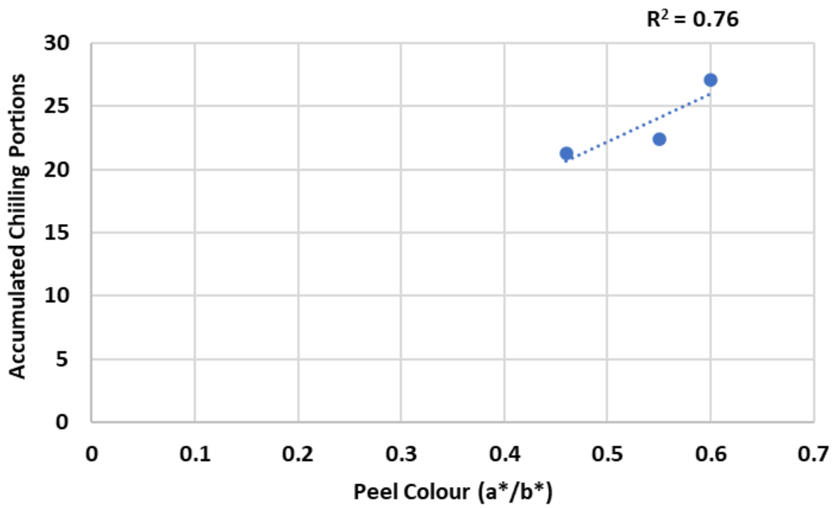

Figure S4. Correlation between accumulated chilling portions (Dynamic model) and the peel colour (a*/b*) of fruit at harvest.

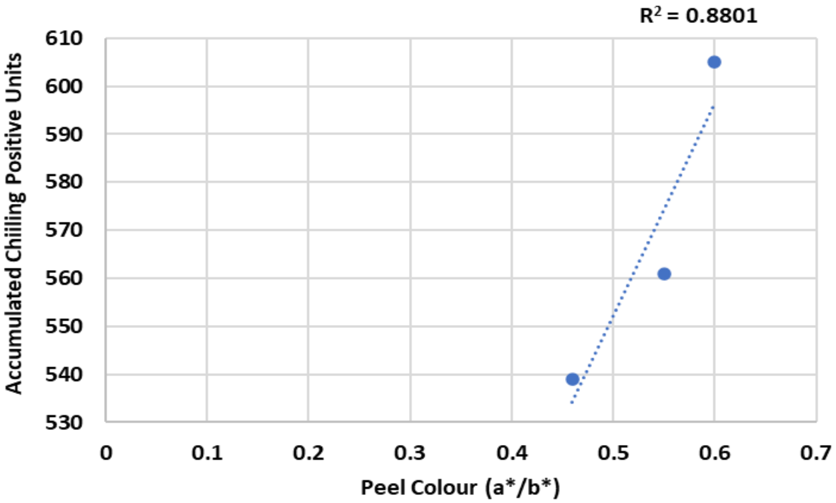

Figure S5. Correlation between accumulated chilling positive units (Positive Utah model) and the peel colour (a*/b*) of fruit at harvest.

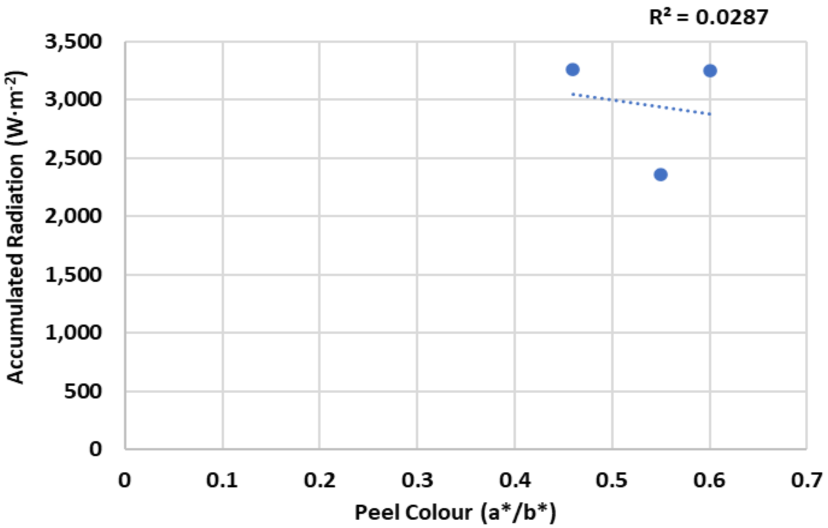

Figure S6. Correlation between the accumulated solar radiation ($W \cdot m^{-2}$) during the period from the onset of anthocyanin accumulation in the peel and the peel colour (a*/b*) of the fruit at harvest.

