Departamento de Química Analítica

Facultad de Ciencias

Universidad de Cádiz

Aplicación de herramientas metabolómicas para el estudio del carácter pungente en pimientos

María de las Mercedes Vázquez Espinosa

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MEMORIA presentada por la graduada Dña. María de las Mercedes Vázquez Espinosa para optar al Grado de Doctora dentro del Programa de Doctorado en Recursos Agroalimentarios por la Universidad de Cádiz.

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En la vida todo llega,

todo pasa, todo cambia

Raquel Aldana

Índice

1. Resumen

En las últimas décadas, el vínculo entre nutrición y salud ha ganado aceptación universal y, por tanto, se ha dado una importancia creciente a los regímenes dietéticos basados en vegetales ricos en antioxidantes. El pimiento es uno de los cultivos de hortalizas de mayor valor a nivel mundial debido a sus propiedades organolépticas, su riqueza en compuestos bioactivos y su fuerte capacidad antioxidante. Su consumo ha aumentado mucho, pero actualmente la sociedad no solo demanda una mayor producción del cultivo tradicional sino versiones mejoradas que contengan compuestos beneficiosos para la salud.

El consumo de pimientos picantes se asocia generalmente con sensaciones picantes, ardientes o punzantes. La pungencia de estos frutos se debe a dos grupos de compuestos químicos conocidos como capsaicinoides y capsinoides. Estos compuestos han exhibido una gran cantidad de propiedades biológicas de relevancia farmacológica, como antioxidantes, antiinflamatorios, analgésicos, antimicrobianos, anticancerígenos o tratamientos antiobesidad. Sin embargo, los capsinoides se caracterizan por tener un nivel de picor significativamente inferior, lo que hace que su uso en aplicaciones culinarias, concentrados e industria farmacéutica tenga una mayor aplicabilidad ya que no se vería limitado por las características pungentes.

El análisis en detalle de estos compuestos se ha visto a menudo desfavorecido tanto por problemas inherentes a los cultivos, como por la ausencia de metodologías asequibles y con el suficiente grado de optimización. Por este motivo, es necesario desarrollar y optimizar técnicas de separación y extracción, que permitan estudiar la composición química de distintas variedades de pimiento e indiquen aquellas que son ricas en los compuestos de interés para obtener extractos de mayor calidad. De esta manera, dichos métodos serían de gran valor para los laboratorios analíticos, así como para empresas o industrias, ya que permiten evaluar la calidad de diferentes variedades y conseguir una mejor selección del producto final gracias a su cuantificación analítica.

En relación a la separación y cuantificación de compuestos bioactivos, las técnicas más utilizadas con diferencia son las de cromatografía líquida de alta eficacia (HPLC) y más recientemente la cromatografía líquida de ultra-alta eficacia (UHPLC).

En cuanto al proceso de extracción de los compuestos químicos de la matriz vegetal, se ha producido recientemente una creciente demanda de técnicas que acorten los tiempos de análisis, reduzcan el consumo de disolventes orgánicos y tengan una mayor eficiencia y rendimiento. Las técnicas verdes nacen para responder a los retos del siglo XXI de proteger tanto al medio ambiente como a los consumidores. Entre estas técnicas se encuentra la extracción asistida por ultrasonidos (UAE) y la extracción asistida por microondas (MAE).

El interés en el estudio de la acumulación de compuestos antioxidantes durante la maduración de la planta está aumentando porque su contenido se considera un parámetro importante con respecto a la calidad de las frutas y verduras. Los pimientos se consumen y cosechan en diferentes etapas de maduración, desde verde inmaduro hasta completamente maduro. A lo largo de la maduración, se producen numerosos cambios bioquímicos, fisiológicos y estructurales. Estas variaciones no solo tienen implicaciones agronómicas bien conocidas (sabor, color, aroma, tamaño), sino que también son relevantes para determinar su uso y calidad.

En la presente Tesis Doctoral se ha llevado a cabo el desarrollo y validación de un método UHPLC, que permita la separación y determinación simultánea de los cinco capsaicinoides mayoritarios (n-DHC, C, DHC, h-C, h-DHC) y los dos capsinoides principales (CTO y DHCTO) en pimientos. Se ha conseguido la separación de todos los compuestos de interés con buena resolución en un tiempo inferior a 2 minutos.

A continuación, se ha optimizado la extracción de los compuestos de interés del pimiento mediante ultrasonidos y microondas. Para evaluar el efecto de las variables independientes sobre la eficacia de extracción, se ha utilizado un diseño estadístico de mezcla y un diseño de experimentos de Box Behnken (BBD) con el fin de determinar las condiciones óptimas, que fueron 5 minutos de tiempo de extracción a una temperatura de 5,5 ºC, pH 8 y empleando un ratio de 0,2:14,5 g:mL para UAE; y 5 minutos de tiempo de extracción a una temperatura de 60 ºC, pH 8 y un ratio de 0,2:15 g:mL para MAE. Los métodos han demostrado ser precisos, reproducibles, económicos y rápidos para obtener altas recuperaciones de los compuestos de interés.

Finalmente, se han estudiado los cambios en la concentración de capsaicinoides y capsinoides a lo largo de la maduración en distintas variedades de pimiento. Se pretende determinar cuál es el momento óptimo de recolección, y si la tendencia es la misma en capsaicinoides y capsinoides, así como en distintas variedades sembradas bajo las mismas condiciones de cultivo, riego, temperatura, humedad y fertilización.

- A diferencia de lo reportado en la bibliografía, en la variedad Naga Jolokia, la máxima concentración de capsaicinoides se alcanza el día 33 de maduración y luego cae drásticamente con una reducción superior al 96%. En el caso del capsiato, alcanza su máxima cantidad el día 19, seguido de una disminución gradual hasta el final de la maduración.

- En el caso de las variedades Habanero, Habanero Roxo y Malagueta, la acumulación de capsiato se incrementa hasta el día 27 de maduración, donde alcanza el mayor valor y luego disminuye drásticamente hasta el final del proceso. Para Bode, el capsiato aumenta desde el inicio hasta el final de la maduración el día 76. Al compararlo con la evolución de capsaicinoides, ya realizado previamente en el grupo de investigación, se puede observar que, en Habanero y Bode, ambas familias de compuestos siguen la misma tendencia; mientras que en Habanero Roxo y Malagueta no.

- Al centrarnos en la variedad ornamental Filius, se ha demostrado la posibilidad de comer frutos de pimiento con similares sensaciones picantes y diferente color y/o forma; así como frutos con el mismo color pero que presentan distinto grado de pungencia.

2. Summary

In recent decades, the link between nutrition and health has gained universal acceptance, and therefore, increasing importance has been given to plant-based diets rich in antioxidants. The pepper is one of the most valuable vegetable crops in the world due to its organoleptic properties, its richness in bioactive compounds and its strong antioxidant capacity. Its consumption has greatly increased, but currently society not only demands a greater production of the traditional crop but also improved versions that contain beneficial compounds for health.

The consumption of spicy peppers (chili peppers) is generally associated with hot, burning or stinging sensations. The pungency of these fruits is due to two groups of chemical compounds known as capsaicinoids and capsinoids. These compounds have exhibited a large number of biological properties of pharmacological relevance, such as antioxidant, anti-inflammatory, analgesic, antimicrobial, anticancer, or antiobesity treatments. However, capsinoids have a much lower level of pungency, which makes them have a greater applicability in culinary applications, concentrates and pharmaceutical industry, since it would not be limited by pungent characteristics.

The detailed analysis of these compounds has often been disadvantaged both by inherent problems to the crops, and by the absence of affordable methodologies with a sufficient degree of optimization. For this reason, it is necessary to develop and optimize separation and extraction techniques that allow studying the chemical composition of different varieties of pepper and indicate those that are rich in the compounds of interest to obtain higher quality extracts. In this way, these methods would be of great value for analytical laboratories, as well as for companies or industries, since they allow evaluating the quality of different varieties and achieving a better selection of the final product thanks to its analytical quantification.

In relation to the separation and quantification of bioactive compounds, by far the most widely used technique are high-performance liquid chromatography (HPLC) and, more recently, ultra-high-performance liquid chromatography (UHPLC).

Regarding the extraction process of the chemical compounds from the plant matrix, there has recently been a growing demand for techniques that shorten analysis times, reduce the consumption of organic solvents and have greater efficiency and performance. Green techniques are born to respond to the challenges of the 21st century to protect both the environment and consumers. These techniques include ultrasound assisted extraction (UAE) and microwave assisted extraction (MAE).

The interest in the study of the antioxidant compounds accumulation during the plant maturation is increasing because its content is considered an important parameter with respect to the quality of fruits and vegetables. Peppers are eaten and harvested at different stages of ripeness, from unripe green to fully ripe. Throughout the maturation, numerous biochemical, physiological, and structural changes occur. These variations not only have well-known agronomic implications (flavor, color, aroma, size, post-harvest sensory properties), but are also relevant in determining their use and quality.

In this Doctoral Thesis, the development and validation of a UHPLC method has been carried out, which allows the simultaneous separation and determination of the five major capsaicinoids (n-DHC, C, DHC, hC, h-DHC) and the two main capsinoids (CTE and DHCTE) in peppers. The separation of all the compounds of interest with good resolution has been achieved in less than 2 minutes.

Then, the extraction of the compounds of interest from the pepper has been optimized by means of ultrasounds and microwaves. To evaluate the effect of the independent variables on the extraction efficiency, a statistical mixture design and a Box Behnken design of experiments (BBD) are used in order to determine the optimal conditions. The optimal conditions for UAE were 5 minutes of extraction time at 5.5 ºC of temperature, pH 8 and using a ratio of 0.2:14.5 g:mL; and for MAE they were 5 minutes of extraction time at 60 ºC of temperature, pH 8 and a ratio of 0.2:15 g:mL. The methods have proven to be accurate, reproducible, inexpensive and fast to obtain high recoveries of the compounds of interest.

Finally, changes in the capsaicinoids and capsinoids concentration throughout maturation in different varieties of pepper has been studied. It is intended to determine which is the optimal harvesting moment, and if the trend is the same in capsaicinoids and capsinoids, as well as in different varieties sown under the same cultivation conditions, irrigation, temperature, humidity and fertilization.

- Unlike what is reported in the literature, in the Naga Jolokia variety, the maximum concentration of capsaicinoids is reached on 33 dpa (days post-anthesis) and then it falls drastically with a reduction of more than 96%. In the case of capsiate, it reaches its maximum amount on 19 dpa, followed by a gradual decrease until the end of maturation.

- In the case of Habanero, Habanero Roxo and Malagueta varieties, the accumulation of capsiate increases until 27 dpa, where it reaches the highest value and then decreases drastically until the end of the process. For Bode, the capsiate increases from the beginning to the end of maturation on 76 dpa. When compared with the evolution of capsaicinoids, already carried out in our research group, it can be observed that, in Habanero and Bode, both families of compounds follow the same trend; while in Habanero Roxo and Malagueta not.

- By focusing on the ornamental variety Filius, the possibility of eating pepper fruits with similar spicy sensations and different color or shape has been demonstrated; as well as fruits with the same color but with a different degree of pungency.

3. Introducción

3.1. PIMIENTOS

3.1.1. Características generales

El pimiento es originario de las zonas tropicales y húmedas de América Central y del Sur. Cristóbal Colón lo introdujo en España en 1493 tras su primer viaje, procedente de Haití. Respecto a su clasificación científica, son los frutos de las plantas del género *Capsicum* y pertenecen a la familia Solanaceae (al igual que el tomate, la patata o la berenjena). El género *Capsicum* incluye 35 especies descritas, pero solo cinco son cultivadas: *C. chinense* Jacq., *C. frutescens* L., *C. annuum* L., *C. baccatum* L. y *C. pubescens* Ruiz & Pav [1].

Los pimientos tienen una amplia diversidad genética y comprenden una cantidad sustancial de variedades que difieren en tamaño (grueso o delgado), color (verde, morado, amarillo, chocolate, naranja o rojo, según la variedad de pimiento y la etapa de maduración), sabor (desde las variedades no picantes hasta las especies más picantes) o forma (redonda, alargada, ancha, estrecha, así como formas especiales como campanillas). Esta versatilidad es la razón de su notable potencial para su uso en preparaciones culinarias de la industria agroalimentaria y se encuentra entre los productos más valorados y comúnmente cultivados [2]. Tradicionalmente, se utilizaban como colorantes naturales y sus principales beneficios provenían de sus características organolépticas como una forma de eliminar la insipidez y de añadir sabor y aroma a muchos alimentos. Hoy en día, no solo se utiliza como colorante y aromatizante en salsas, sopas, carnes procesadas, bocadillos, dulces o refrescos, sino que también se pueden consumir frescos en ensaladas, fritos, hervidos, deshidratados como condimento o incluso como salsa o mermelada. Además, la oleorresina extraída de sus frutos se utiliza como ingrediente en numerosos productos comerciales como repelentes de insectos o incluso aerosoles de autodefensa [3].

Los pimientos no sólo son valorados por sus atributos organolépticos, sino también tienen un papel importante en aplicaciones médicas y farmacéuticas gracias a sus excelentes efectos beneficiosos sobre la salud humana por su alto contenido en compuestos bioactivos como capsaicinoides, capsinoides, carotenoides, compuestos fenólicos, vitaminas, minerales, ácido ascórbico, sustancias antioxidantes, etc [4].

Este hecho los convierte en uno de los productos hortofrutícolas más valorados en todo el mundo, y actualmente se comercializan a nivel internacional. Se encuentra entre las verduras frescas más populares en todo el mundo debido a su combinación de aroma, color, sabor y valor nutricional. Los pimientos son un cultivo económicamente notable, batiendo récords y alcanzando hasta 3,8 millones de hectáreas de tierra de cultivo y una producción de 40,7 mil millones de kg en 2017, ocupando el sexto lugar entre todos los productos hortícolas en términos de producción mundial [5].

Hay que destacar también los llamados "pimientos o guindillas ornamentales", es decir, aquellos que se cultivan por su belleza y generalmente se utilizan en jardinería, decoración de interiores y paisajismo. Normalmente son de color rojo o naranja, pero también existen variedades blancas, amarillas, moradas o verdes; y deben presentar unas características morfológicas que apoyen su valor estético, como frutos pequeños, erectos y coloridos, follaje vivo, facilidad de crecimiento, larga durabilidad y posibilidad de cultivarse en macetas pequeñas [6]. El interés en estas nuevas variedades de pimiento ha aumentado en todo el mundo hasta convertirse en una importante fuente de financiación para los productores [7].

3.1.2. Variabilidad genética del pimiento

Las especies de *Capsicum* cultivadas presentan una amplia diversidad genética generada por la evolución, la selección natural y humana en múltiples ambientes y contextos culturales, así como por la domesticación en diferentes centros y la selección artificial en distintos ambientes agrícolas [8]. La conservación y la utilización sostenible de los recursos genéticos son claves para la mejora continua de los pimientos con el fin de responder al cambio climático y a la creciente demanda mundial de alimentos [9].

La alta biodiversidad de pimiento representa un recurso único que podría utilizarse en programas de mejoramiento para desarrollar nuevas variedades con características agronómicas deseables e identificar formas con mayor resistencia a una serie de factores bióticos y abióticos [10]. En este contexto, las variedades autóctonas bien adaptadas a los entornos agrícolas que han sido cultivadas durante mucho tiempo, representan valiosos reservorios de diversidad genética. Además, se asocian comúnmente con mejores características sensoriales, beneficios saludables y propiedades nutricionales [11].

Las múltiples variedades de pimiento se diferencian por la forma, el tamaño, el grosor de la pulpa (pericarpio), el hábito de crecimiento, las semillas o el color de las flores y frutos. Desde el punto de vista culinario y gastronómico, los frutos de pimiento se clasifican principalmente en dulces y picantes en función de la ausencia o presencia de capsaicinoides. De acuerdo con el nivel de picante, se clasifican en la llamada escala de calor Scoville, que asigna de manera organoléptica y subjetiva una puntuación de picante a cada variedad [12]. Un ejemplo del valor de acritud de las variedades más comunes en todo el rango de picor se muestra en la Figura 1.

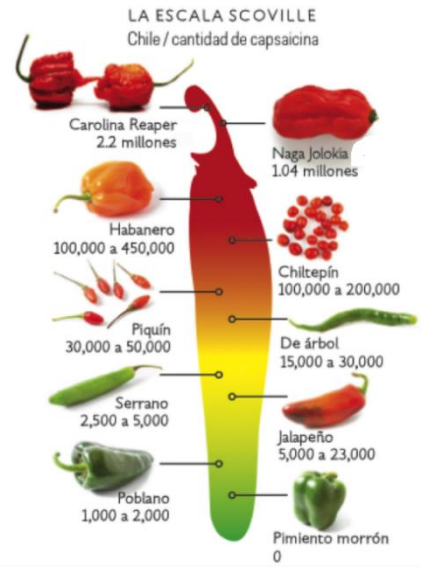


Figura 1: *Representación de la escala Scoville para distintas variedades de pimiento* [13].

3.1.3. Compuestos presentes en los pimientos

Los pimientos son una fuente importante de nutrientes en la dieta humana y contienen una amplia variedad de fitoquímicos, muchos de los cuales presentan unas marcadas propiedades antioxidantes e importantes efectos biológicos [14]. Hay que tener en cuenta que el pimiento posee también un porcentaje muy elevado de agua (aproximadamente un 90%). Los niveles de estos compuestos pueden variar dependiendo del genotipo, del estado de madurez o de las condiciones de crecimiento.

Entre todos ellos, se pueden destacar los siguientes grupos de compuestos:

- **Capsaicinoides**: Responsables de su sabor picante, entre los que destacan la capsaicina y dihidrocapsaicina [15].
- **Capsinoides**, destacando el capsiato y dihidrocapsiato [16].
- **Carotenoides**: Responsables del color rojo intenso de los pimientos, siendo mayoritarios la capsantina, la capsorrubina y la capsantina 5,6-epóxido. Algunos, como el β-caroteno y la β-criptoxantina pueden ser transformados en vitamina A [17].
- **Vitaminas, principalmente C, A y E (tocoferol)**, constituyéndose en uno de los alimentos desintoxicantes más importantes. Si lo comparamos con los cítricos (naranjas, limones, pomelos, etc.), el pimiento nos proporciona más del doble de vitamina C [18].
- **Compuestos fenólicos**: Dentro de esta familia podemos destacar los ácidos fenólicos (cafeico, ferúlico, clorogénico, gálico, cumárico, etc.) y los flavonoides (quercetina, kaempferol, rutina, luteolina, etc.) [19,20].
- **Minerales**, conteniendo principalmente fósforo, potasio, magnesio, hierro y calcio, con cantidades muy bajas de sodio [21].
- **Lípidos** [22].
- **Compuestos volátiles**, como aldehídos, cetonas, alcoholes, pirazinas, etc. [23].

3.2. CAPSAICINOIDES

3.2.1. Características generales

Los capsaicinoides son los compuestos químicos responsables del sabor picante de los pimientos, los cuales son amidas ácidas formadas a partir de ácidos grasos de cadena ramificada C9-C11 y vanillilamina [24]. Su estructura presenta tres secciones claramente diferenciadas, el grupo vanillilo, el grupo carboxamida y la cadena alifática. Los mayoritarios son la capsaicina (C) (*trans*-8-metil-*N*-vanillil-6-nonenamida) y la dihidrocapsaicina (DHC) (8-metil-*N*-vanillilnonanamida), que generalmente representan entre el 77% y 98% del contenido total de capsaicinoides. Algunos otros compuestos relacionados, como nordihidrocapsaicina (n-DHC), homocapsaicina I y II (h-C) u homodihidrocapsaicina I y II (h-DHC), también están presentes en cantidades menores entre los más de 20 compuestos reportados [25,26].

El esqueleto base de los capsaicinoides tiene la siguiente estructura, mostrada en la Figura 2:

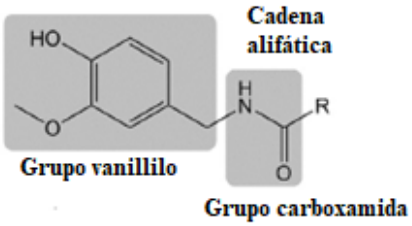
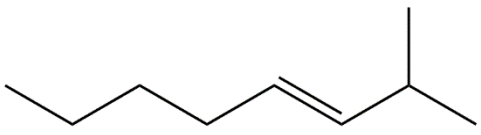
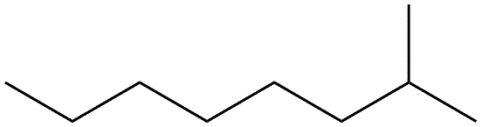
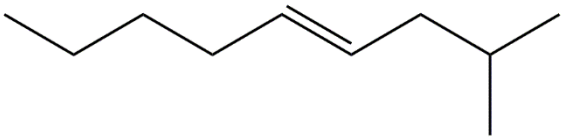
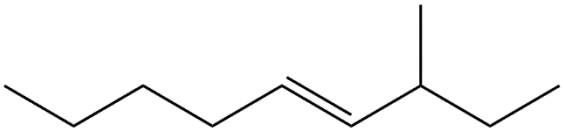
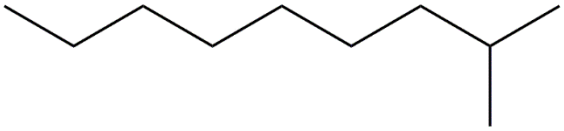
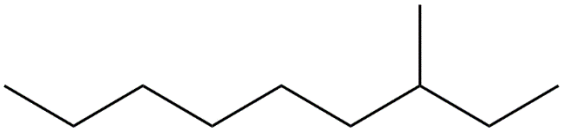
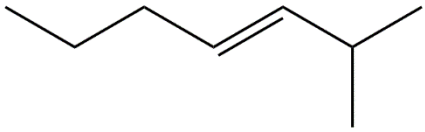
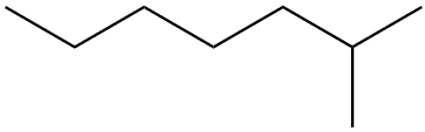
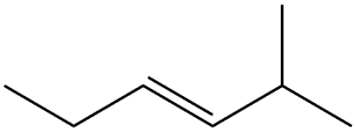
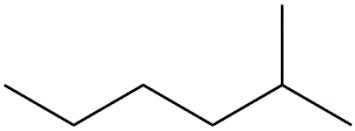


Figura 2: *Estructura general de los capsaicinoides.*

Dependiendo del número de carbonos de la cadena lateral (R) y de los diferentes grados de insaturación, se pueden encontrar los distintos capsaicinoides naturales existentes [27], representando en la Tabla 1 las cadenas alifáticas de los capsaicinoides más abundantes en la naturaleza:

R =	NOMBRE
	Capsaicina

	Dihidrocapsaicina
	Homocapsaicina I
	Homocapsaicina II
	Homodihidrocapsaicina I
	Homodihidrocapsaicina II
	Norcapsaicina
	Nordihidrocapsaicina
	Nornorcapsaicina
	Nornordihidrocapsaicina
	Nonivamida

Solo las frutas del género *Capsicum* sintetizan estos compuestos en la naturaleza. Los capsaicinoides se biosintetizan naturalmente en la placenta del pimiento mediante la condensación de vanillilamina con un ácido graso de cadena ramificada, catalizado por la enzima capsaicina sintasa [28]. La fracción de vanillilamina se sintetiza a partir de fenilalanina mediante la vía de los fenilpropanoides y la parte procedente del ácido graso de cadena ramificada proviene de los aminoácidos de valina o leucina [24]. Una vez sintetizados se acumulan en las vacuolas de las células epidérmicas placentarias hasta que se metabolizan [15]. Además de en la placenta, se han encontrado capsaicinoides en otras partes del fruto, como en el pericarpio o las semillas, e incluso en órganos vegetativos de la planta como las hojas y el tallo, pero siempre en menor cantidad. Esto puede deberse a la adherencia de una pequeña cantidad de los capsaicinoides procedentes de la placenta o a la difusión de los mismos a través de las paredes celulares de la capa epidérmica de la placenta [29].