V. GENERAL DISCUSSION

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This thesis contributes to the understanding of the needs and preferences of citrus fruit consumers, with a particular focus on the two most important crops: mandarins and oranges. In addition, in the case of oranges, and in view of the increasing importance of blood oranges in recent years, the research not only examines what consumers are looking for in blood oranges in comparison to blond oranges, but also goes deeper into the knowledge of the processes of their physiology.

Unveiling key aspects for mandarin consumers

In **Chapter I** of this thesis, a cross-cultural study was carried out with Spanish and Italian consumers with the aim of identifying which are the characteristics that mandarins must have in order to satisfy consumers. The results of this study showed that, for all consumers and regardless of nationality, sensory attributes related to flavour such as 'sweet', 'balance sweet-acid' and 'intense flavour', and related to texture, such as 'juicy', are key to consumer acceptance. Additionally, a group of consumers also consider convenience-related attributes such as 'easy peeling', 'seedless' and 'fibrousness' to be important. Cross-cultural data revealed slight differences between nationalities, with the percentage of consumers demanding convenience-related attributes in addition to mouth-perceived properties being slightly higher in Spain than in Italy.

The crucial role that attributes related to flavour and texture play in consumer acceptance herein observed aligns with the findings from other consumer studies on mandarins (House *et al.*, 2011; Gámbaro *et al.*, 2021, Lado *et al.* 2021). Moreover, Lado *et al.* 2021 found that not all the Uruguayan consumers give the same importance to convenience-related attributes, since, as in our case, these attributes are key to acceptance for only a group of them.

The search for high organoleptic quality and convenience, as demanded by consumers, is one of the main objectives of the IVIA breeding programme, which focuses on obtaining seedless triploid hybrids of superior quality. In **Chapter II** of this thesis, four new mandarin cultivars obtained in this programme were assessed using the Ideal Product technique in combination with the CATA questions. In the sensory evaluation assay, which involved 134 consumers, these cultivars were compared with two commercial parent varieties with coinciding harvest dates. Results from this study corroborated that flavour-related attributes and fribrousness were key attributes for mandarin consumers. Two preference patterns were identified, and although the mandarin varieties preferred by the two consumer groups differed significantly, they used very similar attributes to describe their ideal mandarin. Thus, the “must-have attributes” were ‘refreshing taste’, ‘very aromatic’, ‘sweet’, ‘juicy’, ‘very intense taste’, ‘sour’ and ‘not very fibrous’. This suggests that the use of the attributes to describe mandarins is conditioned by their preferences. These findings are in line with previous studies which have shown that consumers’ use of CATA terms is significantly influenced by their preferences, particularly in the case of terms related to flavour (Ares *et al.*, 2014). Among the new cultivars evaluated in this chapter, an improvement in organoleptic quality was observed in the varieties ‘Pri-89,’ ‘Tri-707,’ and ‘Pri-90.’ These were preferred by consumers over their commercial parent varieties, 'Clemenules' and 'Oronules,' likely because they shared a greater number of organoleptic attributes with the Ideal mandarin than their parent varieties did. The Ideal Product technique, combined with CATA questions, proves to be a useful tool for identifying promising varieties in the advanced phases of the selection process, as well as for identifying parent varieties of interest for crossing.

Among the triploid hybrids developed through the IVIA breeding programme, pigmented mandarins represent a subgroup of particular interest due to their unique characteristics. In this case, beyond the mouth-perceived attributes, such as flavour and fibrousness, and convenience-related traits like seedlessness and ease of peeling, the sense of sight plays a leading role, since the distinguishing characteristic of this variety is its external and internal colouring. In **Chapter III**, the effect of flesh pigmentation intensity on the acceptability of pigmented mandarins was evaluated. As in Chapter I, a cross-cultural study was carried out between Spain and Italy. In this case, the effect of cultural background on consumer behaviour was much more marked, which was associated with the different degree of familiarity with pigmented citrus fruits. Italian consumers, who are very familiar with blood oranges, preferred fruits with slight and medium pigmented flesh, whereas Spanish consumers, with limited exposure to blood oranges, preferred those mandarins with slight pigmentation in the flesh. In line with our results, Lado et al. (2022), after studying the expectations generated among Uruguayan consumers regarding purple sweet potatoes, which were unfamiliar to them, and yellow and orange sweet potatoes, which were familiar, reported that consumers' expectations were strongly linked to familiarity and previous consumption experiences.