Los contenidos relativos de cada uno de los capsaicinoides, así como sus concentraciones totales, pueden variar dentro de límites bastante amplios en las distintas variedades de pimientos. Esta variación de concentración depende de las condiciones nutricionales y de cultivo, del estado de desarrollo del fruto, del propio genotipo y de su interacción con el medio ambiente [30]. Además, se sabe que el contenido en capsaicinoides de los pimientos puede variar entre distintos frutos dentro de la misma planta, recolectados al mismo tiempo después de la floración [31].

3.2.2. Efectos biológicos de los capsaicinoides

Los pimientos además de utilizarse como aditivo alimentario y principio aromatizante en multitud de productos, tienen importantes acciones farmacológicas. Recientemente, se han investigado una serie de propiedades biológicas y efectos beneficiosos para la salud gracias al consumo de pimientos picantes [32]. Sin embargo, son compuestos altamente irritantes. La exposición a altas dosis o a largo plazo tiene un efecto perjudicial sobre la mucosa gástrica y, en última instancia, sobre la salud. Sin embargo, a dosis moderadas estimula las secreciones gástricas, lo que puede ayudar a favorecer la digestión y genera protección en el estómago y la mucosa gástrica [33].

Los estudios epidemiológicos han revelado que el consumo de chiles en grandes cantidades provoca un mayor riesgo de cáncer gástrico. Además, algunos metabolitos de la capsaicina pueden atacar el ADN y desencadenar mutagenicidad y transformación maligna [33]. Sus principales efectos biológicos son:

Antioxidantes

Debido a la exposición a la radicación y otras tensiones oxidativas, se generan especies reactivas de oxígeno (ROS) en los organismos vivos, como superóxido, peróxido de hidrógeno, radical hidroxilo y oxígeno singlete, implicados en enfermedades degenerativas como cáncer, inflamación, aterosclerosis y envejecimiento, así como el deterioro de los alimentos. Los antioxidantes se clasifican como captadores de radicales libres, son compuestos que inhiben o retrasan la oxidación de otras moléculas [34].

El pimiento es un cultivo agrícola importante, no solo por su importancia económica, sino también por el valor nutricional de sus frutos, principalmente porque son una excelente fuente de colorantes naturales y compuestos antioxidantes. Una gran variedad de antioxidantes está presente en los frutos del pimiento como vitamina C o ácido ascórbico, ácidos fenólicos, flavonoides, carotenoides, tocoferoles, capsaicinoides y capsinoides [35].

Se ha demostrado que los capsaicinoides inhiben la peroxidación lipídica catalizada por cationes hierro y la oxidación de las lipoproteínas de baja densidad (LDL) producidas por cationes cobre. La capsaicina y la dihidrocapsaicina fueron capaces de aumentar la resistencia de las LDL a la oxidación al retrasar el inicio de la oxidación y/o ralentizar la velocidad de oxidación. Esta acción estuvo estrechamente relacionada con la capacidad de estos compuestos para formar complejos con estos metales reducidos o su actividad como donadores de hidrógeno [36,37]. La capsaicina también puede prevenir la formación de hidroperóxidos lipídicos provenientes de la auto-oxidación del ácido linoleico [38]. Además, es capaz de prevenir la oxidación del ácido oleico a altas temperaturas [39].

Anti-inflamatorios

Se ha demostrado que la inhalación de capsaicina interfiere en las respuestas neuronales implicadas en la inflamación de los pulmones de las ratas y, tal interferencia, modula la inmunidad a los antígenos inhalados. Además, la capsaicina inhibió el desarrollo de la inflamación inducida por carragenina y la artritis inducida por adyuvantes en ratas, así como la inflamación inducida por etanol [40]. Se sugirió que la liberación de mediadores proinflamatorios, eicosanoides y ezimas hidrolíticas, está asociada con la propiedad antiinflamatoria de la capsaicina [41].

El receptor vainilloide tipo 1 (TRPV1) de capsaicina, está altamente expresado por neuronas nociceptivas. La capsaicina se une a TRPV1 en las neuronas sensoriales para transmitir la sensación de dolor. Además, la capsaicina es inmunológicamente activa para generar más células productoras de anticuerpos y tiene capacidad para modular la proliferación de linfocitos y la producción de inmunoglobulinas [42].

Anticancerígenos o antimutagénicos

La actividad anticancerosa y el potencial mutagénico de los capsaicinoides y los pimientos picantes se ha informado en numerosas investigaciones desde hace mucho tiempo. Se sabe que la proliferación celular juega un papel importante en la carcinogénesis en múltiples etapas y es un sello distintivo fundamental para la prevención del cáncer. Los efectos anticancerígenos y quimiopreventivos de los capsaicinoides están estrechamente relacionados con su capacidad para prevenir la proliferación y migración celular e inducir la apoptosis celular. Tienen la capacidad de reprimir el crecimiento de varias líneas celulares inmortalizadas o malignas mediante la inducción de detención del ciclo, apoptosis, autofagia y/o mediante la inhibición de la actividad metabólica celular [43,44]. También se sugiere que ejercen un efecto quimiopreventivo a través de la modulación del metabolismo de muchos compuestos cancerígenos y mutagénicos y sus interacciones con el ADN.

Por ejemplo, se ha descubierto que la capsaicina y dihidrocapsaicina pueden inhibir una isoforma del citocromo P450, una enzima involucrada en la activación metabólica y la desintoxicación de muchos carcinógenos de bajo peso molecular; o protegen la mutagénesis bacteriana producida por la aflatoxina B_1 [45].

Sin embargo, existe evidencia de que tanto la capsaicina como los extractos de pimientos picantes pueden actuar como cocarcinógenicos o promotores de tumores [46]. Varios estudios epidemiológicos han revelado que los consumidores de pimientos picantes en grandes cantidades tienen un mayor riesgo de padecer cáncer gástrico o úlceras que los no consumidores. Además, los metabolitos de la capsaicina pueden atacar el ADN y desencadenar la mutagenicidad y transformación maligna [47]. Por lo tanto, la capsaicina es un "arma de doble filo" y posee un doble efecto.

Antimicrobianos

Las especies de *Bacillus* son microbios comunes que se encuentran en la mayoría de los entornos naturales (suelo, agua o tejidos vegetales y animales). Tanto *B. cereus* como *B. subtilis* actúan como invasor primario o agente infeccioso secundario en una serie de enfermedades y han sido implicados en algunos casos de intoxicación alimentaria [48]. Las especies de *Clostridium* son organismos anaerobios que se encuentran como células vegetativas o esporas en el suelo, las aguas residuales, los sedimentos acuáticos, los intestinos de los animales y la materia vegetal y animal en descomposición. Pueden causar una serie de enfermedades mortales, como botulismo, gangrena o tétano [49]. Se ha demostrado que los extractos obtenidos a partir de pimientos picantes inhibían el desarrollo de estos patógenos (*Bacillus cereus, Bacillus subtilis, Clostridium sporogenes y Clostridium tetani)* [50].

También se ha demostrado que extractos de capsaicinoides presentan propiedades antibacterianas frente a *Salmonella typhimurium* y *Pseudomonas aeruginosa* [51]. Finalmente, Molina-Torres y colaboradores encontraron que la capsaicina inhibía el crecimiento y desarrollo de *Escherichia coli* y *Pseudomonas solanacearum* [52].

Analgésicos o tópicos contra el dolor

Se ha demostrado que la capsaicina es utilizada terapéuticamente por vía oral o local para reducir el calor inflamatorio y la hiperalgesia química nociva o para reducir ciertos dolores como la artritis reumatoide o la fibromialgia, neuropatías diabéticas y osteoartritis, entre otros [53,54]. Como analgésico, la capsaicina o la oleorresina proveniente de los pimientos picantes se agrega a varias cremas tópicas en una concentración de 0.075% o menos y numerosas pruebas clínicas han revelado que mejoran significativamente el dolor en pacientes con artritis [55].

Recientemente, se está comprendiendo que los mecanismos responsables del efecto de la capsaicina en el alivio del dolor se deben al receptor vanilloide TRPV1. Se trata de un canal catiónico no selectivo activado por una amplia gama de estímulos como calor nocivo, protones y vanilloides. El importante papel de TRPV1 en la sensación de dolor fue validado porque, I) sus agonistas provocan la desensibilización de los canales de TRPV1 que alivia los comportamientos del dolor en especies preclínicas, y II) sus antagonistas alivian los comportamientos de dolor en modelos de roedores de inflamación, osteoartritis y cáncer [56].

Tratamientos antiobesidad

La obesidad se reconoce como una causa de problemas de salud que incluyen resistencia a la insulina, diabetes, hipertensión y enfermedades cardiovasculares o accidentes cerebrovasculares [57]. Es el resultado de un pequeño desbalance energético positivo. Por este motivo, la adopción de una dieta saludable presenta una estrategia altamente rentable para la pérdida de peso, con la posibilidad de menos efectos secundarios negativos que las terapias farmacológicas o las intervenciones quirúrgicas invasivas. Se están buscando alimentos y/o productos para adelgazar con ingredientes bioactivos no calóricos que pueden influir modestamente en el equilibrio energético al alterar el gasto energético, la oxidación del sustrato o las sensaciones apetitivas [58].

La adición de capsaicinoides a la dieta podría tener un efecto beneficioso para el control del peso. Los pimientos picantes están relacionados con un aumento del gasto energético y una disminución tanto de la acumulación de grasa corporal como del colesterol [59]. Existe evidencia de varias vías potenciales para el mecanismo de acción del efecto de los capsaicinoides sobre la ingesta energética. En primer lugar, parece que los capsaicinoides pueden afectar a las hormonas estomacales e intestinales que influyen en el apetito. En segundo lugar, pueden interferir mediante la estimulación del sistema nervioso simpático, provocando la liberación de catecolaminas, dando lugar a una reducción del apetito y, por tanto, de la ingesta de alimentos. En tercer lugar, parecen influir en la elección de los alimentos, habiendo una preferencia por aquellos ricos en carbohidratos sobre los ricos en grasas [60].

Sin embargo, su pungencia o fuerte picor hace que no todas las personas puedan consumirlos en grandes cantidades para perder peso. Además, su propensión a provocar efectos secundarios gastrointestinales limita su consumo.

3.2.3. Evolución del contenido de capsaicinoides durante el proceso de maduración

Los pimientos se cosechan y consumen en diferentes etapas de maduración del fruto, desde verde inmaduro hasta completamente maduro. A lo largo del proceso de maduración, se producen numerosos cambios bioquímicos, fisiológicos y estructurales como la degradación y síntesis de los metabolitos de la fruta [61]. La caracterización de los cambios fitoquímicos que ocurren durante la maduración es particularmente interesante desde el punto de vista dietético y nutricional, ya que no solo tienen implicaciones agronómicas bien conocidas (por ejemplo, sabor, color, tamaño, aroma, propiedades postcosecha, etc.) sino que también son relevantes para determinar la aplicación y calidad de la fruta recolectada, y, en última instancia, la preferencia de los consumidores [62].

El genotipo propio del pimiento claramente juega un papel importante en la concentración y diversidad de estos compuestos bioactivos. Pero también puede verse afectado por las diferentes prácticas agronómicas (como las condiciones de crecimiento o las técnicas de cultivo), el estado de desarrollo del fruto y su interacción con el medio ambiente [32,63,64].

Dentro de los factores medioambientales, la disponibilidad de agua [65] (hay una reducción significativa en el rendimiento de frutos cuando se aplica una cantidad reducida de agua durante los períodos de crecimiento vegetativo, floración y fructificación), la exposición a la luz solar [66] (regula las características morfológicas y actúa como fuente de energía para el metabolismo primario y los procesos fotosintéticos), la temperatura [67] y la suplementación mineral [68] son los que tienen un mayor impacto. Las condiciones de muestreo y almacenamiento deben controlarse de cerca para producir material vegetal de alta calidad para su caracterización y uso posterior [64]. Además, se sabe que el contenido en capsaicinoides de los pimientos puede variar entre distintos frutos dentro de la misma planta, recolectados al mismo tiempo después de la floración [33].

Los cambios en las concentraciones de capsaicinoides individuales y totales durante el período de maduración de la fruta, ya se han analizado en una gran cantidad de variedades de pimiento, incluidas algunas variedades muy picantes, picantes y no picantes. Según lo reportado en la bibliografía, los capsaicinoides comienzan a acumularse desde las primeras etapas del desarrollo del fruto y continúan aumentando su contenido hasta alcanzar una concentración máxima, que suele ser en torno a los 40 días de maduración. En este punto, tiene lugar un cambio brusco en la tendencia, produciéndose una degradación de dichos compuestos [69-71].

Iwai y colaboradores fueron los primeros en sugerir que la producción de capsaicinoides incrementaba durante la maduración hasta un máximo y luego se producía un rápido vuelco en la tendencia, presentándose una degradación de estos compuestos incluso superior al 60% [15]. También estudiaron la estructura celular de la placenta mediante un microscopio observando que se producían ciertos cambios morfológicos en el tejido epidérmico de la placenta durante la maduración. Parece ser por tanto que las células epidérmicas de la placenta son el lugar de acumulación de los capsaicinoides.

Bernal y colaboradores informaron de los primeros datos de oxidación de C y DHC por una peroxidasa y sugirieron que dichas peroxidasas están involucradas en la degradación de los capsaicinoides [72,73]. Las peroxidasas son enzimas que catalizan la oxidación de un gran número de estructuras aromáticas. Concretamente, las peroxidasas básicas pueden estar directamente relacionadas con el metabolismo de los capsaicinoides porque tanto la C y DHC como sus precursores fenólicos, se oxidan fácilamente con esta enzima [74]. La oxidación de los capsaicinoides por la peroxidasa de *Capsicum* depende estrictamente de la presencia de H_2O_2. Además, se ha demostrado que las peroxidasas se localizan principalmente en la placenta y las capas de células epidérmicas más externas de los frutos del pimiento, coincidiendo con el sitio de localización de la mayoría de capsaicinoides [75].

3.3. CAPSINOIDES

3.3.1. Características generales

Los capsinoides fueron reportados por primera vez por Yazawa y colaboradores y se identificaron a finales de la década de 1980 en el cultivar de una variedad de pimientos dulces conocida como *Capsicum annuum* cv. CH-19 Sweet [16]. Hasta la fecha, se han aislado tres capsinoides diferentes: capsiato (CTO), dihidrocapsiato (DHCTO) y nordihidrocapsiato (n-DHCTO), siendo el capsiato el principal ((*E*)-8-metil-6-nonenoato de 4-hidroxi-3-metoxibenzilo)[76]. En 2019, Fayos y colaboradores identificaron tentativamente otros dos capsinoides minoritarios presentes en pimientos mediante HPLC-ESI-MS/MS (QToF). Además del CTO y DHCTO, encontraron un pico cromatográfico (*m/z* 301,1409) que podría atribuirse al ion $[M+Na]^+$ de cualquiera de los isómeros O8C (nornorcapsiato de 4-ene-5-metil, nornorcapsiato de 4-ene-6-metil o vanillil 5-octanoato) y otro (*m/z* 317,1693) correspondiente al ion $[M+Na]^+$ de cualquiera de los isómeros O9C (nordihidrocapsiato de 6-metilo o nordihidrocapsiato de 7-metilo), según su patrón de fragmentación [77].

Posteriormente, estos compuestos se detectaron en otras variedades de pimiento no picante y poco picante, así como en cultivares de pimiento picante y súper picante, aunque en concentraciones considerablemente más bajas que los capsaicinoides [78]. Estos compuestos presentan una estructura química muy similar a la de los capsaicinoides, con la única excepción de su enlace central, que se trata de un grupo amida para los capsaicinoides (amidas de vanillilamina con ácidos grasos de cadena ramificada), mientras que para los capsinoides se trata de un grupo éster (ésteres de alcohol vainíllico con cadenas ramificadas de ácidos grasos). Esta diferencia estructural podría ser la responsable de la menor estabilidad de los capsinoides, que se degradan más fácilmente [79].

El esqueleto base de los capsinoides tiene la siguiente estructura (Figura 3):

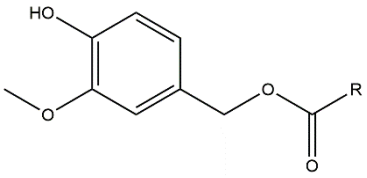
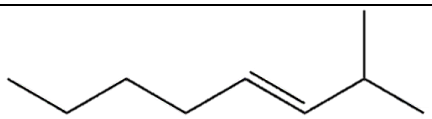
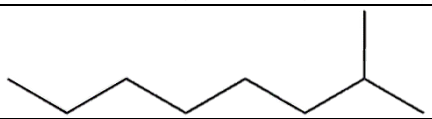
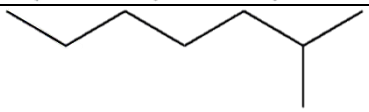


Figura 3: *Estructura general de los capsinoides.*

Al igual que ocurría con los capsaicinoides, el número de carbonos de la cadena lateral (R), la presencia o ausencia de insaturaciones y los grupos metilos ubicados en las diferentes posiciones a lo largo de la cadena, dan lugar a los distintos capsinoides existentes. Esto va a determinar su estructura y, en consecuencia, su bioactividad [77].

R =	**NOMBRE**
	Capsiato
	Dihidrocapsiato
	Nordihidrocapsiato

La cadena carbonada del CTO y DHCTO son las mismas que en la C y DHC respectivamente, diferenciándose solamente en la parte aromática, siendo vanillilamina para los capsaicinoides y alcohol vainíllico en los capsinoides.

La presencia de capsaicinoides y capsinoides en los pimientos está controlada por el gen dominante Pun1 [80]. Sin embargo, la acumulación de uno de los compuestos sobre el otro parece deberse a la acción del gen putativo de la aminotransferasa (p-AMT), que cataliza la formación de vanillilamina a partir de vainillina en la vía biosintetica de capsaicinoides. Se ha descrito que diferentes mutaciones en el gen p-AMT dan como resultado una pérdida de función, con el consiguiente incremento de la producción de alcohol vainíllico sobre la vanillilamina y, en consecuencia, la producción de capsinoides domina sobre la de capsaicinoides [81,82].

Por lo tanto, el alelo p-AMT podría considerarse un gen útil para controlar el contenido de capsaicinoides y capsinoides en programas de mejoramiento de pimientos [83].

Uno de sus principales beneficios es que son compuestos menos irritantes, no pungentes y más apetecibles. Su acritud es evaluada como aproximadamente 1000 veces menor en comparación con la de los capsaicinoides. A pesar de su menor acritud, exhiben las mismas propiedades farmacológicas beneficiosas para la salud humana mencionadas anteriormente para los capsaicinoides, como analgésicos, antioxidantes, anticancerígenos, antiinflamatorios, antimicrobianos o antimutagénicos, pero sin sensación de ardor ni efectos negativos en los organismos de los consumidores [84,85]. También hay que destacar que mejoran el metabolismo de la glucosa y pueden aumentar el gasto de energía y la temperatura corporal central, convirtiéndose así en una herramienta efectiva para los tratamientos antiobesidad, causante de enfermedades cardiovasculares, diabetes, accidentes cerebrovasculares y ciertos tipos de cáncer [86]. Esto los hace más atractivos para el consumo en concentraciones más altas en aplicaciones agroalimentarias, lo que a su vez favorece su inclusión tanto en complementos alimenticios como dietéticos, así como en otros productos con fines medicinales, ya que no presentan los efectos secundarios de irritación o sensación de ardor [87]. Las diferencias en la pungencia percibida entre los capsaicinoides y capsinoides están relacionadas con el sitio receptor vanilloide tipo-1 (TRPV1), que se encuentra en las terminaciones nerviosas sensoriales. Es el receptor responsable de la transducción del dolor y provoca la sensación de ardor que se siente después de la ingestión de estos compuestos. Los capsaicinoides activan estos receptores en la lengua del consumidor, mientras que los capsinoides tienen la capacidad de activarlos en el intestino con un efecto fisiológico similar, lo que resulta en la ausencia de la sensación de ardor [88].

Aunque están presentes de forma natural en determinadas variedades de pimientos, la forma tradicional de producción por aislamiento de la fuente natural ha sido abandonada debido a que es laboriosa e ineficaz en términos de rendimiento del producto. Por este motivo, la síntesis química es una alternativa.

En el grupo de investigación del que formo parte ("Herramientas Analíticas en Vitivinicultura, Agroalimentación y Química Forense (AGR-291)), se encuentra patentado y publicado el procedimiento para la síntesis química de este tipo de compuestos. Consta de cuatro reacciones selectivas de elevado rendimiento: protección del grupo hidroxilo de la vainillina, reducción del carbonilo y posterior esterificación y desprotección de los capsinoides protegidos [89,90].

3.3.3. Evolución del contenido de capsinoides durante el proceso de maduración

A diferencia de lo que ocurría con los capsaicinoides, el número de estudios realizados que se pueden encontrar en la bibliografía sobre la evolución del contenido de capsinoides durante la maduración en diferentes variedades de pimiento es muy escaso. Además, la mayoría de ellos se centraron solo en cuatro momentos específicos del período de maduración del fruto. Se pudieron observar tendencias similares en todas las variedades analizadas, donde la acumulación de capsinoides comenzó entre los 10 y 20 días de maduración y luego aumentó hasta alcanzar su concentración máxima en las etapas intermedias del desarrollo del fruto, es decir, entre los 30-40 días. Finalmente, se produce una inversión en la tendencia y se observa una disminución, marcada o gradual, dependiendo de la variedad [91-93]. Esta reducción en el contenido de capsinoides en las últimas etapas del desarrollo del fruto podría estar asociada a una reducción en la biosíntesis de capsinoides según las condiciones específicas de cultivo [94] o, alternativamente, al efecto de las enzimas peroxidasas que se pueden encontrar en los pimientos. La peroxidasa básica se encuentra en las células epidérmicas placentarias y en las vacuolas, donde se sintetizan los capsaicinoides, y tienen la capacidad de oxidar los precursores fenólicos implicados en la biosíntesis de capsaicina [69,95]. El capsiato también se acumula en las paredes celulares y las vacuolas. Basándose en este hecho, Lema y colaboradores, sugirieron que las mismas peroxidasas de chile que oxidan los residuos de vainillilo de los capsaicinoides eran capaces de oxidar los residuos de vainillilo de los capsinoides. El uso de diferentes inhibidores permitió confirmar que esta peroxidasa realmente tiene la capacidad de realizar dicha oxidación.

Estos resultados apoyan firmemente la suposición de que las peroxidasas básicas que se pueden encontrar en *C. annuum* podrían ser responsables de la oxidación del contenido de capsiato [96].

También se debe tener en cuenta la inestabilidad de estos compuestos en diferentes disolventes. Los capsinoides son ésteres de ácido graso y alcohol vainíllico, por lo que son estables en disolventes poco polares como el acetato de etilo, pero se descomponen fácilmente en disolventes polares como el agua o el metanol [97]. Por lo que, si se desea obtener la mayor concentración de estos compuestos de interés y sus beneficios para la salud, debido a esta inestabilidad en agua y también cuando se someten a altas temperaturas, los pimientos verdes deben consumirse crudos, es decir, se debe evitar la cocción [98].

En general, el patrón de acumulación del contenido de capsiato durante las etapas de maduración del fruto sigue tendencias similares. Sin embargo, dependiendo del genotipo del pimiento, así como de las condiciones de crecimiento y factores ambientales, dicho contenido puede variar y evolucionar de manera diferente. En este sentido, sería necesario monitorear cada detalle de las condiciones de cultivo, ya que pueden influir mucho en el producto final y su composición [61,99]. Por esta razón, sería necesario completar estudios más profundos donde se analicen un mayor número de variedades y bajo una gama más amplia de diferentes condiciones. Además, sería interesante analizar también la posible correlación entre los patrones de acumulación de capsinoides y capsaicinoides durante el desarrollo del fruto. Las conclusiones obtenidas podrían ayudar a determinar el momento óptimo de cosecha que permita obtener el máximo valor añadido de los cultivos.

3.4. DETERMINACIÓN DE COMPUESTOS BIOACTIVOS EN PIMIENTOS

3.4.1. Técnicas de extracción

Uno de los puntos críticos en el estudio de compuestos bioactivos en frutas y vegetales es el proceso de extracción de estos compuestos de la matriz vegetal durante el proceso de determinación analítica. La extracción es una etapa muy importante que envuelve el aislamiento y separación de los compuestos de interés, aquellos que queramos cuantificar y analizar, de la muestra, mediante la utilización de un disolvente adecuado. En este caso, se llevará a cabo una extracción sólido-líquido, que consiste en la transferencia selectiva de la especie de interés presente en la matriz sólida hacia una fase líquida, aprovechando las diferencias de solubilidad de los componentes de dicha mezcla en un disolvente adecuado [100]. De esta manera, se obtienen dos partes claramente diferenciadas, la solución extraída en su disolvente (extracto) y el residuo sólido. El objetivo principal de un proceso de extracción es maximizar el rendimiento del compuesto objetivo y minimizar la extracción de compuestos que pueden interferir en la determinación [101].

El proceso de extracción de las sustancias de interés depende mucho de las condiciones a las que se someta la muestra. Por ello, su eficiencia viene condicionada por diversos parámetros como son el disolvente de extracción, la temperatura, el tiempo o el pH del medio. Por tanto, la determinación y optimización de los parámetros claves son importantes para maximizar el rendimiento [102].

A la hora de preparar la muestra para la extracción, generalmente se desechan el pedúnculo y las semillas de los pimientos, separando posteriormente la placenta y el pericarpio, que se pueden analizar juntos o separados [103,104]. Los pimientos se pueden someter a la extracción de forma fresca, después de ser secados en hornos de aire caliente a determinadas temperaturas o tras un proceso de liofilización [105]. Antes de realizar la extracción de la muestra de pimiento, ya sea fresca, seca o liofilizada, es conveniente aplicar un proceso de triturado o molienda (normalmente con un molinillo eléctrico convencional) con la finalidad de conseguir una correcta homogeneización y elevar la superficie de contacto con el disolvente, mejorando así rendimiento y tiempo.

Con respecto a las técnicas de extracción empleadas, se pueden observar las técnicas convencionales, como Soxhlet, maceración, infusión o hidrodestilación. Sin embargo, recientemente han cobrado una gran importancia nuevas técnicas de extracción de compuestos bioactivos en plantas [106]. Entre éstas, podemos encontrar la extracción asistida por ultrasonidos (UAE), la extracción asistida por microondas (MAE), la extracción mediante fluidos supercríticos (SFE) y la extracción mediante líquidos presurizados (PLE). En comparación con los métodos convencionales, presentan muchas ventajas como su sencillez, una mayor eficacia y rendimiento y, además, son más respetuosas con el medio ambiente, más rápidas y reducen el consumo de disolventes orgánicos y el gasto energético con un mejor control de la temperatura [107,108]. Son conocidas también como técnicas verdes o limpias, basadas en procesos de extracción que reducen el consumo de energía y disolventes, permiten el uso de disolventes alternativos y productos naturales renovables, que garantizan un extracto seguro y de alta calidad [109].

Con el aumento de los costes energéticos y el impulso para la reducción de los gases del efecto invernadero, las industrias de alimentos y de productos químicos de origen vegetal se enfrentan al reto de encontrar nuevas tecnologías para reducir el consumo de energía y cumplir con los requisitos legales sobre seguridad y control de los productos y procesos, así como para reducir los costes y aumentar la calidad y funcionalidad [110,111].

Se han desarrollado y optimizado una gran cantidad de metodologías de extracción novedosas para los capsaicinoides presentes en los pimientos [64,70,71]. Sin embargo, no existe un estudio exhaustivo y en profundidad del desarrollo de metodologías analíticas para la extracción de capsinoides. Debido a sus excelentes propiedades biológicas mencionadas previamente, sería interesante el estudio de estas técnicas de extracción, aplicadas a los capsinoides procedentes de pimientos o de otros tipos de matrices como pueden ser alimentos procesados a partir de pimientos, como salsas, pimentón, encurtidos, etc.

3.4.1.1. Extracción asistida por ultrasonidos (UAE)

- **Principios y mecanismos de la extracción asistida por ultrasonidos**

El sonido surge como resultado del desplazamiento de la energía mecánica a través de la materia, dando lugar a una onda sinusoidal que produce alternadamente los fenómenos de compresión y rarefacción. El período (T) es el tiempo necesario para completar un ciclo y el número de ciclos completos por unidad de tiempo es la frecuencia del sonido. La diferencia fundamental entre los ultrasonidos y el sonido es la frecuencia de onda (es de 500 a 1000 veces mayor que el sonido que normalmente oímos), que se puede dividir en: Ondas audibles, correspondientes al rango de sensibilidad del oído humano (16 Hz – 20 kHz) y ondas infrasónicas y ultrasónicas, es decir, aquellas con frecuencias inferiores y superiores, respectivamente, del rango audible [112].

A diferencia de las ondas electromagnéticas, las ondas sonoras requieren de un medio elástico para ser transmitidas. El ultrasonido se aplica generalmente en medios sólidos/fluidos; las aplicaciones en sistemas de sólidos/gases no son frecuentes porque la absorción del aire dificulta la transmisión [113]. Los ultrasonidos son ondas sonoras de una frecuencia muy elevada, superior a los 20 kHz, que se transmiten por el material con el que se encuentran en contacto, provocando su contracción y posterior expansión y, en consecuencia, la transmisión de la energía por el material. La velocidad de propagación viene determinada, en gran medida, por la resistencia del medio a la compresión, que depende a su vez de la densidad, la rigidez y la elasticidad del medio. Así, la velocidad de propagación aumenta con la rigidez y disminuye con el aumento de la densidad [114].

La mejora en la eficiencia de extracción de compuestos biológicos mediante ultrasonidos se atribuye a la cavitación que se produce dentro del disolvente. La principal fuerza motriz de la extracción mediante sonicación es la llamada ‘cavitación acústica’ (Figura 4).

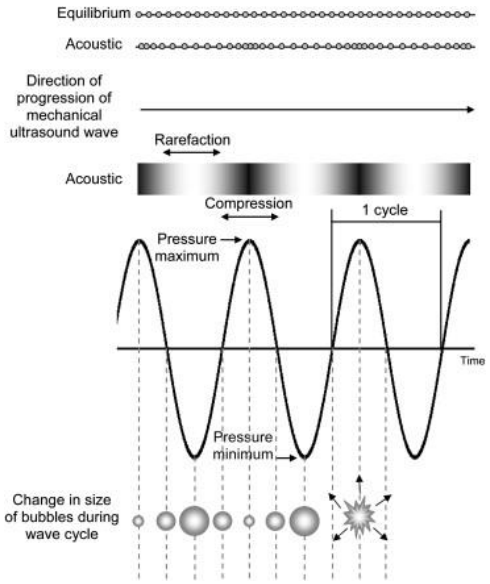


Figura 4: *Ilustración gráfica sobre el fenómeno de la cavitación acústica* [115].

Cuando los ultrasonidos se propagan a través de cualquier medio, inducen una serie de compresiones y rarefracciones en él. Durante el ciclo de compresión, la distancia intermolecular se acorta y se alarga nuevamente en el ciclo de rarefracción. Esto da lugar a la formación de burbujas, las cuales aparecen, crecen y finalmente colapsan dentro del disolvente (Figura 5). En el momento en que una cavidad completa su ciclo de vida, esta implosiona y libera energía. Gran parte de esa energía se convierte en un "jet" o chorro que actúa sobre las zonas próximas a dicha cavidad. Esto genera turbulencias a nivel microscópico, produce un flujo local en el que hay movimientos y colisiones de alta velocidad entre partículas dentro del volumen y consecuentemente este flujo crea una microagitación que es superior a la producida por la tradicional agitación magnética. Los efectos combinados de la microagitación con la transferencia de masas mejorada, dan como resultado un método de extracción más eficiente para la extracción de compuestos naturales [115,116]. Además, el colapso de estas burbujas provoca también la ruptura de las paredes celulares de la matriz vegetal, lo que favorece la penetración del disolvente, la transferencia de masa y la liberación de los analitos, aumentando así el rendimiento de extracción [117].

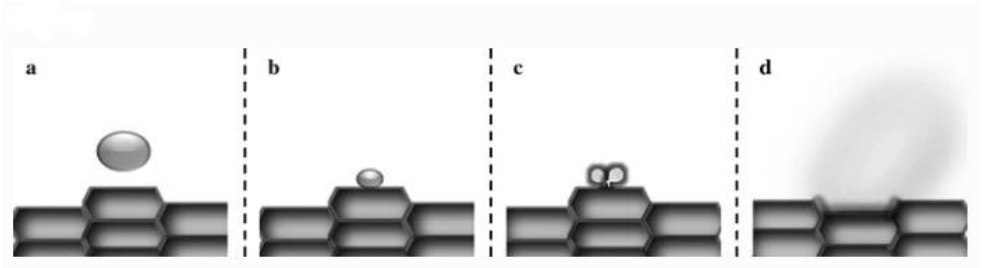


Figura 5: *Representación esquemática de una burbuja de cavitación que se genera en la superficie del material vegetal (a), se comprime (b) y colapsa cerca de la interfaz solución/tejido biológico generando ondas de choque y microchorro (c) que pueden romper la pared celular liberando el material intracelular en la solución (d)* [118].

Por tanto, la UAE se basa en el uso de la energía derivada de los ultrasonidos para facilitar la extracción de compuestos de la matriz de la muestra mediante un disolvente orgánico según la naturaleza de los solutos a extraer [119].

- **Factores que influyen en la extracción asistida por ultrasonidos**

Es necesario considerar la influencia de distintas variables en el proceso de extracción, como la potencia ultrasónica, la frecuencia, la temperatura, el diseño del reactor (baño o sonda), los disolventes seleccionados, la relación muestra/disolvente o el tiempo de extracción [120]. El objetivo del proceso no siempre es lograr el mayor rendimiento de extracción, sino el menor consumo de recursos monetarios y energéticos. Por tanto, la optimización de las variables es un paso muy importante [121].

Potencia ultrasónica

Los efectos de la potencia aplicada varían en función de la densidad de potencia, y ésta a su vez no es la misma para todo el volumen, siendo su distribución en el campo estacionario un factor importante a considerar. Por lo general, el rendimiento de extracción suele aumentar en tiempos más cortos y de forma lineal con la intensidad de potencia aplicada. Se ha demostrado que los ultrasonidos intensificaron tanto la transferencia de masa en la película líquida alrededor de las partículas sólidas como la difusión intrapartícula con el aumento de la intensidad ultrasónica [122]. Sin embargo, es posible que la densidad de potencia aplicada no sea suficiente para afectar

la resistencia externa/interna y se observará un umbral, donde por encima de un cierto valor ya no hay aumento en el rendimiento de extracción [123]. La potencia aplicada no solo influye en la cantidad total de compuestos extraídos, sino que puede afectar la proporción de especies extraídas debido a su efecto en la tasa de extracción [124].

En algunos casos, el uso adecuado del modo pulso de ultrasonido puede reemplazar la irradiación continua por ultrasonidos para obtener mejores rendimientos de extracción o para reducir el consumo de carga eléctrica [125].

Frecuencia ultrasónica

Cuanto menor es la frecuencia, mayor es la burbuja de cavitación. Por tanto, las frecuencias más bajas de ultrasonidos de alta potencia logran implosiones de burbujas más violentas y, en consecuencia, una mayor eficiencia en el proceso de extracción. Pero el efecto de la frecuencia no solo está relacionado con el tamaño de la burbuja de cavitación, sino también con su influencia en las resistencias externas e internas a la transferencia de masa, que depende de la estructura sólida / porosidad y las propiedades texturales específicas de cualquier matriz [126].

Temperatura

En general, el aumento de la temperatura provoca un aumento en el rendimiento de extracción, relacionado con un aumento de la difusividad del disolvente en las células y con la mejora de la transferencia de masa, la desorción y la solubilidad [127]. Sin embargo, también es común observar una disminución en el rendimiento a medida que aumenta la temperatura, especialmente en el caso de compuestos inestables o volátiles. Por tanto, el rendimiento de extracción aumenta a medida que lo hace la temperatura, hasta llegar a un umbral, donde éste disminuye. Además, el uso de temperaturas más altas por encima de la temperatura umbral podría resultar en la aceleración de la volatilización del disolvente, costes de energía más altos y la mejora de la extracción de impurezas [128]. Esto implica que, el rendimiento máximo de extracción se logra a temperaturas significativamente diferentes para cada compuesto dependiendo de su estabilidad y volatilidad.

Hay que tener en cuenta que durante el proceso de extracción se produce inevitablemente un aumento de la temperatura debido a la energía del sonido y, en la mayoría de los casos, el aumento de la temperatura aumenta la velocidad de extracción y la solubilidad de los compuestos, como se ha mencionado anteriormente. Para obtener unos resultados reproducibles es necesario controlar la temperatura haciendo circular un refrigerante durante la extracción, para evitar problemas con compuestos termolábiles y mantener una cavitación controlada [129].

Características del reactor

En la actualidad, los sistemas de extracción más utilizados a escala de laboratorio son los baños ultrasónicos o las sondas de ultrasonido, mostrados ambos dispositivos en la Figura 6.

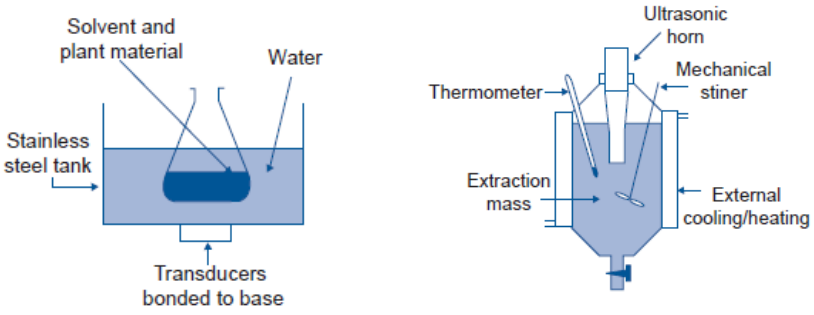


Figura 6: *Representación de un baño (a) y sonda (b) de ultrasonidos* [130].

Baño de ultrasonidos: Consta de un tanque, generadores, transductores y termostato. Consiste en matraces o vasos abiertos sumergidos en baños de ultrasonidos y se utilizan principalmente para la extracción, limpieza y desgasificación [130]. En estos sistemas, la intensidad del ultrasonido es menor porque las ondas tienen que viajar primero por el líquido que contenga el baño y atravesar la pared del recipiente que contenga la muestra antes de llegar a ella [131].

Sonda de ultrasonido: Consta de un generador, un transductor y una sonda propiamente dicha, fabricadas por lo general de titanio o una aleación de éste tolerante al calor y a la corrosión [130]. En este caso, la sonda está sumergida directamente en la solución, permitiendo que los ultrasonidos se transmitieran con una mayor energía al medio, sin ninguna barrera, proporcionando así una mayor intensidad [131].

Relación muestra / disolvente

La selección del disolvente generalmente se basa en lograr una alta afinidad molecular entre el disolvente y el soluto, pero también es necesario considerar los factores que afectan a la cavitación, como la presión de vapor, la tensión superficial la toxicidad, la viscosidad y la polaridad y pH del disolvente [132]. En disolventes orgánicos o medios acuosos, el colapso cavitacional no solo da como resultado la fragmentación de las moléculas, sino también la formación de radicales orgánicos. Los disolventes acidificados pueden intensificar la formación de radicales libres en soluciones acuosas debido a la concentración de H^+, lo que podría conducir a la degradación de los compuestos [133]. Para una cantidad fija de muestra, cuanto más disolvente se utilice, es decir, cuanto menor sea la relación muestra-disolvente, mayor efecto de gradiente de concentración se logra, obteniendo así una tasa de extracción más rápida, hasta llegar a un límite en el que los compuestos no se puedan analizar por tener una concentración demasiado pequeña [134].

Tiempo de extracción

Se requiere un tiempo considerable para que se produzca una cavitación eficiente y provoque el efecto necesario en la estructura de la matriz para que los compuestos puedan ser extraídos al medio. Habría que buscar una situación de compromiso, ya que tiempos cortos pueden dar lugar a una extracción incompleta y tiempos largos pueden producir reacciones de degradación en nuestros compuestos [135]. El objetivo es conseguir las mayores recuperaciones posibles de los compuestos de interés en el menor tiempo posible.

- **Aplicaciones de la extracción asistida por ultrasonidos**

Una de las características que hace que este sea uno de los métodos elegidos por excelencia es que consigue altas eficiencias y rendimientos de extracción, con un mínimo consumo de disolvente y en tiempos inferiores a muchas otras técnicas de extracción. Todo esto se consigue gracias a que la superficie de contacto entre la fase sólida y líquida es mucho mayor, lo que a menudo se ve traducido en un menor coste,

una disminución del tiempo requerido y una mejor conservación del medio ambiente. Además, se trata de una técnica sencilla, fácil de usar, versátil, flexible y requiere poca inversión en comparación con otras técnicas de extracción [113, 136].

Por todo ello, es una técnica ampliamente extendida, que se ha utilizado recientemente con mucha frecuencia para extraer una amplia variedad de compuestos de interés biológico (como compuestos fenólicos, ácidos orgánicos, capsaicinoides, pesticidas, hidrocarburos aromáticos, carotenoides, entre otros) sobre frutas y verduras [119, 135]. El objetivo de los investigadores es emplear esta técnica para desarrollar un método reproducible y cuantitativo de extracción que les proporcione un mayor rendimiento en menos tiempo y con un coste mínimo.

Respecto a los capsaicinoides se han desarrollado dos métodos de extracción mediante ultrasonidos, donde se han optimizado todos los parámetros operativos que influyen en el proceso [122,137]. Esta técnica también se ha aplicado para extraer capsaicinoides de la pulpa de pasta de pimiento picante basado en un concepto de biorrefinería [138]; así como en pimientos dedo moça para estudiar sus efectos sobre la matriz vegetal [139]. Finalmente, se ha empleado para analizar los cambios de estos compuestos bioactivos durante el desarrollo del fruto en distintas variedades de pimiento como Cayena [70] o Malagueta [140].

3.4.1.2. Extracción asistida por microondas (MAE)

- **Principios y mecanismos de la extracción asistida por microondas**

Las microondas son ondas electromagnéticas (EM) no ionizantes de alta frecuencia, situadas entre el rango de radiofrecuencias e infrarrojos (de 0,30 a 300 GHz). Consiste en un campo eléctrico y un campo magnético que oscilan perpendicularmente. Al exponerse a un campo electromagnético oscilante de frecuencia apropiada, las moléculas polares intentan alinearse en fase con el mismo, pero debido a fuerzas intermoleculares, experimentan inercia y son incapaces de seguir el campo. Esto resulta en un movimiento aleatorio de las moléculas de la muestra, lo que genera calor [141, 142].

La transferencia de energía se produce mediante dos mecanismos, por conducción iónica y rotación de dipolos, es decir, por medio del desplazamiento de iones cargados presentes tanto en el soluto como en el disolvente y de inversiones de dipolos [143]. En la conducción iónica, los iones en disolución migrarán cuando se aplique el campo electromagnético. Esta migración de iones disueltos aumenta la penetración del disolvente en la matriz, facilitando así la liberación de los compuestos de interés. La rotación dipolar supone un realineamiento de los dipolos con el campo aplicado. Estos movimientos moleculares forzados producen una "fricción molecular" y un calentamiento [144].

La eficiencia del calentamiento por microondas depende del factor de disipación del material, tan δ, que mide la capacidad de la muestra para absorber energía y disipar calor a las moléculas circundantes, que se expresa mediante la siguiente ecuación:

tan δ = ε" / ε´, donde, ε" es la pérdida dieléctrica (medida de la eficacia de transformación de energía de microondas en calor) y ε´ es la constante dieléctrica (mide la capacidad del material para absorber energía de microondas) [144].

Cuanto mayor sea el momento dipolar del disolvente más rápido se calentará el mismo bajo irradiación de microondas. Las moléculas polares y soluciones iónicas absorben fuertemente las microondas porque tienen un momento dipolar permanente, el cual interactúa con las microondas. La radiación excita principalmente el enlace OH presente en disolventes como metanol o etanol. Al absorber energía, pasa rápidamente del estado fundamental a uno excitado, lo que eleva la energía de las moléculas y su temperatura, lo que a su vez permite la extracción de los compuestos de interés de la matriz vegetal [141]. Sin embargo, disolventes apolares como el hexano no se calientan cuando se exponen a las microondas.

A diferencia del calentamiento convencional donde el calor penetra lentamente desde el exterior al interior del objeto, el microondas es conocida como una técnica "fría", es decir, el calentamiento aparece en el núcleo del objeto y se extiende desde el interior del cuerpo. La radiación de microondas puede enfocarse directamente en la muestra y, al calentar selectivamente solo las moléculas de interés, es mucho más

eficiente y homogénea y el consumo de energía y tiempo de extracción son mucho menores [142,145]. Además, la MAE permite una reducción significativa en el consumo de disolventes orgánicos, así como la posibilidad de analizar varias muestras simultáneamente [146].

Cada sistema de microondas consiste en tres partes básicas: la fuente de microondas (es un magnetrón, que consiste en un tubo de vacío con un cátodo central de emisión de electrones de potencial altamente negativo, que está rodeado por un ánodo estructurado que forma cavidades, acoplados por los campos de franjas y tienen la frecuencia de resonancia de microondas), la guía de ondas y el aplicador [142].

Actualmente, se han desarrollado dos tipos de sistemas de extracción asistida por microondas disponibles comercialmente: el sistema de recipiente cerrado y abierto.