The effect on consumer acceptance of providing information about the antioxidant properties associated with the anthocyanin content of these pigmented varieties was also investigated. Health claims of this type are classified as credence attributes, which refer to information provided by sellers that consumers cannot directly verify post-purchase, such as production methods, environmental and social consideration or certification systems (Moser *et al.*, 2011; Fernqvist and Ekelund, 2014; Wu *et al.*, 2021). Our results showed that informing Spanish consumers about the health benefits of pigmented mandarins had a positive effect, with approximately 20% more Spanish consumers expressing an interest in purchasing one of the pigmented varieties after receiving the information. In addition, their perception of naturalness and healthiness increased, as did their acceptance of the appearance and expected liking of the mandarins. In contrast, for Italian consumers, the information provided did not have a significant impact on their purchase intention and perception of pigmented mandarins. Italian consumers showed a higher level of acceptance of pigmented mandarins regardless of whether they were previously informed about their beneficial properties or not. This is likely due to the greater familiarity of Italian consumers with pigmented citrus fruits, which increases their awareness of the health benefits associated with these mandarins. This information can be of great value for breeding programs to include flesh colour as a selection criterion and to pursue commercial strategies that highlight the health benefits of these varieties.

Understanding the needs of blood orange consumers and physiological aspects linked to anthocyanin accumulation

The citrus sector's commitment to diversifying its supply has brought blood oranges back into the spotlight. Thus, one of the objectives of this doctoral thesis was to understand how consumers perceive this type of orange in comparison to blond varieties. In order to examine this in depth, and taking into account the current liberalisation of markets, a cross-cultural study was carried out in **Chapter IV,** involving four citrus-producing countries (China, Italy, Mexico and Spain). This study corroborated the effect of cultural background on consumer attitudes towards a particular food product. A clear example of this are the different barriers to consumption found for participants from different countries. While the prize was one of the main barriers for oranges consumption in China, it was not a relevant impediment in the other three countries. In the specific case of blood oranges, in all the evaluated countries 'I prefer other fruits' was the main barrier for consumption. In Spain 13% of the people who declared not consuming have never taste these oranges. Therefore, a higher promotion should be one of first actions to take.

We concluded that blond and blood oranges can coexist in the market with high consumption rates. However, because of these cultural differences, interventions specific to each market are needed to ensure the commercial success of pigmented varieties. Despite the cultural differences, we also found patterns common to all consumers. When consumers in the four countries were asked what their main reasons for consuming blood oranges were, in all cases they chose liking the orange ("I like them a lot") and recognising their health benefits ("because of their healthy properties").

Oranges liking was undoubtedly linked to fruit juiciness and flavour. This relationship became evident when we look at the importance of sensory attributes for fruit repurchase. Thus, consumers in the four countries prioritised the juiciness ("being juicy") and flavour-related attributes ("intense flavour", "sweet", "sweet-acid balance" and "being aromatic") more than those related to the convenience of the fruit such as "being seedless", "not being messy when eating" or "being easy to peel". In terms of health properties, citrus fruits are well-known for being rich in certain bioactive compounds such as vitamin C, minerals and carotenoids (Abakpa and Adenaike, 2021).

When it comes to blood oranges, the anthocyanin content plays a key role in these two aspects that consumers regard as key, the health-related properties and flavour liking. As previously explained, anthocyanins are water-soluble pigments that belong to the flavonoid family and are known for their antioxidant properties and potential effects against several diseases (Li *et al.*, 2017). Regarding flavour, the results from **Chapter V** of this thesis corroborates the relationship between pigmentation intensity and soluble solids content, as demonstrated by Tarancón *et al.* (2022) in 'Sanguinelli'. This relationship was observed across all three varieties evaluated ('Sanguinelli', 'Moro' and 'Tarocco Rosso'). Thus, in each cultivar, the higher anthocyanin content was associated with a higher concentration of sugars, with sucrose being the one whose level increased most in the intensely pigmented fruit. These findings support the idea of a metabolic connection between sugar content and anthocyanin synthesis, a relationship previously observed at the genetic level (Solfanelli *et al.*, 2006). It has been suggested that the increase in sugars in parallel with anthocyanin accumulation may play a crucial role in providing the carbon skeletons required for anthocyanin biosynthesis (Muccilli *et al.*, 2009).