-Sistema cerrado: Las extracciones se realizan en un recipiente sellado con diferentes modos de radiación de microondas. Se usan principalmente para la extracción en condiciones drásticas como alta temperatura, y constan de un magnetrón, un horno, donde los recipientes de extracción se colocan sobre un plato giratorio, dispositivos para controlar la temperatura y la presión, y una serie de componentes electrónicos de potencia [147]. La alta presión y temperatura de trabajo del sistema permiten una extracción rápida y eficiente con un menor consumo de disolvente debido a la capacidad del disolvente para absorber la energía del microondas. La presión opera por encima de la presión atmosférica y la temperatura se puede regular por encima del punto de ebullición del disolvente de extracción [148]. Sin embargo, es susceptible a pérdidas de compuestos volátiles con un rendimiento de muestra limitado.

-Sistema abierto: Funciona en condiciones más suaves ya que opera a presión atmosférica y a temperaturas no tan elevadas. Por este motivo, se considera más adecuado para la extracción de compuestos termolábiles. Tiene una mayor producción de muestras y se puede agregar más disolvente al sistema. Además, la parte superior del recipiente está conectada a una unidad de reflujo para condensar cualquier disolvente vaporizado [149].

- **Factores que influyen en la extracción asistida por microondas**

La eficiencia de la MAE depende en gran medida de la selección de las condiciones de operación y los parámetros que afectan los mecanismos de extracción y el rendimiento. Es importante comprender los efectos y las interacciones de estos factores.

Potencia de microondas

La potencia de microondas controla la cantidad de energía suministrada a la muestra que se convierte en energía térmica en el material dieléctrico para aumentar su temperatura. Afecta las interacciones y la tasa de equilibrio y controla la partición de analitos entre la muestra y el disolvente [150].

En general, el rendimiento de extracción aumenta proporcionalmente con el aumento de la potencia de microondas y dará como resultado un tiempo de extracción más corto; hasta un límite antes de que el aumento se vuelva insignificante o disminuya [151]. Una alta potencia de microondas puede aumentar la temperatura del sistema hasta un valor demasiado alto, provocando la disminución del rendimiento de extracción debido a que los compuestos termosensibles podrían sufrir daños y descomposición o degradación térmica [150].

Naturaleza del disolvente

La selección del disolvente adecuado depende de la solubilidad del analito objetivo, las propiedades de absorción de las microondas por el disolvente y la interacción con la matriz de la muestra y su constante dieléctrica [151]. En general, cuanto mayor es la constante dieléctrica y la pérdida dieléctrica, mayor es la capacidad del disolvente para absorber energía del microondas, lo que puede conducir a una tasa más rápida de calentamiento del disolvente con respecto al material vegetal. Además, sus propiedades se pueden modificar combinando diferentes disolventes, lo que conduce a una selectividad hacia diferentes compuestos objetivos [152]. También hay que tener en cuenta la toxicidad del disolvente utilizado [153]; así como el pH, que puede afectar significativamente la diferente eficiencia de extracción [154].

Generalmente, se utilizan disolventes orgánicos para extraer los compuestos orgánicos presentes en las matrices vegetales. En muchas ocasiones se suele adicionar un poco de agua a las muestras de extracción, ya que así pueden absorber de manera más eficaz las radiaciones de microondas, lo que hace que la separación de los compuestos de interés se produzca en menos tiempo. El agua también se emplea en muchas ocasiones como disolvente de extracción a la hora de extraer compuestos orgánicos solubles en agua [155].

Los líquidos iónicos a temperatura ambiente (RTIL) están ganando atención debido a sus excelentes propiedades como disolvente: presión de vapor insignificante, amplia gama de líquidos, buena estabilidad térmica, viscosidad ajustable, miscibilidad con agua y disolventes orgánicos convencionales, buena solubilidad y extractabilidad de compuestos [156]. Han resultado ser más eficientes que los disolventes comunes y son preferibles para compuestos que se degradan fácilmente, ya que gracias a su alto poder disolvente puede acelerar la extracción y reducir el riesgo de sobreexposición al calentamiento por microondas [157].

Relación muestra / disolvente

Una relación óptima de disolvente y muestra sólida asegura un calentamiento homogéneo y eficaz. Un exceso de disolvente provoca un calentamiento deficiente, ya que la radiación de microondas sería absorbida por el disolvente y se requeriría energía adicional. Una baja cantidad de disolvente en el sólido promueve una barrera de transferencia de masa ya que la distribución de compuestos activos se concentra en ciertas regiones, lo que limita el movimiento de los compuestos fuera de la matriz celular [153]. También habría que considerar el tamaño del recipiente. Con la misma proporción de disolvente y muestra, el recipiente más pequeño tiende a generar una presión interna más alta en comparación con el más grande, lo que puede acelerar la extracción [158].

Temperatura

El aumento de la temperatura provoca un aumento de las interacciones intermoleculares dentro del disolvente, dando lugar a un movimiento molecular más alto que aumenta la solubilidad. También puede causar una acumulación de presión celular que provoca la ruptura y posterior apertura de la matriz y, como resultado, una mayor disponibilidad de los componentes en la solución para ser extraídos. Además, a alta temperatura, disminuye la viscosidad del disolvente, aumentando su movilidad y solubilidad y, con ello, la eficiencia de extracción [159]. Por tanto, el rendimiento de extracción aumenta con el aumento de la temperatura. Sin embargo, a partir de un límite se produce el efecto contrario, disminuyendo la cantidad extraída al aumentar la temperatura como consecuencia de la degradación de los compuestos de interés [160]. Hay que tener en cuenta que la temperatura de degradación de cada compuesto es diferente, por tanto, la temperatura óptima de extracción dependerá de la estabilidad del compuesto activo deseado.

Tiempo de extracción

Por lo general, el rendimiento de la extracción tiende a aumentar con el aumento del tiempo de extracción, pero llega un cierto límite en el que comienza a disminuir. Se ha demostrado que la sobreexposición a la radiación de microondas incluso a baja temperatura o baja potencia operativa disminuye el rendimiento de extracción debido a la pérdida de la estructura química de los compuestos activos. Para evitar el riesgo de degradación térmica, oxidación o hidrólisis, el tiempo de extracción de la MAE suele variar desde pocos minutos hasta media hora [161].

Si se requiere un tiempo de extracción más largo, las muestras se extraen en múltiples pasos usando ciclos de extracción consecutivos, reduciendo así el riesgo de degradación térmica. De esta manera, se vuelve a alimentar la muestra con disolvente nuevo y se repite el paso de extracción para garantizar la finalización de la extracción. El uso de lotes frescos de disolvente evita su saturación con soluto, aumentando la transferencia de masa y la cinética de extracción y, con ello, el rendimiento [162].

El número total de ciclos requeridos difiere en función del proceso y debe ser justificado para ahorrar en el tiempo total de extracción y el consumo de disolvente.

Efecto de agitar la muestra

La barrera de transferencia de masa creada por los compuestos activos concentrados en una región localizada debido a la insuficiencia de disolvente se puede minimizar, lo que da como resultado un mejor rendimiento de extracción. La agitación acelera la velocidad de extracción al acelerar la desorción y disolución de los compuestos activos unidos a la matriz de la muestra [163].

- **Aplicaciones de la extracción asistida por microondas**

Esta técnica ha adquirido un gran desarrollo recientemente gracias a su alto rendimiento en comparación con las técnicas convencionales, la reducción tanto de la cantidad de disolventes empleados como del tiempo de extracción y el grado de automatización que permite [145].

En un principio, la mayoría de las aplicaciones se centraban en la determinación de distintos contaminantes (como pesticidas, metilmercurio o hidrocarburos aromáticos) en diversas matrices como sedimentos, agua o suelos [164,165]. Hoy en día, se utiliza de manera rutinaria para la digestión por microondas de determinadas matrices para análisis elementales, usando ácidos minerales acuosos [166]. Además, se está observando un gran incremento en su empleo para el desarrollo y optimización de métodos de extracción de compuestos nutracéuticos y de interés biológico, así como sustancias antioxidantes en una gran cantidad de matrices vegetales y alimentarias [141, 147].

Al igual que para ultrasonidos, se han desarrollado dos métodos donde se han optimizado los factores que afectan en la extracción de capsaicinoides mediante microondas [167,168]. Además, esta técnica verde se ha empleado para la extracción de los capsaicinoides principales presentes en distintas variedades como pimientos picantes secos (*Capsicum frutescens* Linn.) [169] o chiles habaneros [170].

3.4.2. Métodos de separación y análisis

El primer método fiable desarrollado para cuantificar la pungencia o picor general de los pimientos fue publicado en 1912 por Wilbur Scoville, conocido como prueba de calor Scoville [13]. Se trata de un método organoléptico y subjetivo basado en la determinación de la dilución más alta que se puede realizar de un extracto de chile para que la sensación de picor pueda ser percibida o detectada por un panel de degustación humano [171]. A pesar de sus limitaciones debido a errores humanos inherentes ya que no existe un estándar analítico para comparar, su popularidad y difusión mundial lo convirtieron en una escala de acritud o pungencia común [172].

La determinación exacta de los niveles de capsaicinoides y capsinoides se ha hecho imprescindible debido a la creciente demanda de estos productos por los consumidores en alimentación o productos medicinales y cosmético, así como en aerosoles forenses y de autodefensa o incluso como adsorbentes para remover contaminantes [173]. Además, es necesario disponer de procedimientos instrumentales analíticos estándar que sean reproducibles y seguros. Se han aplicado numerosos métodos rápidos para la determinación del contenido de capsaicinoides y capsinoides, los cuales son muy útiles para comparar niveles de pungencia entre diferentes muestras, incluyendo electroforesis capilar [174], cromatografía en capa fina [175], cromatografía de gases [176], cromatografía líquida de alta resolución con detección espectrofotométrica [64], de fluorescencia [168] o espectrometría de masas [77] y cromatografía líquida de ultra alta resolución [177], siendo las técnicas cromatográficas las más utilizadas [178].

3.4.2.1. Cromatografía líquida de alta eficacia (HPLC)

Es un tipo de cromatografía en columna, que permite la separación de los componentes de una muestra entre una fase móvil (el disolvente) y una fase estacionaria (el empaque de la columna), basándose en sus propiedades físicas y/o químicas. Una de las características esenciales del proceso cromatográfico es que la fase móvil donde se encuentra disuelto el extracto, fluya a través de la fase estacionaria mediante la aplicación de una presión elevada [179].

Estas transferencias de masa entre ambas fases deben ser rápidas y frecuentes para conseguir el equilibrio en el sistema cromatográfico, dando lugar así a una alta eficacia de la columna. Para ello, las distancias de difusión deben ser pequeñas y el área de contacto superficial entre las dos fases, grande. En función de la naturaleza de los constituyentes de la muestra, éstos serán más afines a una de las dos fases, por lo que algunos de ellos quedarán más retenidos en la columna y otros saldrán más rápido de la misma, provocando así su separación [180].

Existen diferentes modalidades dependiendo del tipo de fase estacionaria y la polaridad del disolvente [179]:

- Cromatografía en fase normal: La fase estacionaria es de naturaleza polar (sílice o alúmina) y la fase móvil es no polar (hexano o acetato de etilo generalmente). De esta forma, las muestras polares son retenidas más fuertemente por la columna, permitiendo la elución de los compuestos no polares en primer lugar.

- Cromatografía en fase reversa: La fase estacionaria es de naturaleza no polar (columnas de hidrocarburos) y el disolvente de elución es polar (mezclas de agua con acetonitrilo o metanol acidificado generalmente). En este caso, los compuestos no polares serán retenidos más tiempo en la columna y saldrán primero los polares.

A su vez, cuando la composición de la mezcla de disolventes permanece constante a lo largo de la etapa de elución, se denomina elución isocrática; mientras que cuando ésta varía, se conoce como elución en gradiente (de mayor resolución).

Su éxito se debe a la posibilidad de actuar de forma muy precisa sobre la selectividad entre los compuestos, a través de la elección de la columna y de la composición del eluyente, es decir, al sacar partido de las interacciones disolución/fase móvil/fase estacionaria.

3.4.2.2. Cromatografía líquida de ultra alta eficacia (UHPLC)

Esta técnica de separación tiene su base en la cromatografía líquida de alta eficacia (HPLC), pero, gracias a los avances tecnológicos que se han llevado a cabo en este campo, permite obtener separaciones de mayor resolución y sensibilidad en períodos de tiempo mucho más cortos (llegando a reducir el tiempo de análisis hasta 10 veces), con un menor consumo de disolvente. Esto se consigue gracias a la reducción del tamaño de las partículas de la fase estacionaria, con diámetros inferiores a 2 µm. En consecuencia, se requieren sistemas capaces de operar a presiones mucho mayores (a veces superiores a 100 bar) para vencer la resistencia del flujo de disolventes y que la fase móvil fluya a través de la columna [181,182]. Presenta varias ventajas entre las que destacan la óptima separación de los componentes de una muestra con alta sensibilidad, el ahorro tanto de muestra como de tiempo o la fácil adaptación a determinaciones cuantitativas. No obstante, presenta el inconveniente de que es necesario realizar varios estudios preliminares, variando el gradiente, el porcentaje de cada disolvente, los tiempos…, con el fin de encontrar las condiciones óptimas de trabajo [183].

Hoy en día se requieren análisis rápidos y económicos para muchas aplicaciones, incluyendo análisis farmacéuticos y de alimentos, a fin de aumentar el número de análisis a realizar, reduciendo tiempo y costes; pero sin un sacrificio significativo en la eficiencia o la resolución de la separación en la columna [184]. Acortando la longitud de la columna y/o incrementando la velocidad de flujo de fase móvil, se consigue una reducción del tiempo de retención de los analitos, pero sacrificando la eficiencia en la separación. Trabajando a alta temperatura se reduce la viscosidad de la fase móvil, mejorando la difusividad y permitiendo trabajar a velocidades de flujo mayores, lo que hace posible el uso de columnas con menor tamaño de partícula para incrementar la eficiencia en la separación. Sin embargo, el aumento de la temperatura puede producir la degradación de los analitos de interés. A su vez, el empaquetado de la columna con partículas pequeñas permite una mayor eficiencia y velocidad en la separación, pero conlleva un aumento considerable de la presión, lo que limita su uso con bombas convencionales [185].

La UHPLC es la técnica analítica más habitual para la separación y determinación de capsaicinoides y capsinoides en muestras de pimientos o en otras matrices, como se puede comprobar en la bibliografía existente. Barbero y colaboradores [177] desarrollaron un método para la separación de los principales capsaicinoides presentes en pimientos (n-DHC, C, DHC, h-C y h-DHC), con buena resolución y en algo menos de 3 minutos. Schmidt y colaboradores [186] realizaron la determinación de C y DHC en diversos chiles picantes provenientes de Austria, consiguiendo la separación de ambos compuestos en tan solo 1,7 minutos. Barbosa y colaboradores [187] determinaron el perfil de capsaicinoides para la caracterización y clasificación de pimentón con atributos de denominación de origen protegida (DOP). Bhandari y colaboradores [188] evaluaron el contenido de capsaicinoides en cuatro extractos de *Capsicum chinense* Jacq, para analizar sus potenciales farmacológicos y estudiar su genotoxicidad. Arrizabalaga-Larrañaga y colaboradores llevaron a cabo la determinación de capsaicinoides para la caracterización y autenticación del origen geográfico del pimentón [189]. Todos ellos emplearon como fase móvil un disolvente binario de agua con mezclas hidroalcóholicas ligeramente acidificados. Además, trabajaron en gradiente y en fase reversa, consiguiendo separaciones y determinaciones de alta eficacia y reproducibilidad.

3.4.2.3. Columnas analíticas

Uno de los objetivos de la cromatografía en las últimas décadas ha sido la separación rápida de los compuestos de interés, sin sacrificar significativamente la eficiencia o la resolución de la columna. Para ello, se busca emplear columnas cortas con altas velocidades de flujo de fase móvil que permitan reducir el tiempo de análisis, pero también provoca una reducción de la eficiencia. Otra posibilidad es emplear una alta temperatura, que provoca la disminución de la viscosidad y con ello, una mayor difusividad, permitiendo el uso de columnas más largas o partículas más pequeñas para aumentar la eficiencia; sin embargo, puede provocar la degradación de los analitos y el deterioro de la columna. Otro enfoque es la reducción del tamaño de partícula del relleno de la columna, pero conlleva un incremento considerable de la presión [184].

Primero, se desarrollaron las columnas empaquetadas, ampliamente utilizadas en el campo de las ciencias biomédicas y ambientales. Están fabricadas con un tubo de vidrio, metal o teflón, rellenos de un material sólido compacto (compuesto principalmente por partículas esféricas, inerte a elevadas temperaturas y debe humectarse homogéneamente con el paso de la fase líquida) cuyo objetivo es retener y ubicar la fase estacionaria con el fin de conseguir la mayor superficie de contacto posible con la fase móvil. Este tipo de columnas proporcionan una alta eficiencia de separación y una fácil adaptabilidad de la muestra, al mismo tiempo que reduce el consumo de disolvente, muestra, coste de operación y contaminación. La presión de cabeza de la columna capilar empaquetada es proporcional a la longitud de la columna. Las columnas largas tienen una mayor eficiencia en la separación, sin embargo, su preparación es bastante difícil debido a la alta presión de relleno requerida [190].

A finales de los años 80, aparecieron las columnas monolíticas que permiten separaciones rápidas sin comprometer la eficiencia o la resolución. La corta longitud del camino de difusión y la alta porosidad suministrada por los grandes poros, disminuyen la resistencia hidráulica del flujo de la fase móvil, lo que reduce la caída de presión, permitiendo operar a velocidades de flujo mayores y en columnas relativamente más largas; cosa que no se podía conseguir con las columnas empaquetadas convencionales [191].

Estas columnas constan de un esqueleto de sílice y dos tipos de poros. Los macroporos (aproximadamente 2 µm) son responsables de una baja resistencia al flujo, lo que permite la aplicación de caudales elevados, manteniendo la contrapresión de la columna en niveles bajos. Los mesoporos (alrededor de 12 nm) están interconectados, formando una extensa red de canales, asegurando suficiente área de superficie para una separación eficiente. Otra ventaja práctica es que se necesita poco tiempo para equilibrar la columna cuando se utiliza una fase móvil en gradiente, acortando aún más el tiempo de análisis [192].

Se introdujeron por primera vez para polímeros orgánicos, que demostraron ser muy útiles en estudios proteómicos con separaciones de alta eficiencia y detección de péptidos de alta sensibilidad. Sus principales ventajas son el fácil control del proceso de polimerización, la fácil preparación de un capilar y la disponibilidad de una gran cantidad de tipos de monómeros. Sin embargo, estos materiales presentan una gran desventaja debido a que se hinchan o encojen cuando están en contacto con disolventes orgánicos y tienen poca estabilidad mecánica [193]. Estos inconvenientes no estarían presentes en las columnas monolíticas de sílice, introducidas posteriormente y fabricadas con la tecnología sol-gel, lo que permite la formación de un material altamente poroso.

Finalmente, han surgido otras columnas cromatográficas muy utilizadas gracias a sus ventajas en la separación, conocidas como "Fused Core" (Figura 7). Estas columnas permiten separaciones muy rápidas, como consecuencia no solo de su pequeño tamaño de partículas, sino que, además, éstas están formadas por un núcleo sólido recubierto de una capa de sílice porosa de 0,5 µm [194]. De esta manera, el analito no penetra en el núcleo sólido, sino que solo puede difundirse en la capa de sílice porosa, lo que conduce a una ruta de difusión más corta, minimizando así el ensanchamiento de los picos cromatográficos, mientras que se mantiene un diámetro total suficientemente grande para evitar la generación de una contrapresión tan alta. En consecuencia, esta estructura permite reducir la transferencia de masa, lo que aumenta la eficiencia, la sensibilidad y la resolución máxima para lograr el análisis en tiempos más cortos [195,196].

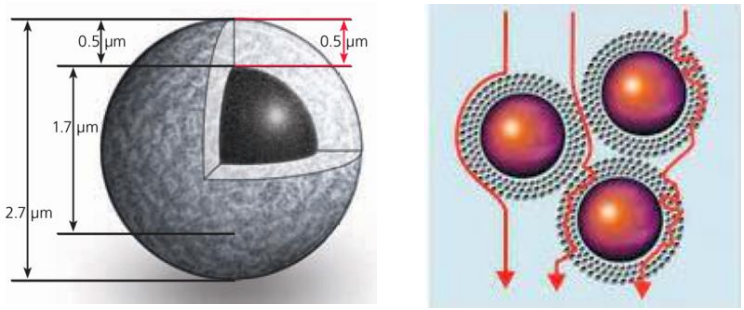


Figura 7: *Estructura de las partículas de las columnas "Fused Core"* [197].

3.4.3. Métodos de identificación y cuantificación

3.4.3.1. Espectroscopía de absorción ultravioleta-visible (UV-Vis)

La espectroscopía UV-Vis es una técnica que utiliza la radiación electromagnética de las regiones visible, ultravioleta cercana e infrarroja cercana del espectro, es decir, una longitud de onda entre 180 y 1100 nm [198]. Esta región del espectro de absorción aporta poca información estructural, pero presenta multitud de aplicaciones en análisis cuantitativo. La radiación absorbida por las moléculas desde esta región del espectro provoca transiciones electrónicas que pueden ser cuantificadas, y relacionadas, mediante la aplicación de la ley de Lambert-Beer, con la concentración del compuesto de interés presente en la muestra. Esto constituye la base de los métodos designados bajo el término general de colorimetría [199].

La cromatografía líquida, con frecuencia, se encuentra acoplada a detectores UV-Vis, que permiten llevar a cabo la determinación y cuantificación de los compuestos de interés, una vez obtenido el cromatograma con los distintos picos cromatográficos separados. Se debe tener en cuenta la diferencia entre los detectores UV-Vis y PDA (fotodiodos alineados). El primero de ellos, solo es capaz de detectar una única longitud de onda; mientras que el segundo, puede hacer un barrido y escanear un rango completo de longitudes de onda, de manera que será capaz de detectar al mismo tiempo todos los compuestos que puedan absorber a cualquier longitud de onda dentro de dicho rango [200].

Esta técnica ha sido empleada en múltiples ocasiones para la detección y cauntificación de los capsaicinoides y capsinoides presentes en distintas variedades de pimientos. Se ha comprobado que la longitud de onda de máxima absorción para estos compuestos bioactivos es 280 nm [201,202]. No obstante, en un principio se vio que el máximo de absorción estaba a 200 nm; sin embargo, a esta longitud de onda tan pequeña, el coeficiente de absorción de la fase móvil es tan grande que enmascara los valores de la absorbancia de los compuestos de interés. Por este motivo, se elige el otro máximo relativo, a una longitud de onda mayor (280 nm), donde los disolventes empleados en la fase móvil no posean absorbancia [104].

3.4.3.2. Espectrometría de masas

Es una técnica analítica que permite la determinación de los pesos moleculares de los compuestos de la muestra analizada, lo que permite obtener información sobre su composición, estructura o naturaleza y, por tanto, su identificación. Se basa en la separación y detección de los iones formados en una fuente de ionización o en una cámara de colisión a partir de los compuestos a analizar. Las moléculas presentes en la muestra a analizar se ionizan al pasar por un campo eléctrico y/o magnético, de manera que las especies portadoras de carga modificarán su velocidad y trayectoria de manera diferente, en función de su relación masa/carga [203].

Para llevar a cabo esta técnica, se requiere de varios componentes colocados en serie: una fuente de ionización, elegida en función de la muestra a analizar y la aplicación que se va a realizar; un analizador, encargado de la separación de los iones que salen de la fuente de ionización; y un detector. El proceso consiste en la introducción de la muestra a analizar, la evaporación e ionización de las moléculas, la aceleración de los iones formados, su separación en función de la relación masa/carga y finalmente su detección [204].

Es una de las tecnologías más versátiles gracias a su alta sensibilidad y selectividad, y a su menor tiempo de respuesta con respecto a otras técnicas. Se considera como un detector universal capaz de proporcionar una gran cantidad de información sobre las características estructurales de los analitos de interés. No obstante, hay que tener en cuenta que se trata de una técnica destructiva, aunque solo es necesaria una cantidad de muestra ínfima [205].

Esta metodología ofrece además la posibilidad de realizar estudios MS/MS o incluso MS^n, de manera que permite ver el patrón de fragmentación de los compuestos de interés presentes en la muestra a analizar. El proceso consiste en la generación de los iones de la muestra, la fragmentación del ion del compuesto que queramos analizar y el aislamiento de la relación masa/carga del fragmento que nos interese, para volver a realizar de nuevo su fragmentación. Dependiendo del analizador, este proceso se realiza de diferentes formas.

En el caso del triple cuadrupolo (QqQ) se consigue colocando los analizadores en serie. En primer lugar, se produce la ionización de la muestra que se desea analizar mediante una descarga eléctrica. En el primer cuadrupolo se selecciona y aisla el ion padre, en el segundo se produce la fragmentación de dicho ion y en el tercero se aisla el fragmento que nos interese maximizando la sensibilidad y especificidad. Si tenemos una trampa iónica, los iones entran en el analizador y se fragmentan por la aplicación de diferentes voltajes. Luego, atrapa al ion que nos interese seguir fragmentando y los demás salen con diferente trayectoria dependiendo de su relación *m/z*. Finalmente, el QToF consta de la combinación de un cuadrupolo (Q), que discrimina los iones en función de su relación *m/z* mediante el efecto de un campo eléctrico; y un analizador de tiempo de vuelo (ToF), que discrimina los iones en función de las diferentes velocidades a las que se desplazan a lo largo del tubo de vuelo [206].

Esta técnica suele estar acoplada a métodos de separación, generalmente cromatográficos, que permite separar las moléculas de interés antes de su llegada a la fuente de iones y así facilitar su detección y cuantificación [207].

Actualmente, se ha empleado ampliamente para el análisis de capsaicinoides, empleando distintas fuentes de ionización como ESI, APCI o presión atmosférica [208,209], así como distintos analizadores como la trampa de iones [209] o el cuadrupolo de tiempo de vuelo QToF [210]. Además, se ha utilizado para estudiar el patrón de fragmentación de estos compuestos haciendo uso de la metodología MS/MS [211].

3.5. BIBLIOGRAFÍA

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4. Hipótesis y objetivos

Esta Tesis Doctoral se incluye dentro de las actividades previstas en el proyecto de investigación del "Programa Estatal de I+D+i Orientados a los Retos de la Sociedad" y titulado "Aplicación de herramientas genómicas y metabolómicas para el estudio del carácter pungente en pimiento y cebolla", financiado por el INIA (Instituto Nacional de Investigación y Tecnología Agraria y Agroalimentaria) (código RTA 2015-00042-C02-01). Además, se realiza en colaboración con el Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA-Aragón).

Tanto los capsaicinoides como los capsinoides son compuestos ampliamente utilizados en la alimentación, bien mediante su consumo directo en forma de pimientos picantes frescos o mediante otros alimentos preparados a partir de ellos como salsas, pimentones, encurtidos, etc. Además, ambas familias de compuestos presentan excelentes características bioactivas de gran interés biológico, definidas principalmente por sus propiedades anticancerígenas, antioxidantes, antitumorales, antiinflamatorias o analgésicos tópicos contra el dolor.

Tanto la extracción asistida por ultrasonidos como por microondas han demostrado ser metodologías altamente eficaces para llevar a cabo la extracción de capsaicinoides. Por otro lado, la cromatografía líquida de ultra-alta eficacia es la técnica analítica más habitual para la separación y determinación tanto de capsaicinoides como de capsinoides, gracias a su alta resolución y sensibilidad.

En base a la bibliografía existente y al interés biológico del estudio de capsaicinoides y capsinoides, se plantearon las siguientes hipótesis de partida:

- La optimización de la extracción asistida por ultrasonidos y de la extracción asistida por microondas va a permitir llevar a cabo la extracción y determinación de capsinoides de forma rápida, limpia y eficaz.
- La optimización del método de separación mediante cromatografía líquida de ultra-alta eficacia va a permitir llevar a cabo la separación y cuantificación simultánea de capsaicinoides y capsinoides de forma rápida y eficaz.

- Una vez optimizados dichos métodos de extracción y análisis se podrían utilizar para evaluar el contenido de capsaicinoides y capsinoides en una gran cantidad de variedades de pimientos, para encontrar aquellas que son ricas en dichos compuestos y obtener extractos de gran calidad; así como para estudiar la evolución del contenido de capsaicinoides y capsinoides durante la maduración del fruto, con el fin de determinar el momento óptimo de recolección aprovechando al máximo sus propiedades beneficiosas para la salud.

De acuerdo con las hipótesis anteriormente expuestas, el objetivo general del presente proyecto de tesis es: Desarrollo de metodologías rápidas, eficientes y reproducibles de extracción y análisis de capsaicinoides y capsinoides con el fin de evaluar el perfil metabolómico asociado al carácter pungente en pimiento, mediante técnicas analíticas.

Para conseguirlo, se plantean los siguientes objetivos específicos:

- Identificar los compuestos de interés presentes en los pimientos mediante cromatografía líquida de ultra alta eficacia acoplada a un espectrómetro de masas con cuadrupolo de tiempo de vuelo (UHPLC-Q-ToF-MS).

- Desarrollar, optimizar y validar técnicas de análisis novedosas para la separación y cuantificación de capsaicinoides y capsinoides mediante cromatografía líquida de ultra alta eficacia en fase inversa (rp-UHPLC-PDA).

- Desarrollar y optimizar técnicas que permitan la extracción de capsaicinoides y capsinoides de la matriz vegetal. Para ello se evaluarán los parámetros más influyentes de dos técnicas de extracción: extracción asistida por ultrasonidos (UAE) y extracción asistida por microondas (MAE).

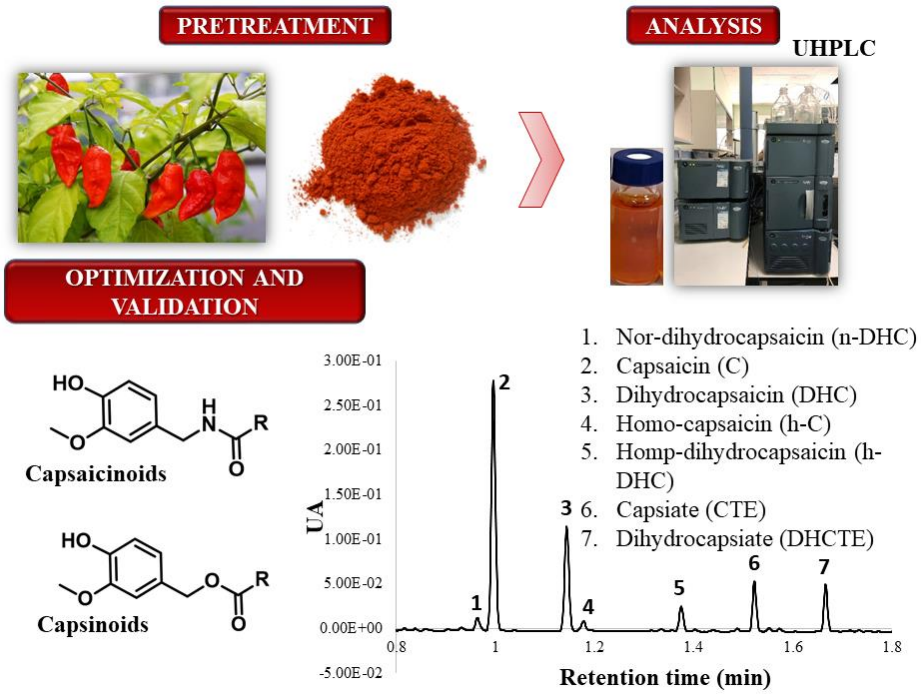
- Estudiar la evolución de la concentración de capsaicinoides y capsinoides en distintas variedades de pimientos a lo largo de la maduración del fruto, para obtener información del momento más apropiado para su recolección en función del interés del agricultor, además de determinar qué variedades presentan una mayor concentración de estos compuestos bioactivos de interés.

5. Resultados

5.1. Desarrollo y optimización de un método para la separación rápida y eficaz de los principales capsaicinoides y capsinoides en pimientos

Los resultados presentados en este capítulo se han publicado en:

Vázquez-Espinosa, M.; González-de-Peredo, A.V.; Espada-Bellido, E.; Ferreiro-González, M.; Barbero, G.F.; Palma, M. Simultaneous determination by UHPLC-PDA of major capsaicinoids and capsinoids contents in peppers. *Food Chem*. **2021**, *356*, 129688. doi.org/10.1016/j.foodchem.2021.129688



- **Simultaneous determination by UHPLC-PDA of major capsaicinoids and capsinoids content in peppers**

Abstract

Capsaicinoids and capsinoids compounds have been a focus of special attention for their health benefits. An effective and rapid Ultra-High-Performance Liquid Chromatography (UHPLC-PDA) method has been developed and validated for the simultaneous separation and quantitative determination of the major capsaicinoids and capsinoids present in peppers. The separation of all the compounds of interest was achieved in less than 2 min by means of an ACQUITY UPLC BEH rp-C18 column (100 mm × 2.1 mm i.d., 1.7 µm particle size). The variables that have been optimized are the mobile phase (water as solvent A and acetonitrile as solvent B, both acidified by adding 0.1% acetic acid), separation gradient, column temperature (35 – 70 ºC), flow rate (0.6 – 0.95 mL min^{-1}), and injection volume (2.5 – 3.5 µL). The evaluation of the chromatographic performance revealed excellent resolution, retention factor, and selectivity. The method was satisfactorily validated in terms of linearity, detection and quantification limits, precision, and robustness.

1. Introduction

Peppers are fruits produced by plants in the *Capsicum* genus from the Solanaceae family. Its first use dates from the year 7000 BCE, and at that time its main benefits came from its organoleptic characteristics and physiological effects (da Silva Antonio, Moreira Wiedmann & da Veiga Junior, 2019). They have been for centuries and are still a very popular spice in our food industry for culinary applications. Peppers are an efficient way to eliminate insipidity and to add flavor and color to many foods. In addition to their organoleptic value, peppers also have tent in antioxidants and other bioactive compounds (Baenas, Belović, Illic, Moreno & García-Viguera, 2019). This fact makes them one of the most valued horticultural products all over the world, and they are currently internationally commercialized. Peppers are an economically notable crop worldwide, breaking records and reaching up to 3.8 million hectares of cultivation land and a production of 40.7 billion kg in 2017, ranking sixth among all horticultural products in terms of world production (FAO, 2018).

One of the main characteristics of chili peppers is their spicy taste and their intense pungency, which is caused by capsaicinoids and capsinoids. Capsaicinoids are biosynthesized in the placenta of the fruits through the condensation of a vanillilamine linked to an amide group with a long fatty acid chain (Lu, Ho & Huang, 2017). Capsaicin (C) and dihydrocapsaicin (DHC) are the predominant capsaicinoids found in peppers, representing up to 97% of the total amount in some cases, but other minor capsaicinoids can also be found (Schweiggent, Carle & Schieber, 2006). Capsinoids were most recently found in the fruits of a low-pungent cultivar of *Capsicum annuum* L. (CH-19 Sweet). They have a very similar structure but with an ester group instead of an amide group being much less irritating compounds. The major compounds within this family are capsiate (CTE) and dihydrocapsiate (DHCTE) (Rosa et al., 2002). Both families of compounds have been a focus of special attention for their improvement of health conditions, due to their antioxidant, anticancer, anti-inflammatory, analgesic or antidiabetic properties, as well as blood glucose regulation and prevention of neurodegenerative diseases (Rosa et al., 2002; Bogusz et al., 2018; Luo, Peng & Li, 2011).

Since these compounds are present in many foods, and some medicinal and cosmetics products, as well as in forensic and self-defense sprays or even as adsorbents to remove contaminants (Barbosa, Campmajó, Saurina, Puignou & Nuñez, 2020; Korkmaz, Atasoy & Hayaloglu, 2020), a large number and diversity of techniques have been proposed for their extraction and determination. The first organoleptic and subjective method developed to quantify the general pungency of peppers, known as Scoville heat test (Gillette, Appel & Lego, 1984) was published in 1912 by Wilbur Scoville. Despite its limitations due to human inherent errors since there is no analytical standard to compare, its popularity and worldwide diffusion turned it into a common acre scale (Korel, Bağdatlioğlu, Balaban & His¸il, 2002).

Taking into account the variability of the capsaicinoids and capsinoids contents depending on the pepper variety, the part of the fruit, the state of maturity and the growing conditions, more sophisticated and updated analytical methods are needed. Numerous methods have been applied for the determination of capsaicinoids and capsinoids contents, including capillary electrophoresis (Liu, Chen, Liu, Deng, Duan & Tan, 2010), thin layer chromatography (María-Ioana, Constantin, Delia, Cornelia-Anca & Constantin, 2004), gas chromatography (Hawer, Ha, Hwang & Nam, 1994), high performance liquid chromatography (HPLC) with spectrophotometric detection (de Aguiar, Coutinho, Barbero, Godoy & Martínez, 2016), fluorescence detection (Barbero, Palma & Barroso, 2006) or mass spectrometry detection (Fayos, Savirón, Orduna, Barbero, Mayor & Garcés-Claver, 2019) and ultra-high-performance liquid chromatography (UHPLC) (Barbero, Liazid, Ferreiro-González, Palma & Barroso, 2016).

By far, the most often used technique for the determination and quantification of these compounds is reverse phase HPLC, but it consumes large amounts of solvents as a result of the high flow rates. In addition, more rapid, sensitive and accurate methods are needed (Rostagno et al., 2011). UHPLC results in higher resolution and sensitivity separations in very short periods of time and, therefore, with lower solvent consumption. All of this is achieved thanks to the reduction in particle size, with

diameters less than 2 μm. Consequently, systems capable of operating at high pressure levels, sometimes exceeding 100 bar (Klejdus, Vacek, Lojková, Benešová & Kubáň, 2008) are required. The separation is generally carried out by means of reverse phase C-18 analytical columns and using a gradient mobile phase with binary solvents of different polarity (Manchón et al., 2011).

Due to the excellent biological properties of these compounds present in peppers, it would be necessary to have an analytical method that allows their separation and subsequent determination and quantification. All the methods reported in the literatura carry out the optimization of the separation of capsaicinoids and capsinoids independently, so there is currently no method that allows the determination of both families of compounds at the same time. Some of the existing methods determine only the two major compounds of each family, that is, C and DHC (Ha et al., 2010) or CTE and DHCTE (Singh et al., 2009), using analysis times of more than 4 and 10 min for their separation, respectively. Other methods achieve the separation of the majority and minority compounds from each group, however the time required is moderately long (Coutinho, Barbero, Godoy, Palma & Barroso, 2016; Coutinho et al., 2015). It should also be mentioned an optimized method that allows the separation of 8 major capsaicinoids in Brazilian *Capsicum chinense* fruits in just 4 min (Sganzerla, Coutinho, Tavares de Melo & Godoy, 2014) and other two methods that separate the main capsaicinoids in just 3 min (Barbero, Liazid, Ferreiro-González, Palma & Barroso, 2016; Stipcovich, Barbero, Ferreiro-González, Palma & Barroso, 2018). However, in none of them capsinoids are determined. Therefore, the aim of this work is getting to develop and validate a rapid and reliable UHPLC-PDA method which allows the simultaneous separation and quantitative analysis, not only of the five major capsaicinoids (nordihydrocapsaicin (*n*-DHC), capsaicin (C), dihydrocapsaicin (DHC), homocapsaicin (h-C), and homodihydrocapsaicin (h-DHC)), but also the two main capsinoids (capsiate (CTE), and dihydrocapsiate (DHCTE)). Moreover, the proposed method should accomplish a total time reduction for the separation and determination of each group of compounds without compromising the chromatographic peaks resolution.

2. Materials and methods

2.1. Biological material

Methanol extracts from the Naga Jolokia variety (*Capsicum chinense*), obtained by ultrasound-assisted extraction (UAE), have been used for the development and validation of the chromatographic separation. The Naga Jolokia peppers were sown in October 2017 and harvested by mid- March 2018 from the Agronomic Institute of Campinas (IAC) (Campinas, SP-Brazil). This variety has been the focus of attention for the world scientific community due to its extremely high pungency and unique aroma. It has been rated at over 1 million Scoville Heat Units (SHUs) and it is used as a spice in either fresh or dry presentations.

2.2. Sample preparation

As a first step, the peppers were lyophilized by means of a laboratory freezer dryer LYOALFA 10/15 (Telstar Technologies, S.L., Terrasa, Barcelona, Spain), and crushed using a conventional electric grinder (Mandine MCG2013B-16, Carrefour, Madrid, Spain). For its extraction, a UP200S ultrasonic probe (Hielscher Ultrasonics, Teltow, Germany) and a double wall vessel coupled to a thermostatic bath with temperature controller (7 Liter Refrigerated Circulator, PolyScience, Niles, IL, USA) were used. The extraction was carried out according to the conditions described for the method developed and optimized by Vázquez- Espinosa et al. (Vázquez-Espinosa et al., 2019). For the chromatographic analysis, a certain volume of the extract was filtered through a 0.22 μm syringe filter (Nylon Membrane Filter, FILTERLAB, Barcelona, Spain). Both the lyophilized sample and the extracts were frozen at -20 ºC prior to analysis.

2.3. Chemicals and reagents

Milli-Q water was obtained by a Millipore purification system (Bedford, MA, USA). Glacial acetic acid was purchased from Merck (Emsure, Darmstadt, Germany). Acetonitrile and methanol were HPLC grade and were obtained from Panreac Química S.A.U. (Castellar del Vall´es, Barcelona, Spain).

The reference standards for capsaicin (97%) and dihydrocapsaicin (90%) were supplied by Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The capsiate and dihydrocapsiate standards were synthesized according to the methodology described by Barbero et al. (Barbero, Molinillo, Varela, Palma, Macías & Barroso, 2010).

2.4. UHPLC equipment

The UHPLC separation of the 7 compounds studied was carried out by means of an ACQUITY UPLC® H-Class System (Waters Corporation, Milford, MA, USA) coupled to an ACQUITY UPLC Photodiode Array Detector (PDA) set at a wavelength of 280 nm, since it corresponds to the maximum absorption level of these compounds. The equipment consisted on an ACQUITY UPLC H-Class Auto Sampler, an ACQUITY UPLC Quaternary Pump System and a Waters ACQUITY UPLC BEH rp-C18 column (100 mm × 2.1 mm i.d., 1.7 μm particle size, Waters Corporation, Milford, MA, USA). The compounds of interest were identified by mass spectrometry. In addition, those compounds whose standards were available, had their resulting retention times and UV–vis absorption spectra as obtained from the tests performed compared against their reference standards. Finally, the entire system was controlled by Empower^{TM} 3 Chromatography Data Software (Waters Corporation, Milford, MA, USA).

2.5. Identification of the capsaicinoids and capsinoids by UHPLC-Q-ToF-MS

The capsaicinoids and capsinoids were identified by means of an Ultra-High-Performance Liquid Chromatographer (UHPLC) coupled to a quadrupole-time-of-flight mass spectrometer (Q-ToF-MS) (Synapt G2, Waters Corp., Milford, MA, USA) and the method described by Stipcovich et al. was applied (Stipcovich, Barbero, Ferreiro-González, Palma, & Barroso, 2018). Full-scan mode was used (*m*/*z* = 100–600). The following *m*/*z* ratios were used for the identification of the protonated molecular ions $[M + H]^+$: *n*-DHC, 294; C, 306; DHC, 308; h-C, 320; h-DHC, 322; CTE, 307; and DHCTE, 309.

2.6. Development of the chromatographic method

Methanol extract samples from Naga Jolokia peppers were employed for all the chromatographic tests. The samples already contained the five main capsaicinoids and capsiate, and a proportional amount of dihydrocapsiate standard was added before the analysis. The different variables, specifically the solvents used as mobile phase (Milli-Q water as eluent A and methanol or acetonitrile as eluent B, both acidified with 0.1% of acetic acid), the operating temperature (35 – 70 ºC), the flow rates (0.6 – 0.95 mL min^{-1}), and the injection volume (2.5 – 3.5 µL) were optimized. The criterion to select the best chromatographic separation was based on optimal chromatographic properties: retention time (t_R), resolution (Rs; (A)), retention factor (K; (B)) and selectivity (α; (C)), which were calculated according to the following equations:

$$\textbf{(A)}\ R_S = \frac{2(t_{R(B)} - t_{R(A)})}{W_{b(A)} + W_{b(B)}} \qquad \textbf{(B)}\ K = \frac{t'_R}{t_M} \qquad \textbf{(C)}\ \alpha = \frac{t'_{R(B)}}{t'_{R(A)}} = \frac{K(B)}{K(A)}$$

where: t_R (A) and t_R (B) are the retention times of two adjacent peaks A and B, respectively; W_b (A) and W_b (B) are the peak widths at the base of two adjacent peaks A and B, respectively; t'R is the predetermined retention time ($t'_R = t_R - t_M$); and t_M is the dead time, that is, the time required to eluate a compound that is not retained by the stationary phase. All of these properties were calculated by means of Empower™ 3 Software. The integration of the compounds of interest was carried out manually by integrating the "valley to valley" peak.

2.7. Validation process

The chromatographic method was validated in accordance with ICH Guideline Q2 (R1) (Ich, 2005). Specifically, a validation protocol was performed to ensure an adequate detection, identification and quantification of the 7 compounds of interest. The parameters evaluated were: linearity, detection (LODs) and quantification (LOQs) limits, precision (repeatability and intermediate precision), and robustness. All of them were calculated by means of Microsoft Office Excel 2016.

2.7.1. Linearity

The method's linearity was calculated by constructing the calibration curves corresponding to C, DHC, CTE, and DHCTE based on their standard solutions. Due to there are no standards available for *n*-DHC, h- C, and h-DHC and they present similar molecular structures, these compounds were quantified according to DHC (*n*-DHC and h-DHC) and C (h-C) calibration curves and to their individual molecular weights. For this purpose, different concentrations (0.1, 0.5, 1, 5, 25, 100, 200 mg L^{-1}) were presented against the peak area. Their coefficients of determination (R^2), which quantify the degree of linear correlation, were calculated according to their calibration curves. This specific parameter is particularly significant to confirm a linear correlation between the results obtained and the actual concentration of each compound within the investigated range.

2.7.2. Limits of detection and quantification

Based on the calibration curves generated for each one of the compounds of interest, the detection limits (LODs) and the quantification limits (LOQs) were determined by dividing 3 and 10 times respectively, the standard deviation of the blank sample by their respective regression slope.

2.7.3. Precision

The precision of the method was determined by performing repeatability and intermediate precision studies in relation to the following chromatographic properties: retention time, peak area, peak width, peak height, and peak resolution for the 7 compounds of interest. A total of 30 independent UHPLC analysis were performed on similar samples on three consecutive days (10 analyses per day). In this way, the intermediate precision was evaluated by determining the coefficient of variation (CV) of each parameter according to the results obtained from the 30 tests. Similarly, the repeatability of the method was determined by means of the CV corresponding to the data obtained from the analyses completed on the same day.