Furthermore, this chapter presents, for the first time, evidence that the composition of volatile compounds varies with the intensity of fruit pigmentation, even when the fruits are at the same stage of maturity. We observed that the intensely pigmented fruits of 'Tarocco Rosso', 'Moro', and 'Sanguinelli' had higher concentrations of certain compounds than slightly pigmented fruits. In particular, four compounds (ethyl acetate, ethyl-2-methyl butanoate, ethyl-2-butenoate and ethanol) were found in higher levels in the fruits with the higher anthocyanin concentration. This observation leads us to hypothesise that not only is there a metabolic connection between sugars and anthocyanins, but there may also be a link between the biosynthetic pathways of anthocyanins and aromatic compounds.

Although in blood orange varieties greater pigmentation implies changes in the aroma profile, our results also demonstrated that contrary to what had been previously hypothesised (Näf *et al.*, 1996; Moufida and Marzouk, 2003; Arena *et al.*, 2006), blood oranges do not have their own profile of volatile compounds that distinguishes them from blond varieties. Thus, we found that some of the blood orange varieties had an aromatic profile more similar to that of the blond varieties than to that of the other blood oranges; this was true for both slightly pigmented and intensely pigmented fruits.

Moreover, a combined analysis of the volatile compounds detected in this study and those reported in previous research by Arena *et al.* (2006) supports this conclusion. Thus, compounds previously suggested by Arena *et al.* (2006) as unique to blood oranges, namely methyl butanoate and ethyl octanoate, were present in the blond varieties we evaluated. Moreover, ethyl-2-methyl butanoate, which was only detected in the blood varieties in our study, were present in the blond varieties analysed by Arena *et al.* (2006). This finding underscores the importance of evaluating a range of varieties when investigating traits linked to a specific group, as well as considering data from prior research when analyzing and interpreting our results, allowing for a more comprehensive integration of existing knowledge.

Chapter VI examines in greater depth the metabolism of volatile compounds in oranges. We evaluated the main changes during ripening of the blood variety 'Tarocco Ippolito', which has recently attracted significant interest due to its intense internal colouration and flavour. In this chapter, it was confirmed that the volatile profile of oranges at commercial maturity stage is primarily composed of monoterpenes and esters, with alcohols, aldehydes and sesquiterpenes also contributing to their aromatic characteristics. Three evolution patterns were observed in the volatile compounds during fruit ripening: 1) compounds whose concentration decreases as fruit ripens, mainly aldehydes associated with immature odour notes; 2) compounds that remain stable in concentration during fruit ripening, among which we found some key citrus aroma compounds such as valencene and D-limonene; 3) compounds whose concentration increases as the fruit ripens, mainly monoterpenes and esters. Among the compounds following the last pattern, we identified ethyl-2-methyl butanoate, ethyl-2-butenoate and ethyl acetate. As described in Chapter V, these three compounds were associated with higher anthocyanin concentrations when comparing slightly pigmented fruit versus intensely pigmented fruit. Therefore, the association between these compounds and anthocyanin accumulation was observed not only at the commercial maturity stage (slightly *versus* intensely pigmented fruit), but also during the accumulation of anthocyanins from the onset in early stage of maturity to the final concentration at the commercial maturity stage.

Finally, **Chapter VII** addresses another key aspect of the physiology of blood oranges. It is well known that not all climatic conditions are favourable for the synthesis of anthocyanins, which can affect the viability of blood orange cultivation in certain regions. In this chapter, we investigated an aspect that had not been assessed previously, namely, the effect of thermal amplitude, chilling temperatures and solar radiation on anthocyanin biosynthesis.

Our results showed no direct association between thermal amplitude or solar radiation and anthocyanins accumulation. Chilling was identified as the primary factor that promotes the onset of anthocyanin synthesis. While the cold-induced activation of the genes responsible for anthocyanin production has been demonstrated in various post-harvest studies (Carmona *et al.*, 2020, 2022; Chen *et al.*, 2023), this is the first time that the chilling requirements for the initiation of this process while the fruit is still on the tree have been quantified. We employed a novel approach to calculate these chilling requirements, using models developed for dormancy release in plants.