In accordance with the AOAC manual for Peer-Verified Methods program (AOAC, 2012), a CV below 10% was taken as the reference to confirm the precision of the method.

2.7.4. Robustness

This parameter denotes the ability of a method to remain unaffected by minor but continuous variations of the method variables. In our case, the robustness of our method was evaluated by implementing 5% variations to the column temperature range, the flow rate, and the injection volume. The robustness tests intended to determine the way such variations would affect several chromatographic properties; particularly, retention time, peak area, and peak resolution. Each variable was evaluated at three different levels and for each level a total of four repetitions were completed.

Tuckey's test was used for the statistical analysis, assuming 0.05 as level of significance. This procedure was handled by means of Statgraphic Centurion statistical software Ver. XVII (Statgraphics Technologies, Inc., The Plains, VA, USA) plus an analysis of variance.

3. Results and discussion

3.1. Development of the UHPLC method

For the development of the UHPLC-PDA method, Naga Jolokia extract already containing the 5 capsaicinoids (*n*-DHC, C, DHC, h-C, h- DHC), and CTE plus the manually added DHCTE, was injected into the UHPLC system. The objective was to obtain well-resolved chromatographic peaks easily integrable, specifically the highest resolution of the closest ones (in this case *n*-DHC – C and DHC – h-C, which are the two most complex pairs of peaks to elucidate), and where separation times and column pressure values were not excessively high. For this purpose, a step-by-step strategy for the optimization of the chromatographic variables (mobile phase, gradient, column temperature, flow rate, and injection volume) was implemented so that a rapid and reproducible method could be attained.

Mobile phase: The mobile phase was selected according to the results from a previous series of experiments where acidified water was used as solvent A and acidified methanol or acetonitrile as solvent B (both with the addition of 0.1% acetic acid). Several runs were performed using a linear gradient from solvent A to solvent B (0 – 100%) and the time gradient was modified from 4 to 8 min at 1 min intervals. The flow rate and column temperature were maintained constant at 0.8 mL min^{-1} and 50 ºC, respectively. Although good results were obtained from both solvents, due to its lower viscosity as well as the lower back pressure generated in the system, acetonitrile was selected as the optimal solvent for the mobile phase B. Thanks to such lower pressure, higher flow rates could be used and would allow for a reduction in the time required for each analysis (Rostagno, Debien, Vardanega, Nogueira, Barbero & Meireles, 2014). In addition, acetonitrile has a high sensitivity to short UV wavelengths, which reduces the noise in UV–Vis detection (Protti & Mezzetti, 2015). It should be noted that, in general, when acetonitrile was used, peaks were obtained more rapidly and with better shapes in comparison to those obtained when methanol was used as the solvent.

Separation gradient: Several trial-and-error experiments were carried out using the established optimal solvent (acidified water and acetonitrile) in order to determine the gradient that would provide the best separation and in the shortest possible time. An increase in the amount of solvent B lead to a reduction in time, but caused a slight overlap of some of the chromatographic peaks, together with a lesser drop in resolution (Fig. 1S). On the contrary, a smaller amount of solvent B would prevent such peak overlaps and a higher resolution would be accomplished. Nevertheless, a considerably longer analysis time would be required (Fig. 2S). For this reason, the objective was to find an intermediate solution, which could consist on injecting phase B earlier and at lower percentage. After several tests, the best separation of the main capsaicinoids and capsinoids present in peppers was achieved with the following gradient: 0 min, 0% B; 0.1 min, 45% B; 0.4 min, 45% B; 0.7 min, 50% B; 0.9 min, 55% B; 1.3 min, 75% B; 1.8 min, 75% B; 2.4 min, 100% B; 4.4 min, 100% B; and 4.6 min, 0% B. This gradient allows the successful separation of the compounds of

interest in less than 2 min. The experiments were also performed with a column temperature of 50 ºC and a flow rate of 0.8 mL min^{-1}.

Column temperature: The column temperature was gradually increased from 35 ºC to 70 ºC at 5 ºC intervals to determine the effect of temperature rising on retention time and peak chromatographic resolution (Stipcovich, Barbero, Ferreiro-González, Palma, & Barroso, 2018). It was essential not to surpass the maximum operating temperature of the column (90 ºC), since that might significantly reduce its useful life. In this case, because of the degradation of capsinoids at higher temperatures, no levels higher than 70 ºC would be applied (Ohyama, Nogusa, Shindoa, Suzuki, Bannai, & Kajimura, 2016). On the other hand, high temperatures may attain more rapid analysis because of the subsequent reduction of both viscosity and back pressure values and, in turn, the possibility of implementing higher flow rates; a factor that would contribute to further reducing retention time values. In addition, a lower viscosity and greater diffusivity of the mobile phase at high temperatures would result in a considerably lower mass transfer resistance (Nováková & Vlćková, 2009). Higher temperatures would lead to a clear trend to produce taller and narrower peaks, which means a better separation between peaks and with improved resolution. Having said that, it should also be noted that at 70 ºC a slight degradation of the capsiate takes place and this negatively affects peak resolution. Consequently, 65 ºC was selected as the optimal temperature for its short retention time and efficiency level. The chromatogram obtained at 70 ºC has been represented in Fig. 3S so that it can be compared to the one obtained at the optimal temperature (Fig. 4S). Similar results were obtained by other authors, where a better separation of the compounds of interest were achieved up to a certain temperature, and then, as temperature increased above that particular level, peak resolution would gradually fall (Barbero, Liazid, Palma & Barroso, 2008).

Flow rate: The high temperature level that had been selected as the optimum value allowed to reduce the back pressure of the column and to explore the consequences of using greater flows to shorten the analysis time (Osorio-Tobón, Carvalho, Barbero, Nogueira, Rostagno, & de Almeida Meireles, 2016).

Accordingly, the flow rate was increased from 0.6 to 0.95 mL min^{-1} by 0.05 mL min^{-1} intervals. The maximum flow rate was conditioned by the system pressure's limit of 15000 psi. Then, it was observed that, as the flow rate was increased, the analysis time would be shorter and the peaks' width was reduced, which meant an optimal separation of the seven chromatographic peaks. Hence, the best resolved chromatogram was obtained by implementing a flow rate of 0.95 mL min^{-1}. No higher flows were tested to ensure that the maximum pressure allowed by the equipment was not reached at any time.

Injection volume: Finally, the optimal injection volume was optimized. The method was tested for 2.5, 2.8, 3.0, 3.3, and 3.5 μL injection volumes and the resolution level of the chromatograms that were obtained presented hardly any differences. An injection volume of 3.0 μL was chosen as the optimal value, since it was sufficient to quantify each of the chromatographic peaks while sample waste was minimized.

3.2. Characteristics and validation of the developed UHPLC method

In summary, the optimal conditions for the UHPLC-PDA method developed in this study employ the following gradient: 0 min, 0% B; 0.1 min, 45% B; 0.4 min, 45% B; 0.7 min, 50% B; 0.9 min, 55% B; 1.3 min, 75% B; 1.8 min, 75% B; 2.4 min, 100% B; 4.4 min, 100% B; and 4.6 min, 0% B. The column temperature was adjusted to 65 ºC, the selected flow rate was 0.95 mL min^{-1} and the injection volume was 3 μL. Under these conditions, the best balance between analysis time and best separation of the compounds of interest was achieved. The total analysis time was 4.6 min, including the return to the initial conditions and the re-equilibration of the column. However, a time length of less than 2 min was enough for the successful separation of the five capsaicinoids and the two major capsinoids under study. The chromatogram obtained under the established optimal conditions can be seen in Fig. 1.

The best possible separation, that is, this optimal chromatogram allowed the calculation of several chromatographic properties of the peak studied (retention time (t_R), peak width (w_R), resolution (Rs), retention factor (K), and selectivity (α)) (Table 1).

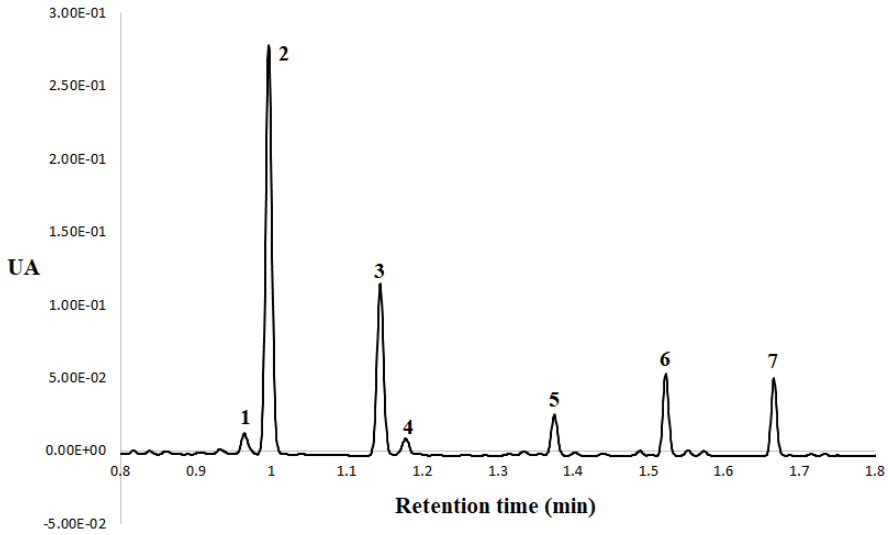


Fig. 1. Chromatogram of Naga Jolokia pepper extract obtained according to the optimized conditions of the developed method (λ = 280 nm). Nordihydrocapsaicin (n-DHC), capsaicin (C), dihydrocapsaicin (DHC), homocapsaicin (h-C), homodihydrocapsaicin (h-DHC), capsiate (CTE), and dihydrocapsiate (DHCTE).

Table 1 Chromatographic characteristics of the developed UHPLC method.

	Retention time (min)	Width (seg)	Retention factor (K*)	Resolution (Rs)	Selectivity (α)
n-DHC	0.964	2.000	6.1940		
C	0.996	2.900	6.4328	1.0958	1.0386
DHC	1.114	2.650	7.3134	2.5514	1.1369
h-C	1.178	2.800	7.7910	1.4092	1.0653
h-DHC	1.375	1.800	9.2612	5.1391	1.1887
CTE	1.522	1.900	10.3582	4.7676	1.1185
DHCTE	1.666	1.500	11.4328	5.0824	1.1037

The results that were obtained confirmed a superior chromatographic performance with regards to the successful separation of both families of compounds; capsaicinoids and capsinoids.

In relation to resolution, values greater than 1.5 are the usual objective, since that would keep peak overlapping at just 0.3%. In contrast, values of just 1 would cause 4% overlapping between two adjacent chromatographic peaks. In our study in particular, values greater than 1 were obtained in all the cases, including the separation of *n*-DHC and C, the two compounds that presented the most significant difficulties for a successful separation. With respect to selectivity, values between 1 and 2 were desired and, in fact, all the determined compounds presented values that lied within this range. Therefore, the superior selectivity of the method was confirmed. Improvements in selectivity are rather relevant, since they may lead to improvements in resolution and/or to shorter analysis. Finally, and regarding the retention factor, the values should ideally remain between 1 and 10, although, sometimes, this range could be extended to 0.5 – 20. In this case, all the capsaicinoids presented values within the ideal range, while capsinoids would be included in the extended range. Nevertheless, both values would be really close to 10 in any case.

The method was validated in accordance with ICH Guideline Q2 (R1) (Ich, 2005). Thus, linearity, precision, limits of detection and quantification as well as robustness were evaluated as described below.

Linearity: The linearity of the developed chromatographic method was verified according to the coefficients of determination (R^2) obtained from the calibration curves of each compounds of interest. The linearity of the method was satisfactory for all the compounds in the studied range (0.01 – 200 g mL^{-1}), since the values obtained were very close to or even equal to 1 in all the cases. The calibration curves and the R^2 values are shown in Table 2.

Limits of detection (LOD) and quantification (LOQ): The LOD and LOQ for capsaicinoids and capsinoids were estimated based on the standard deviation of the blank and the regression slope of each compound, as already explained in the Materials and Methods section. The values obtained were in the order of ppm, which are very similar to the results found in the literature for the same compounds (Kuzma et al., 2015).

The results are shown in Table 2. Lower values can be observed for capsaicinoids when compared against those obtained for capsinoids.

Table 2 Validation parameters for the developed UHPLC method

	Calibration curve	R^2	LOD (ppm)	LOQ (ppm)
n-DHC	y = 1461.1240x – 641.5089	1.0000	0.095	0.316
C	y = 1619.7031x – 563.4586	0.9998	0.165	0.551
DHC	y = 1394.4911x – 641.5089	1.0000	0.112	0.373
h-C	y = 1548.6132x – 563.4586	0.9998	0.173	0.576
h-DHC	y = 1333.6290x – 641.5089	1.0000	0.137	0.456
CTE	y = 1561.3768x – 1302.1331	0.9996	0.211	0.704
DHCTE	y = 1383.4561x – 888.0054	0.9998	0.160	0.533

Precision: The repeatability and intermediate precision of the developed chromatographic method were established as a function of the retention time, peak area, peak width, peak height, and peak resolution corresponding to each compound, expressed as the coefficient of variance (CV). CV values were lower than 5% for all the peaks and for all the evaluated chromatographic properties, as can be seen in Table 3. These precision values were within the acceptable limits established by the AOAC manual for Peer-Verified Methods Program (AOAC, 2012). It can, therefore, be confirmed that the method is quite precise, being the retention time the parameter that presented the highest precision.

Robustness: The robustness of a method is its ability to remain unaffected by lesser deliberate variations in the method variables. In this work, it was evaluated by testing a variation of ± 5% in column temperature, flow rate, and injection volume. The effect of these variables on the resulting values corresponding to three properties, i.e., retention time, peak area, and chromatographic resolution was verified. The statistical comparison was performed using Tuckey's test, and the results are shown in Table 4, where the different letters in the same row for each parameter indicate that the values were considered to be statistically different (*p*-value less than 0.05).

Table 3 Intraday (%) and interday (%) precisión (RSD) with respect to different chromatographic properties.

	Retention time		Peak area		Peak width		Peak height		Peak resolution	
	Intraday	Interday	Intraday	Interday	Intraday	Interday	Intraday	Inetrday	Intraday	Interday
n-DHC	0.128	0.161	0.132	0.583	1.052	2.041	0.368	0.402		
C	0.113	0.147	0.187	0.317	0.465	1.737	0.176	0.187	0.734	2.011
DHC	0.126	0.149	0.196	0.347	1.067	3.023	0.107	0.143	0.505	1.734
h-C	0.128	0.147	0.377	0.756	0.351	1.517	0.206	0.330	0.988	2.004
h-DHC	0.081	0.123	0.280	0.370	2.297	2.638	0.638	0.656	0.931	1.382
CTE	0.055	0.118	0.306	0.319	1.704	2.941	0.197	0.257	1.671	2.236
DHCTE	0.041	0.083	0.816	3.321	3.261	4.673	0.609	3.417	2.241	3.283

Table 4 Robustness of the developed UHPLC method.

		Temperature (°C)			Flow rate (mL min^{-1})			Injection volumen (µL)		
		60.00	**65.00**	**70.00**	**0.90**	**0.95**	**1.00**	**2.50**	**3.00**	**3.50**
Retention time	**n-DHC**	58.440^{a}	57.390^{b}	56.340^{c}	59.820^{a}	57.180^{b}	54.570^{c}	57.255^{a}	57.300^{a}	57.210^{a}
	C	60.420^{a}	59.310^{b}	58.185^{c}	61.785^{a}	59.085^{b}	56.415^{c}	59.160^{a}	59.220^{a}	59.130^{a}
	DHC	69.510^{a}	68.100^{b}	66.630^{c}	70.635^{a}	67.815^{b}	64.995^{c}	67.890^{a}	67.950^{a}	67.815^{a}
	h-C	71.595^{a}	70.125^{b}	68.610^{c}	72.645^{a}	69.840^{b}	66.975^{c}	69.900^{a}	69.930^{a}	69.825^{a}
	h-DHC	83.925^{a}	82.110^{b}	80.085^{c}	84.585^{a}	81.780^{b}	78.975^{c}	81.795^{a}	81.780^{a}	81.735^{a}
	CTE	92.715^{a}	91.110^{b}	89.220^{c}	93.330^{a}	90.840^{b}	88.305^{c}	90.840^{a}	90.825^{a}	90.795^{a}
	DHCTE	101.355^{a}	99.765^{b}	97.995^{c}	101.970^{a}	99.495^{b}	97.155^{c}	99.555^{a}	99.525^{ab}	99.480^{b}
Peak area	**n-DHC**	7337.00^{a}	7526.75^{b}	7692.25^{c}	7481.75^{a}	7280.50^{b}	7027.75^{c}	6094.50^{a}	7324.00^{b}	8591.50^{c}
	C	138396.5^{a}	138332.5^{a}	138315.3^{a}	145670.5^{a}	137996.0^{b}	131062.5^{c}	115107.0^{a}	138364.3^{b}	161446.3^{c}
	DHC	60945.25^{a}	60595.00^{b}	60831.75^{c}	63578.75^{a}	60452.00^{b}	57406.25^{c}	50384.00^{a}	60576.00^{b}	70731.00^{c}
	h-C	6595.25^{a}	5772.25^{b}	5979.75^{c}	6034.25^{a}	5774.50^{b}	5452.00^{c}	4809.25^{a}	5784.00^{b}	6746.75^{c}
	h-DHC	13906.25^{a}	14034.00^{b}	13921.25^{a}	15488.25^{a}	14547.00^{b}	13507.25^{c}	12161.25^{a}	14617.25^{b}	17065.00^{c}
	CTE	25363.50^{a}	25549.00^{b}	25627.50^{b}	26947.00^{a}	25559.50^{b}	24303.00^{c}	21315.00^{a}	25602.75^{b}	29915.00^{c}
	DHCTE	4323.75^{a}	4597.25^{b}	4772.00^{c}	4735.00^{a}	4549.00^{b}	4456.25^{c}	3825.00^{a}	4526.25^{b}	5290.75^{c}
Peak resolution	**n-DHC**									
	C	0.7696^{a}	0.7918^{a}	0.8974^{b}	0.8784^{a}	0.8659^{ab}	0.8458^{b}	0.8564^{a}	0.8654^{a}	0.8630^{a}
	DHC	3.1649^{a}	3.5253^{b}	3.5861^{b}	3.5492^{a}	3.5460^{a}	3.5033^{a}	3.5634^{a}	3.5730^{a}	3.5362^{a}
	h-C	0.9643^{a}	1.0801^{b}	1.0636^{b}	1.0730^{a}	1.0874^{a}	1.0602^{a}	1.0865^{a}	1.0668^{a}	1.0757^{a}
	h-DHC	6.9961^{ab}	7.1833^{a}	6.9035^{b}	5.8608^{a}	5.5375^{b}	5.5654^{b}	5.5817^{a}	5.5283^{a}	5.5403^{a}
	CTE	4.8006^{a}	5.0352^{b}	5.0408^{b}	3.9526^{a}	3.9723^{a}	4.0903^{b}	4.0213^{a}	4.0218^{a}	3.9955^{a}
	DHCTE	4.7525^{a}	5.2074^{b}	4.8416^{a}	5.1035^{a}	5.2089^{a}	4.9887^{a}	5.1116^{a}	5.2420^{a}	5.1712^{a}

The developed method showed a total robustness regarding retention times and resolution when the injection volume changed from 1.8 to 3.3 μL, with the only exception being DHCTE. The peak area was not taken into account since it varies depending on the volume injected. Therefore, it is essential to have an exact control on the amount of sample injected.

With respect to the effect that the controlled variations had on the chromatographic resolution, the method proved to be practically robust (*p* greater than 0.05) when the temperature or the flow rate were modified, with some exceptions affecting C or h-DHC. As far as the influence that the variations of the column temperature and the flow rate would have on the method, significant differences (*p* less than 0.05) resulted with regards to retention times and peak areas. This means that the developed method is highly sensitive and susceptible to be affected by any change in these two parameters. They should, therefore, be adequately controlled and a precautionary statement should accompany the method documentation. Nevertheless, as long as an adequate set up and conditioning of the equipment is performed, the method has proven its capacity to perform a highly accurate and distinctive separation of the chromatographic peaks (Shabir, 2003).

3.3. Implementing the method to the analysis of real sample

The developed and validated UHPLC-PDA method was applied to the analysis of several pepper varieties in order to quantify their major capsaicinoids and capsinoids contents. The results can be seen in Table 5. Firstly, it should be noted that the presence of DHCTE was not observed in any of the varieties studied. C and DHC were the major compounds in all the analyzed samples, followed by CTE, with C showing the highest concentration in all the peppers studied. The samples evaluated showed a great variability with respect to the total content of these compounds of interest (0.15 – 9.6 mg g^{-1}). The largest concentrations were registered in Naga Jolokia peppers, where total concentration reached up to 9.6549 mg g^{-1}. Finally, we should mention that Biquinho pepper, a sweet variety, did not present any capsaicinoid or they were below the detection limit. On the other hand, among the compounds studied, only capsiate could

be detected in Biquinho samples. These results corroborate the efficiency of the developed method and its reliability for the simultaneous determination of capsaicinoids and capsinoids in pepper extract samples.

Table 5 Concentration (mg g^{-1}) of the studied compounds in several pepper sample varieties (n = 3)

Sample	n-DHC	C	DHC	h-C	h-DHC	CTE	DHCTE	Total
Habanero Roxo	0.0748 ± 0.0069	3.5504 ± 0.2442	0.5917 ± 0.0315	0.0217 ± 0.0035	0.0341 ± 0.0075	0.0918 ± 0.0012	-	4.3644 ± 0.2948
Bode	0.0334 ± 0.0042	0.9505 ± 0.1329	0.3925 ± 0.0246	0.0139 ± 0.0017	0.0306 ± 0.0041	0.2536 ± 0.0143	-	1.6745 ± 0.2158
Naga Jolokia	0.1846 ± 0.0088	6.0586 ± 0.1939	2.9649 ± 0.0930	0.1301 ± 0.0037	0.0958 ± 0.0024	0.2209 ± 0.0012	-	9.6549 ± 0.3009
Malagueta	0.1218 ± 0.0010	1.6369 ± 0.0287	0.7367 ± 0.0069	0.0818 ± 0.0011	0.0733 ± 0.0006	0.3799 ±0.0199	-	3.0304 ± 0.0364
Filius Blue Green Variety	0.1028 ± 0.0005	1.3534 ± 0.0013	0.5086 ± 0.0010	0.0387 ± 0.0004	0.0389 ± 0.0018	0.3365 ± 0.0204	-	2.3789 ± 0.0099
Filius Blue Purple Variety	0.1178 ± 0.0029	1.2709 ± 0.0198	0.6393 ± 0.0046	0.0289 ± 0.0006	0.0445 ± 0.0028	0.4904 ± 0.0358	-	2.5918 ± 0.0219
Habanero	0.0449 ± 0.0012	1.3971 ± 0.0196	0.2861 ± 0.0061	0.0157 ± 0.0015	0.0075 ± 0.0009	0.1378 ± 0.0071	-	1.8891 ± 0.0224
Biquinho	-	-	-	-	-	0.1756 ± 0.0055	-	0.1756 ± 0.0055

4. Conclusion

The rp-UHPLC-PDA method developed is a rapid and reproducible analytical tool for the first simultaneous determination of capsaicinoids and capsinoids contents in peppers. The separation of all the compounds of interest was achieved in less than 2 min. This means a significant improvement and a substantial reduction in the analysis time, solvent and associated costs compared to the methods reported in the literature. The optimal chromatographic variables were as follow: 65 ºC column temperature, 0.95 mL min^{-1} flow rate and 3 μL injection volume. The developed method has exhibited high precision (CV less than 5%); as well as excellent validation results (sensitivity, linearity, LOD and LOQ). In this sense, it can be concluded that the combination of UHPLC under carefully optimized chromatographic conditions results in a

considerable performance improvement when compared to other conventional methods. Finally, the proposed UHPLC-PDA method was successfully applied for the determination and quantification of major capsaicinoids and capsinoids content in peppers. This method would be adequate for the determination of other products that may contain *Capsicum* extracts.

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CRediT authorship contribution statement

Mercedes Vázquez-Espinosa: Data curation, Formal analysis, Investigation, Writing - original draft. **Ana V. González-de-Peredo:** Data curation, Formal analysis, Investigation, Writing - original draft. **Estrella Espada-Bellido:** Investigation, Methodology. **Marta Ferreiro- González:** Data curation, Software, Supervision. **Gerardo F. Barbero:** Conceptualization, Investigation, Methodology, Supervision. **Miguel Palma:** Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.129688.

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5.2. Desarrollo y optimización de un método para la extracción de capsinoides presentes en pimientos

Los resultados presentados en este capítulo se han publicado en:

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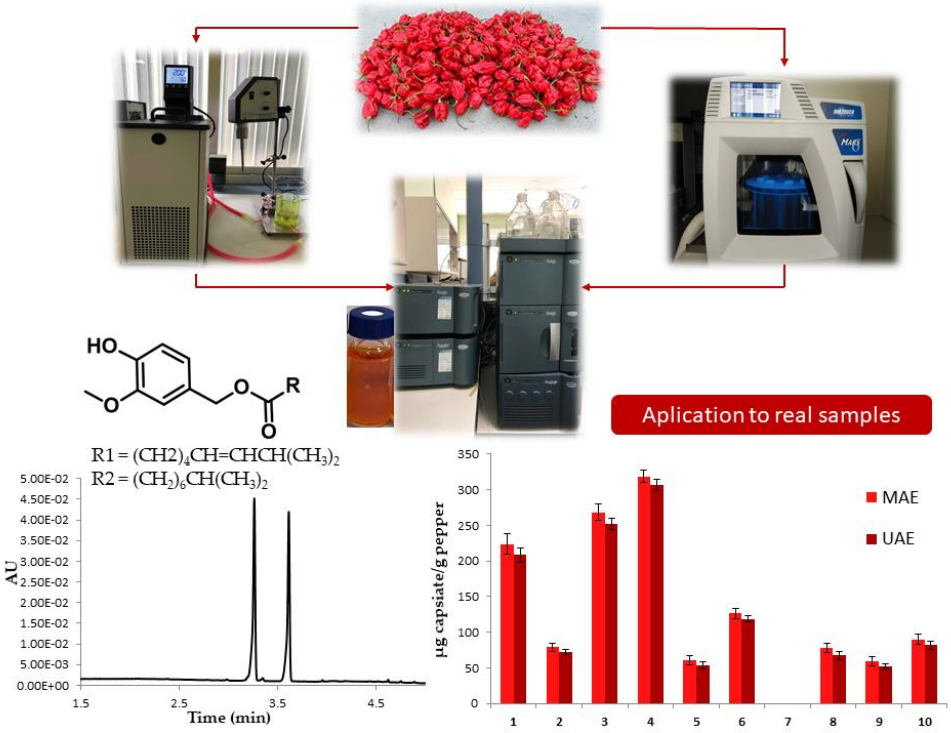

- **Optimizing and Comparing Ultrasound- and Microwave-Assisted Extraction Methods Applied to the Extraction of Antioxidant Capsinoids in Peppers**

Abstract

Capsinoids are very similar antioxidant compounds to capsaicinoids, but less irritating, non-pungent and more palatable, and can thus be used in greater concentrations for food applications. To date, three capsinoids (capsiate, dihydrocapsiate, and nordihydrocapsiate) have been isolated from the pepper fruits. Due to its substantial commercial importance, it would be convenient to determine which pepper varieties have a richer content. Ultrasound- (UAE) and microwave- (MAE) assisted extraction have been implemented and analyzed using multivariate statistical methods. Firstly, different solvents were tested individually. The three best solvents were used in a set mixture design, where 42% methanol and 58% ethyl acetate were determined as the optimum combination for UAE, and 100% methanol for MAE.

Subsequently, a Box–Behnken experimental design with four variables for both UAE and MAE (time, temperature, pH and sample mass:solvent volume "ratio") was performed. The sample mass:solvent volume was the most influential variable in UAE; while for MAE no variable was any more influential than the others. Finally, both optimized extraction methods were successfully applied to different varieties of peppers. Besides, to demonstrate the effciency of both extraction methods, a recovery study was performed. The results prove the potential of both techniques as highly adequate methods for the extraction of capsinoids from peppers.

1. Introduction

Peppers (*Capsicum* spp.) are plants from the Solanaceae family, originating in Central and South American tropical and rainy areas [1]. Nowadays, the pepper is a well-known fruit due to its high content in bioactive compounds and its strong antioxidant capacity. It is among the most popular fresh vegetables worldwide due to its combination of aroma, color, flavor, and nutritional value [2]. Peppers are not only valued for their sensory attributes, but also have a significant role in medical and pharmaceutical applications [3]. This fruit is a great source of nutraceutical compounds such as capsaicinoids and capsinoids, bioactive components that support a healthy diet [4].

One of the main features of red peppers is their pungency, which is caused by a specific type of chemical compounds known as capsaicinoids [5]. They have three clearly differentiated sections, the vanillyl group, the carboxamide group and the aliphatic chain [6]. These compounds have exhibited a large number of biological properties of pharmacological relevance such as antioxidant, anti-inflammatory, analgesic, antimicrobial and anticarcinogenic [7,8]. Furthermore, they are related to an increase in the energy of the body and decrease in both body fat and cholesterol, which leads to a reduction in cardiovascular diseases, diabetes or cerebrovascular accidents [9]. One of its negative aspects is that long term or high dose exposures have a detrimental effect on the users' gastric mucosa and ultimately on the health [10]. On the contrary, capsinoids, although very similar to capsaicinoids, are less irritating, non-

pungent and more palatable, so a larger amount of them can be consumed on a daily basis without any negative consequences. In addition, they have the same above-mentioned beneficial properties as capsaicinoids on human health; particularly their antioxidant properties, but without any burning feeling or negative effects on consumers' organisms [11–13]. They have a very similar chemical structure except for their central bond, which is an ester group instead of an amide group. This structural difference could be responsible for the lower stability of capsinoids [14]. The difference in pungency is associated to vanilloid type-1 receptor (TRPV1), which is the component responsible for the burning sensation. Capsaicinoids activate TRPV1 on the tongue, while capsinoids have the ability to activate it after it has reached the intestine, which results in the absence of burning sensations, although with similar effect [15]. The chemical structures are shown in Figure 1.

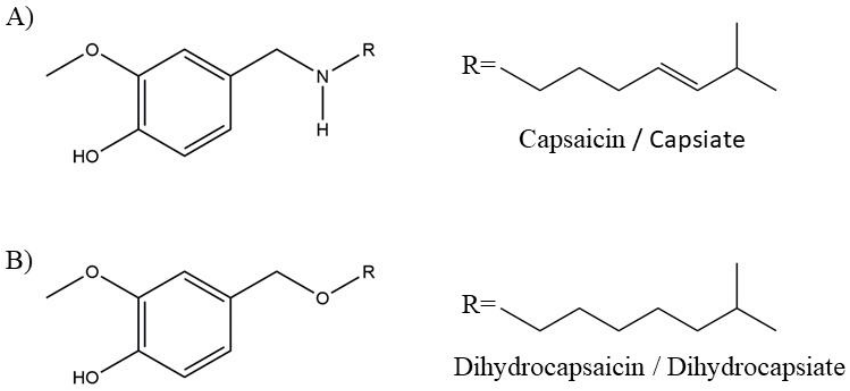

Figure 1. Chemical structures of capsaicinoids (A) and capsinoids (B).

Extraction is a very important step for the isolation and separation of the compounds of interest from the solid matrix and there is no single and standard extraction method. The extraction process depends on the conditions to which the sample is treated. Capsinoids extraction is of great importance due to their excellent beneficial properties for health, their high antioxidant capacity, and their great comercial importance agri-food applicability thanks to the lower pungency compared to that of capsaicinoids. There has been a recent growing demand for extraction techniques that shorten extraction times, and reduce the consumption of organic solvents [16]. Among other extraction methods, ultrasound-assisted extraction (UAE)

and microwave-assisted extraction (MAE) are considered excellent green alternatives that have been widely used for the extraction of bioactive compounds. When compared to conventional methods they present some advantages such as their simplicity, greater effciency and performance. They are also more environmentally friendly, faster, and reduce solvent and energy consumption with an improved control of temperature [17].

UAE is based on the use of energy derived from ultrasounds (sound waves with frequencies higher than 20 kHz) to facilitate the extraction of compounds from the simple matrix by an organic solvent according to the nature of the solutes to be extracted [18]. The improvement in biological compounds extraction effciency achieved by means of ultrasounds is attributed to the cavitation that occurs within the solvent. When ultrasounds are applied, cavitation bubbles are produced and compressed. The increase in the pressure and temperature leads to the collapse of the bubble, which results in a 'shock wave' that breaks the solid matrix's cell walls, which, in turn, favors the penetration of the solvent, the mass transfer and the release of the analytes, thus increasing the extraction performance [19,20]. It is a widely employed technique, recently used to extract different varieties of compounds of biological interest from potatoes [21], grapes [22] and blueberries [23].

MAE consists of an electric and magnetic field that oscillate perpendicularly (high frequency non-ionizing electromagnetic waves between 0.3–300 GHz) [24]. The molecules in the sample try to align themselves in phase with the electromagnetic field of the appropriate frequency, but due to the intermolecular forces, they suffer inertia and are unable to align with the waves. This leads to a random movement of them, which generates heat. This selective form of heating is much more efficient and homogeneous and the energy consumption is much lower. In addition, the migration of disolved ions increases the penetration of the solvent into the matrix and favors the release of analytes [25,26]. Microwaves have also been used to extract antioxidant substances from a large number of matrices, such as tomato [27], oil [28] or blackberries [29].

There is no standard protocol for UAE and MAE, and several parameters, such as the solvent concentration, ratio, extraction time and temperature, may significantly affect the extraction efficiency. Therefore, the determination and the optimization of key parameters are important for maximizing the yield [30].

Although a large number of studies have been carried out on the extraction of capsaicinoids [31–33], using a considerable variety of solvents, scarce studies on the extraction of capsinoids have been reported in literature. Moreover, most of them analyze the implementation of an extraction method specifically developed for capsaicinoids, to the extraction of capsinoids. However, capsinoids have a lower polarity, and it is, therefore, necessary to develop new and specific extraction methods for them [34].

Multivariate statistical techniques have been previously used to optimize different processes such as extraction and chromatographic separation of antioxidant compounds. The main benefit obtained from the optimization of these processes is the shortening of time and the reduction in the number of experiments required with the subsequent economic and energy consumption savings [35]. In this work, the optimization process includes a mixture design—a special class of response surface experiments where the investigated factor (in this case the extraction solvent) is made up of several components. The solvent components interact with each other and these solvent–solvent interactions are taken into account to determine the optimal mixture by improving several responses at the same time [36]. Subsequently, a Box–Behnken experimental design (BBD) was used; a response surface factorial design that avoids performing experiments under extreme conditions. Moreover, BBD allows for the development of mathematical models that facilitate the assessment of the statistical significance of each factor analyzed, as well as their interactions. When interaction effects are detected, the optimal conditions between univariate and multivariate optimization will be different [37,38].

Capsinoids are compounds with superior health benefits, as well as major antioxidant properties, but they degrade easily and in a short time [39].

For this reason, it is necessary to develop extraction and analysis techniques that indicate those pepper varieties that are rich in the compounds of interest to obtain higher quality extracts. Since an exhaustive analytical method for the extraction of capsinoids has not yet been developed, the aim of the work described here is to apply multivariate statistical techniques to optimize the extraction of capsinoids from peppers by both UAE and MAE methods.

2. Materials and Methods

2.1. Reagents

Methanol, ethanol, acetone, ethyl acetate and acetronitrile were obtained from Panreac Química (S.A.U., Castellar del Vallés, Barcelona, Spain), hexane from Carlo Erba Reagents (Dasit Group, Sabadell, Barcelona, Spain), and acetic acid from Merck (Darmstadt, Germany), all of these substances were of HPLC grade. Milli-Q water was obtained by means of a Millipore water purification system (Bedford, MA, USA). Solutions of hydrochloric acid and sodium hydroxide (Panreac Química, S.A.U, Castellar del Vallés, Barcelona, Spain) were used to adjust the pH values with a Crison GLP 21 pH-meter (Crison, Barcelona, Spain). Capsiate (4-hydroxy-3-methoxybenzyl (E)-8-methyl-6-nonenoate) and dihydrocapsiate (4-hydroxy-3-methoxybenzyl-8-methylnonanoate) standards were synthesized following the method described by Barbero et al. [40].

2.2. Pepper Sample

Biquinho (*Capsicum chinense*) was the pepper variety employed to study the extraction process. The samples were supplied by Germplasm Bank of Zaragoza from the Agrifood Research and Technology Center (CITA) in Aragón (Zaragoza, Spain). The Biquinho variety was chosen because a high content in capsiate had been detected in the preliminary tests. The sample was lyophilized by means of a laboratory freezer dryer LYOALFA manufactured by Azbil Telstar Technologies (Terrasa, Barcelona, Spain); it was then ground in a conventional electric mill to increase the contact surface between the solvent and the fruit, and stored at -20 ºC prior to analysis.

The methods developed were applied to other ten varieties of peppers to determine the amount of capsiate (care was taken to select the fruit of the same generation and with similar size). All of them were sown and harvested at the Agronomic Institute of Campinas (IAC) (Campinas, SP-Brazil), and stored at -20 ºC.

2.3. Extraction Equipment and Procedure

2.3.1. Ultrasound-Assisted Extraction (UAE)

The extraction was performed using a UP200S ultrasonic system (Hielscher Ultrasonics, Teltow, Germany) which allows the control and modification of both the amplitude and the cycle. The sample was immersed in a thermostatic bath coupled to a temperature controller (7 Liter Refrigerated Circulator, PolyScience, Illinois, United States) to maintain the desired extraction temperature. Lyophilized sample (approximately 0.2 g) was weighed in a 50 mL "Falcon" and the necessary volume of the appropriate solvent was added based on the experimental design. Then, it was introduced into the double-walled vessel to control the temperature and the ultrasonic probe was also inserted while avoiding any contact with the vessel's walls and bottom. The extraction was started under controlled UAE conditions. After the extraction, the sample was allowed to cool down at room temperature. The extract was centrifuged twice for 5 min at 7500 rpm (orbital radius 9.5 cm), transferring the supernatant after each centrifugation cycle to a 25 mL volumetric flask and making up to the mark with the appropriate solvent. Finally, the extract was transferred to a vial and stored in a freezer at -20 ºC prior to analysis.

2.3.2. Microwave-Assisted Extraction (MAE)

A MARS 6 240/50 (One Touch Technology, CEM Corporation, Matthews, NC, USA) was used for MAE. Similarly to the UAE described above, the necessary amount of sample (0.2 g) was weighed and put into an extraction chamber (high pressure Teflon containers). Then, the corresponding amount of the optimized solvent was added, it was closed by means of a teflon cap to prevent gas leaks that may be generated because of the high temperatures and it was then placed in the rotary carousel.

Once all the cameras had been prepared, the carousel was placed into the extraction equipment and closed to start the corresponding extraction program as configured. The rest of the procedures after the extraction were the same as for the previously explained UAE.

2.4. UHPLC Analysis

An UHPLC Acquity Ultra Performance LC Class (Waters Corporation, Milford, MA, USA) system was used for the separation and quantification of capsinoids. It was equipped with an ACQUITY UPLC®H-Class autosampler set at 15 ºC, an ACQUITY UPLC quaternary solvent manager, an ACQUITY UPLC Photodiode Array Detector set at 280 nm, and an ACQUITY UPLC BEH C-18 reverse phase column (100 mm x 2.1 mm; 1.7 µm particle size) set at 50 ºC. The mobile phase consisted of Milli-Q water as solvent A and acetonitrile as solvent B, both acidified by adding 0.1% acetic acid. Theses solvents were filtered through a 0.22 µm nylon filter (Nylon Membrane Filter, FILTERLAB, Barcelona, Spain) and degassed using an ultrasonic bath (Elma S300 Elmasonic, Singen, Germany). The gradient employed was as follows: 0 min, 0% B; 0.50 min, 45% B; 1.60 min, 45% B; 1.95 min, 50% B; 2.45 min, 55% B; 2.80 min, 63% B; 3.00 min, 63% B; 4.00 min, 100% B; 6.00 min, 100% B. An injection volume of 3 µL and a flow of 0.8 mL min^{-1} were applied. The UHPLC chromatogram is shown in Figure S1.

A calibration curve was used to quantify the compounds of interest. Capsiate and dihidrocapsiate at known concentrations between 0.01 and 200 mg L^{-1} were used as reference patterns. The calibration curves and the limits of detection and quantification were as follows: y = 2715.2076x – 526.9713, LOD = 0.079 mg L^{-1}, LOQ = 0.265 mg L^{-1} / y = 2498.4568x – 382.2437, LOD = 0.096 mg L^{-1}, LOQ = 0.320 mg L^{-1}, for capsiate and dihidrocapsiate respectively, both with a linear regression coefficient (R^2) of 0.9997. The results were expressed as micrograms of capsinoids per gram of dry pepper.

2.5. Experimental Design and Data Treatment

Two statistical designs were proposed for the optimization of the capsinoid extraction methods from peppers. The first method consists of selecting the best extraction solvent based on a combination of water, acetonitrile, methanol, ethanol, acetone, hexane or ethyl acetate. Then, a statistical mixture design with three components (the three best solvents for each method) was used to determine the optimal mixture of the solvents.

After the optimal extraction solvent was selected, a Box–Behnken experimental design was carried out to determine the best equipment conditions in relation to time, temperature, pH, and ratio. During the optimization procedures, two types of variables were considered: the responses or dependent variables, and the factors or independent variables. The latter could take three posible values, which were coded as -1, 0, and 1 representing their minimum, medium, or maximum value, respectively. An experimental design matrix with 27 trials was performed in duplicate with six repetitions of the center point. Both responses obtained from the different extractions were entered into a second-order polynomial equation to correlate them with the independent variables including the linear, quadratic and interactive components [41,42]:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} X_i X_j + \varepsilon$$

where y is the response, β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for the terms of intersection, linear, quadratic and interaction, respectively; and i and j are the independent coded variables that affect the response.

These coefficients as well as the statistical significance of the model were determined by the analysis of variance (ANOVA). For the experimental design, data analysis and model building, the Statgraphic Centurion Version XVII (The Plains, Fauquier, Virginia, United States) was employed.

3. Results and Discussion

3.1. Selecting the Extraction Solvent

3.1.1. Solvent Selection Procedure for UAE

A mixture design was performed in order to determine the optimal extraction solvent. The rest of the variables were set as follows: 10 min extraction time, 40 ºC temperature, 80% of the total power (200 W) and 10 mL volume. These initial conditions had been previously analyzed to verify that they were valid and that the extraction of capsinoids was feasible. These results are shown in Figure S2.

After setting up these conditions, the extraction efficiency of different single component solvents was tested (Milli-Q water, acetonitrile, methanol, ethanol, acetone, hexane, and ethyl acetate). All the extractions were carried out in duplicate and the results are represented in Figure 2.

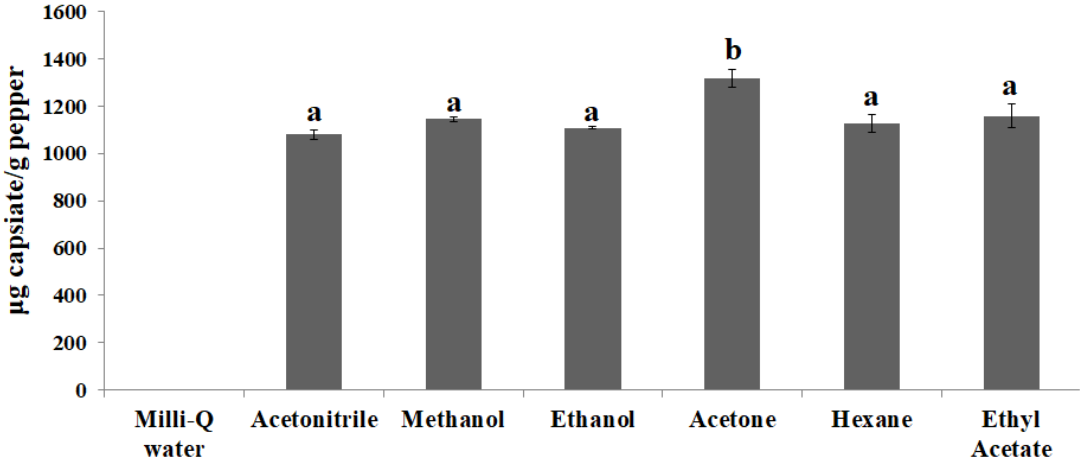

Figure 2. Capsiate concentration (µg capsiate/g pepper) obtained with different individual solvents by ultrasound-assisted extraction (UAE). The use of different letters indicates that there is a significant difference according to Tukey's test ($p < 0.05$).

It can be seen that no capsiate was extracted when wáter was used as the only solvent. Due to its high polarity, it is not a good solvent for the extraction of this type of compounds, which have an aromatic ring and a hydrocarbon chain in their structure.

Hexane is a very volatile solvent, so a large amount of it was lost by evaporation during the extraction procedure, which resulted as detrimental for its extraction efficiency. Furthermore, it has a lower polarity than capsiate, which makes extraction difficult. For these two reasons, i.e., polarity and affinity, acetone, ethyl acetate and methanol proved to be the most suitable solvents to extract capsiate by producing the highest extraction yields.

These three solvents were, therefore, selected for the mixture design (Table S1). Statgraphic Centurion was the statistical program used and Simplex Centroid Extended model with 10 experiments was employed. Linear, quadratic and cubic mathematical models were tested to explain the results. Each mathematical model was validated by means of an analysis of variance (ANOVA), as shown in Table 1. In turn, the optimal mixture of solvents was observed in the region that can be seen in Figure 3.

Table 1. Analysis of variance (ANOVA) of the mathematical models of the statistical mixture design obtained by ultrasound-assisted extraction.