From the four models evaluated (Chilling Hours, Utah, Positive Utah and the Dynamic model), only two, the Positive Utah and Dynamic models, showed a consistent pattern of chilling requirements under our agroclimatic conditions: the onset of anthocyanin accumulation in the flesh was associated with 4-5 chilling portions and ≈ 128 chilling P-units, while the initiation of the process in the rind was established with ≈ 10 chilling portions and ≈ 240 chilling P-units. Our findings provide a basis for applying this approach to other anthocyanin-producing fruits, such as grapes.

In addition to stablishing the chilling requirements for the onset of anthocyanin synthesis, this chapter also highlights that a greater accumulation of cold results in a more intense red colouration of the rind at harvest. A recent study by Serna-Escolano *et al.* (2024) found that fruits located at the top of the canopy exhibited more intense external colouration compared to those at the lower part of the canopy. The authors hypothesised that this might be due to lower temperatures at the top of the canopy, which is in line with our findings.

As a whole, all the information generated in this thesis represents a significant advance in the understanding of citrus consumer profiles and the physiology of blood oranges. Beyond its scientific importance, it can be translated into practical applications and have positive implications for the sector, in terms of increased fruit quality and competitiveness in the markets.

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VI. CONCLUSIONS

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1. Two different types of mandarin consumers were identified in Spain and Italy: “Consumers focused on in-mouth perceptions” and "multisensory consumers". Attributes such as ‘sweet’, ‘balance sweet-acid’, ‘intense flavour’, ‘juicy’ and 'not fibrous' were found to be crucial to the repurchase intention of both groups. However, ‘pulp colour’ and attributes mainly related to convenience such as ‘easy peeling’ and ‘seedless’ were more important for the “multisensory” group. The proportion of “multisensory consumers” was higher in Spain (59%) than in Italy (48%).

2. The use of CATA (Check-All-That-Apply) questions in combination with the description of the ideal product is an effective tool for selecting new mandarin varieties from breeding programmes. Two groups of consumers with different preference patterns were identified. The new varieties ‘Pri-89’ and ‘Tri-707’ were the preferred choices for one profile of consumers, implying an improvement over ‘Clemenules’. ‘Pri-90’ is an appropriate variety for those consumers sensitive to bitterness and who like mandarins with a certain level of sourness, indicating a quality improvement in relation to ‘Oronules’.

3. The flesh colour intensity of pigmented mandarins has a clear impact on consumer acceptance. Italian consumers were generally more receptive to these fruits, probably due to their familiarity with blood oranges, and showed a preference for mandarins with slight and medium pigmentation intensity. Spanish consumers preferred slightly pigmented mandarins. Intensely pigmented mandarins did not appeal to consumers of either nationality. Providing information on the health benefits of these mandarins had a positive effect on the acceptance of Spanish consumers, whereas it did not have a significant impact among Italians.

4. Blood and blond oranges can co-exist in markets with high consumption levels, but this requires tailored marketing strategies for each country since there is an impact of cultural background on consumption barriers and contexts. Liking and healthiness perception are the main drivers of consumption irrespective of the culture.

5. Blood oranges do not have a distinctive volatile profile that distinguishes them from blond varieties, as some blood varieties have an aromatic profile that is more similar to that of blond varieties than to other blood oranges.

6. In the three evaluated varieties, 'Moro', 'Tarocco Rosso' and 'Sanguinelli', the intensely pigmented samples contained higher levels of anthocyanins and sugars compared to their slightly pigmented counterparts. Additionally, the intensely pigmented samples had higher concentrations of ethanol, ethyl acetate, ethyl-2-methyl butanoate and ethyl-2-butenoate. These findings suggest that there is not only a metabolic relationship between sugars and anthocyanins, but there may also be an interplay between the biosynthetic pathways of anthocyanins and aromatic compounds.

7. The ripening of the blood orange 'Tarocco Ippolito' was associated with a decrease in aldehydes and an increase in monoterpenes and esters. In addition, a group of compounds remained constant throughout the ripening process, including key citrus aroma compounds such as valencene and D-limonene.

8. Chilling was identified as the key environmental factor driving anthocyanin synthesis in blood oranges, with specific chilling requirements necessary to initiate this process. Anthocyanin synthesis in the flesh and rind of blood oranges does not occur simultaneously, as it takes longer to begin in the rind. The onset of anthocyanin accumulation in the flesh of 'Tarocco Ippolito' was associated with 4-5 chilling portions (dynamic model) and approximately 128 chilling P-units (positive Utah model), while the initiation of the process in the rind was associated with ≈ 10 chilling portions and ≈ 240 chilling P-units.