	Mathematical Model	*p*-Value	R^2 Coefficient (%)	F-Value
UAE	Linear	0.7579	7.6159	0.29
	Quadratic	0.0051	96.5392	22.32
	Cubic	0.0250	96.7146	14.72

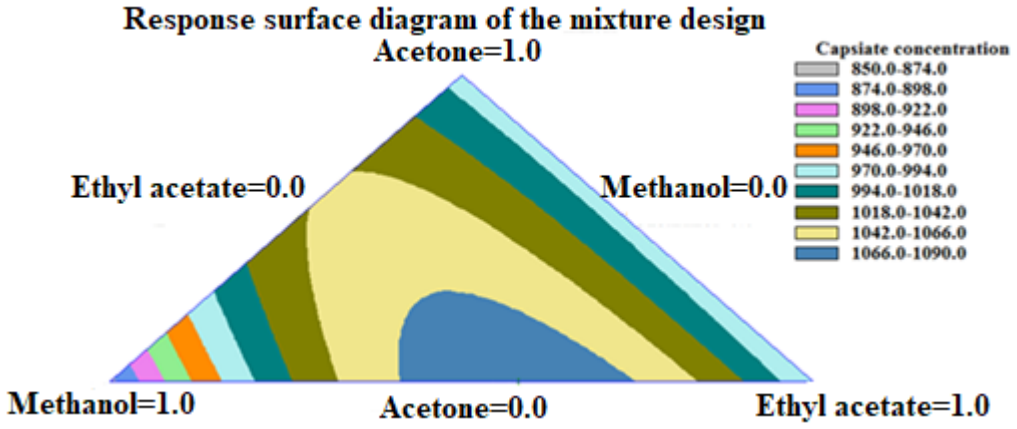

Figure 3. Mixture design diagram obtained by ultrasound-assisted extraction.

As far as UAE is concerned, both the quadratic and the cubic models were significant, since they showed a *p*-value less than 0.05 as well as high F and R^2 values. The same optimum mixture of solvents was obtained for both models: 42% methanol + 58% ethyl acetate.

Similar studies for the extraction of capsaicinoids, where different solvents (methanol, ethanol, acetonitrile, acetone, ethyl acetate and water) had been tested, have been found in literature. A 100% methanol was determined as the optimal extraction solvent [33,43]. Due to the lower polarity of capsinoids in comparison with capsaicinoids [44], it was to be expected that the optimum solvent or solvent mixtures should have a lower polarity than of methanol to facilitate the extraction process. In this sense, Lang Y. et al. used 100% ethyl acetate for the extraction of capsinoids from peppers [45], but, unfortunately, they did not fully develop the method. After all the tests were completed, the optimal solvent for the UAE method was found to be a combination of methanol and ethyl acetate.

3.1.2. Solvent Selection Procedure for MAE

MAE was tested with the same individual solvents used for UAE. In this case, the rest of the variables were adjusted as follows: 5 min extraction time, 800 W power, 10 mL volume and 50 ºC temperature—which was the minimum temperature allowed by the equipment. All the extractions were carried out in duplicate and the results are represented in Figure 4.

As expected, when water was used as the only solvent the amount of compounds extracted was practically negligible due to its high polarity in comparison with that of capsiate. The solvents that obtained the best extraction yields with MAE were metanol and ethanol, followed by acetone.

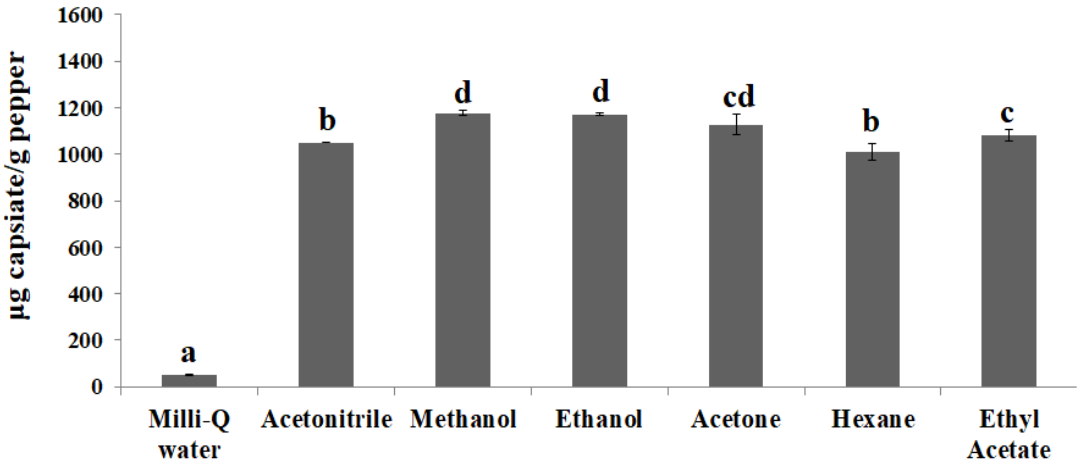

Figure 4. Capsiate concentration (µg capsiate / g pepper) with different individual solvents by microwave-assisted extraction (MAE). The use of different letters indicates that there is a significant difference according to Tukey's test ($p < 0.05$).

A slightly different result was obtained compared to UAE, since both extraction methods have different properties, conditions and operation procedures. In the UAE method, sound waves travel through the material and cause the cavitation phenomena that heat the entire sample evenly over the extraction process [18]. On the contrary, in the MAE method, the heat is generated by the interaction between the radiation and the molecules, as the molecules try to align with the waves of the electromagnetic field. The radiation excites mainly the OH bond in methanol and ethanol. By absorbing energy, it quickly passes from the ground state to an excited one, which raises the energy of the molecules and their temperature, what in turn enables the extraction of the compounds of interest from the plant matrix [25]. Methanol, ethanol and acetone have exhibited the most appropriate characteristics in terms of polarity and affinity for the extraction of capsinoids from peppers by MAE.

These three solvents were, therefore, selected for the mixture design (Table S2). An identical statistical treatment as the used for UAE was applied to MAE, i.e., Simplex Centroid Extended model with 10 experiments. Then, each mathematical model was validated by an analysis of variance (ANOVA), as shown in Table 2. Figure 5 shows the optimal mixture of solvents.

Table 2. Analysis of variance (ANOVA) of the mathematical models of the statistical mixture design obtained by microwave-assisted extraction.

	Mathematical Model	*p*-Value	R^2 Coefficient (%)	F-Value
MAE	Linear	0.3851	23.8650	1.10
	Quadratic	0.2538	71.8957	2.05
	Cubic	0.3299	78.6285	1.84

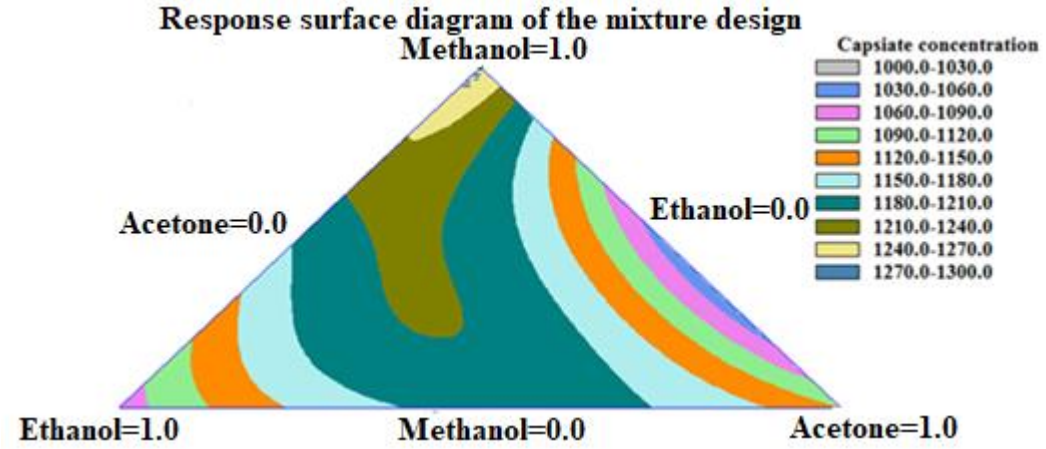

Figure 5. Mixture design diagram obtained by microwave-assisted extraction.

None of the models tested with MAE was particularly relevant, since none of them had a *p*-value lower than 0.05. Therefore, 100% methanol was used as the optimal solvent, since it was the optimum result of the model with the highest R^2 coefficient value. In addition, this solvent is the easiest one to use as it is compatible with the rp-C18 HPLC column and neither evaporation of the solvent from the extract nor redissolution of the compounds of interest are required.

3.2. Determining Optimal Ultrasound Conditions

After the optimal solvent mixture had been determined, a Box–Behnken design with four independent variables (time: 5, 10, 15 min, temperature: 5, 30, 55 ºC, pH: 2, 5, 8, and relation sample mass:solvent volume ratio: 0.2:5, 0.2:10, 0.2:15 g:mL) and concentration of capsiate (µg g^{-1}) as the response was employed.

For the optimization of such experimental variables, 27 experiments had to be completed, the results of which are shown in Table S3.

The analysis of variance in Table 3 generated some mathematical models that intended to evaluate the individual effect of each variable, as well as any possible interactions between them. Those variables or interactions that had a *p*-value lower than 0.05 were considered to have a statistically relevant influence on the response at 95% confidence level. Ratio was the only variable that had an influence on the amount of capsiate extracted.

Table 3. Analysis of variance (ANOVA) for the Box-Behnken experimental design (BBD) experiment design obtained by ultrasound-assisted extraction.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-Value	*p*-Value	Coefficient
A: Time	1	9.90	9.90	0.02	0.8777	-0.9086
B: Temperature	1	1689.52	1689.52	4.22	0.0625	-11.8656
C: pH	1	87.99	87.99	0.22	0.6478	-2.7079
D: Ratio	1	3059.83	3059.83	7.63	0.0172	15.9683
AA	1	155.11	155.11	0.39	0.5455	5.3928
AB	1	775.61	775.61	1.94	0.1894	13.9249
AC	1	4.71	4.71	0.01	0.9155	-1.0849
AD	1	360.88	360.88	0.95	0.3489	9.7581
BB	1	922.46	922.46	2.30	0.1551	13.1515
BC	1	1166.59	1166.59	2.91	0.1137	-17.0777
BD	1	677.18	677.18	1.69	0.2181	-13.0114
CC	1	142.12	142.12	0.35	0.5626	5.1621
CD	1	207.96	207.96	0.52	0.4851	7.2104
DD	1	341.03	341.03	0.85	0.3745	-7.9964
Residual	12	4809.17	400.76			
Total	26	14881.40				

These results were graphically represented by a Pareto chart (Figure 6) for a better understanding.

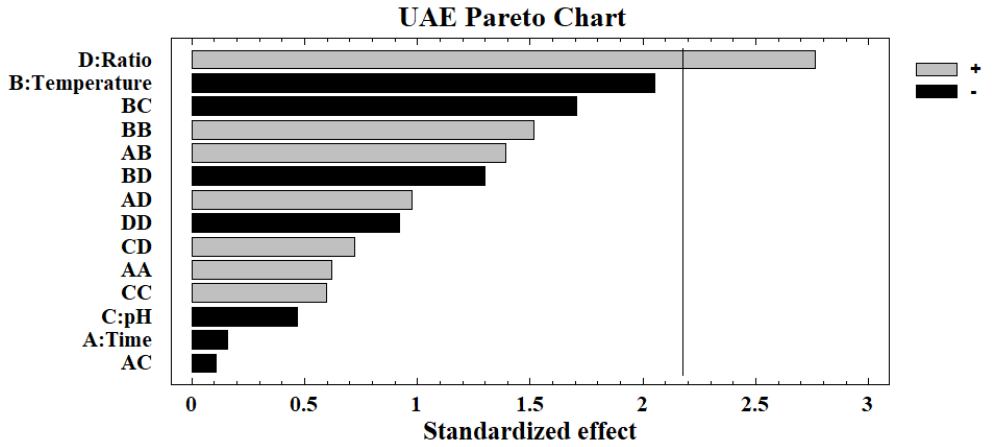

Figure 6. Pareto standardized diagram obtained by ultrasound-assisted extraction.

The significance of each variable and their interactions are easily observed in decreasing order. In turn, positive and negative signs refer to a direct or inverse relationship, between the effect and the response. Again, ratio was the only variable with a significant influence. In addition, the greater the amount of solvent, the greater the amount of capsiate that would be extracted. This is concordant with mass transfer principles, since a higher ratio implies higher concentration gradient between the solid and the bulk of the liquid, resulting in a greater driving force for diffusion of compounds to the solvent [46], but up to a certain amount of solvent, where the extract would be exceedingly diluted for any quantification [47]. The effect of temperature was rather similar to that of ratio, although inversely; i.e., the lower the temperature, the greater the amount of capsiate that would be extracted.

This is due to the easier degradation of this compound at moderately high temperatures [48]. It can be said that a quite robust method has been validated, and the optimized solvent mixture, using this extraction technique, allows for extracting practically the total amount of capsiate present in the pepper samples by means of UAE. The variations in the other factors had hardly any influence on the extraction yields.

After the statistical treatment of the data, the following optimal conditions were determined: 5 min extraction time, 5.5 ºC temperature, pH 8 and 14.5 mL volume by weight.

Although the extraction time was not a significant variable with regard to its influence on the response, the shortest time was selected as the optimal one. For this reason, a univariate study of the extraction kinetics was carried out to find out if the same or an even greater amount of capsiate could be extracted in a shorter time. The extractions were carried out in triplicate for 1, 2, 5 and 10 min under the optimal previous conditions. The results are shown in Figure 7.

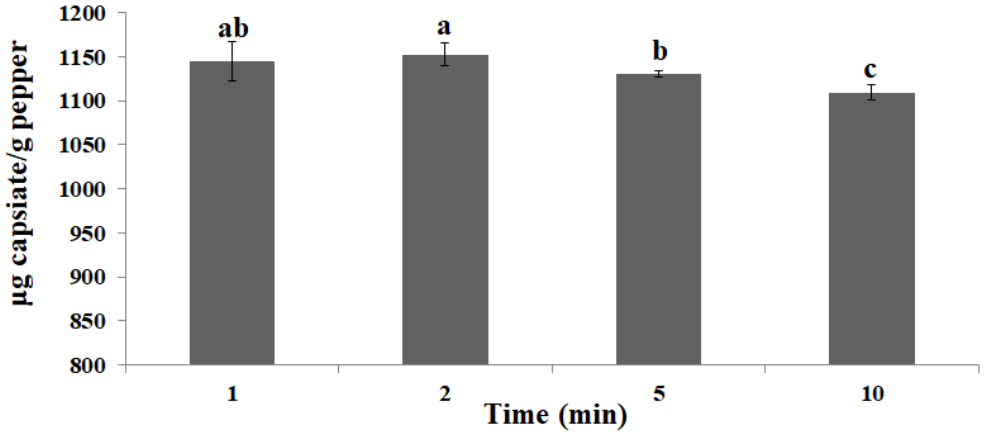

Figure 7. Capsiate concentration (µg capsiate / g peppers) in relation to the extraction time obtained by ultrasound-assisted extraction. The use of different letters indicates that there is a significant difference according to Tukey's test ($p < 0.05$).

It was observed that there were no significant differences between 1 and 2 min, although a greater amount of capsiate extracted at 2 min was visually noticeable. Therefore, it is a fairly rapid method related to the degradability of capsiate.

Finally, intermediate precision and repeatability tests were carried out under the previously determined optimal conditions. For repeatability, 10 extractions were performed on the same day and for intermediate precision, 10 extractions per day were carried out during three consecutive days. The coefficients of variation obtained, 1.60% and 2.08%, respectively supported the precision of this extraction method (Table S4). Both coefficients were below 5%, which is usually considered as the limit to affirm that a method is accurate in this type of studies.

3.3. Determining Optimal Microwave Conditions

A similar statistical treatment as for UAE was applied to determine the optimal conditions for MAE. In this case, the ranges used for each independent variable were as follows: 5, 10, 15 min, 50, 75, 100 ºC temperature, 2, 5, 8 pH, and 0.2:5, 0.2:10, 0.2:15 g:mL sample mass:volume of solvent; i.e., ratio. The extracted capsiate (μg g^{-1}) was quantified as the response. The results are shown in Table S3.

The analysis of the variance (ANOVA) in Table 4 shows that no variable or interaction between them had a *p*-value lower than 0.05, so they did not have a statistically significant influence on the response, at 95% confidence level. In view of these results, they were analyzed with a confidence level of 98%, but again no variables were found to be significant.

Table 4. Analysis of variance (ANOVA) for the BBD experiment design obtained by microwave-assisted extraction.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-Value	*p*-Value	Coefficient
A: Time	1	6647.48	6647.48	2.38	0.1489	-23.5363
B: Temperature	1	74.42	74.42	0.03	0.8731	2.4904
C: pH	1	5.66	5.66	0.00	0.9648	0.6871
D: Ratio	1	1838.72	1838.72	0.66	0.4330	12.3785
AA	1	308.46	308.46	0.11	0.7454	-7.6050
AB	1	129.49	129.49	0.05	0.8331	5.6896
AC	1	76.57	76.57	0.03	0.8713	-4.3752
AD	1	38.29	38.29	0.01	0.9087	3.0938
BB	1	1122.19	1122.19	0.40	0.5381	-14.5056
BC	1	43.93	43.93	0.02	0.9023	-3.3138
BD	1	454.39	454.39	0.16	0.6938	-10.6582
CC	1	251.40	251.40	0.09	0.7693	6.8656
CD	1	2621.09	2621.09	0.94	0.3518	25.5983
DD	1	377.87	377.87	0.14	0.7194	-8.4173
Residual	12	33520.90	2793.41			
Total	26	47794.30				

The Pareto chart in Figure 8 confirmed that no variable or interaction exceeds the value of 2.17 and, therefore, their influence on the response was not significant. Several authors did not obtain large significance values for the variables analyzed with other similar matrices [49,50].

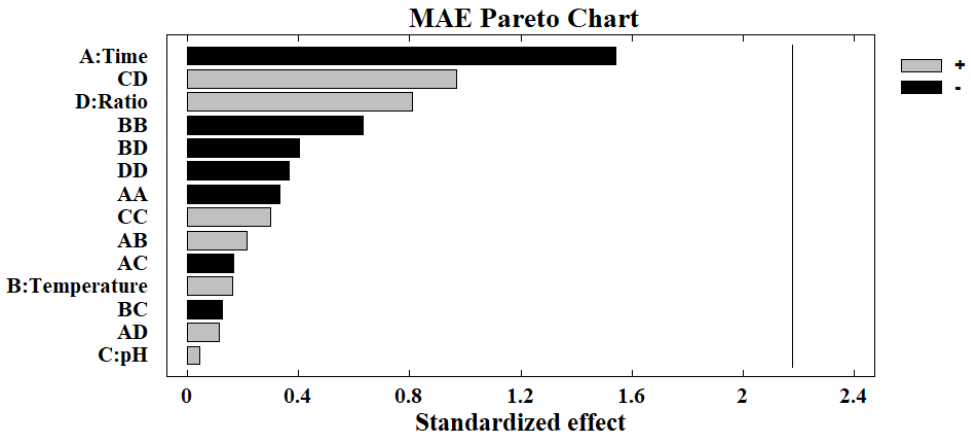

Figure 8. Pareto standardized diagram obtained by microwave-assisted extraction.

Even though no variable was actually significant, the most influential one was the extraction time, with an inverse relation, that is, the shorter the time the greater the amount of capsiate that would be extracted; followed by the ratio, which on the contrary, had a direct influence, i.e., the greater the amount of solvent, the greater the amount of the compound of interest that can be extracted. Finally, the other two variables analyzed, temperature and pH, had almost no influence on the response. These results indicate that MAE is a dependable method for the extraction of this type of compounds of interest present in peppers, since it allows for extracting the highest possible amount of capsiate from the samples regardless of any variations of the factors that have been analyzed.

After the statistical treatment of the data, the following optimal conditions were established: 5 min extraction time, 60 ºC temperature, pH 8 and 0.2:15 g:mL ratio. It can be seen that, for some of the variables extreme values within their intervals were found to be their optimal value. In relation to ratio, a limit value was considered above which the compound of interest would be exceedingly diluted for an accurate quantification. In relation to pH, it had almost no influence on the response.

The extraction time, even though it did not reach the minimum level to be considered as significant, was the variable that had the greatest influence on the response. In a similar way as for UAE, a univariate study of the extraction kinetics was carried out (Figure 9).

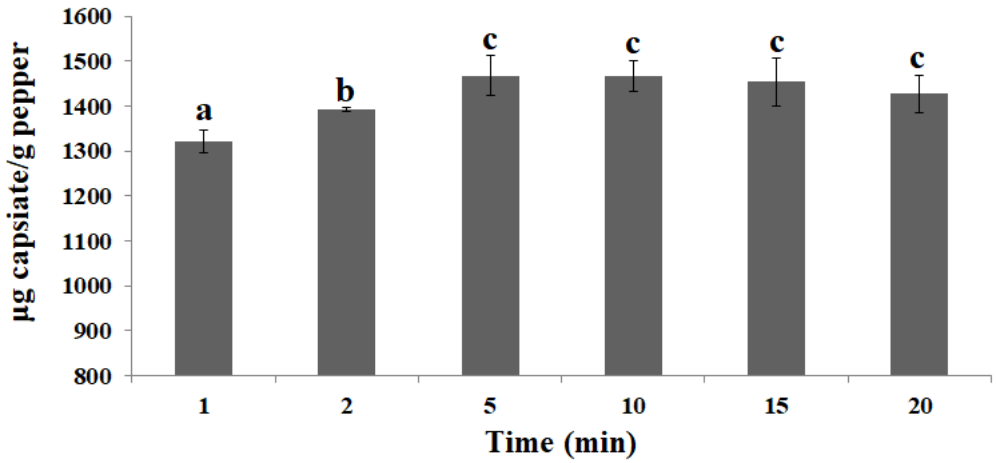

Figure 9. Capsiate concentration (µg capsiate / g peppers) in relation to the extraction time obtained by microwave-assisted extraction. The use of different letters indicates that there is a significant difference according to Tuckey's test ($p < 0.05$).

In this case, the time interval was extended in order to register the trend at higher values. Although no significant differences between 5, 10 and 15 min were registered, 5 min was established as the optimal extraction time for saving reasons.

Finally, a repeatability and intermediate precision study was carried out under the previously selected optimal extraction conditions and in the same way as explained above for UAE. Very accurate results were obtained, since the coefficient of variation were 2.48% for repeatability and 3.96% for intermediate precision (Table S4).

3.4. Real Samples Analysis

Once the optimal conditions for the extraction of capsinoids from peppers had been proposed, the developed method was tested on different pepper samples to verify the presence of these compounds and to quantify them. Lyophilized peppers were extracted in triplicate, then they were analyzed by UHPLC-DAD and the results can be seen in Figure 10.

The extraction method proved to be efficient for the extraction of capsinoids from different varieties of pepper. In addition, it was observed that the compounds of interest were present in both sweet (Biquinho), and spicy (Naga Jolokia or Malagueta) peppers varieties. Capsiate was found in most of the varieties studied, except for Bahiana, which was subjected to both extraction methods (UAE and MAE) without any success; i.e., no dihydrocapsiate was found by either method. Naga Jolokia was the variety of pepper with the greatest capsiate content, with extraction yields of 318.90 µg g^{-1} and 306.60 µg g^{-1} by MAE and UAE, respectively. Finally, it can be said that there were no significant differences between the capsiate extraction yields obtained by either extraction methods. However, visually it can be seen that a slightly larger amount of the compound of interest was extracted by the MAE method with respect to that of UAE.

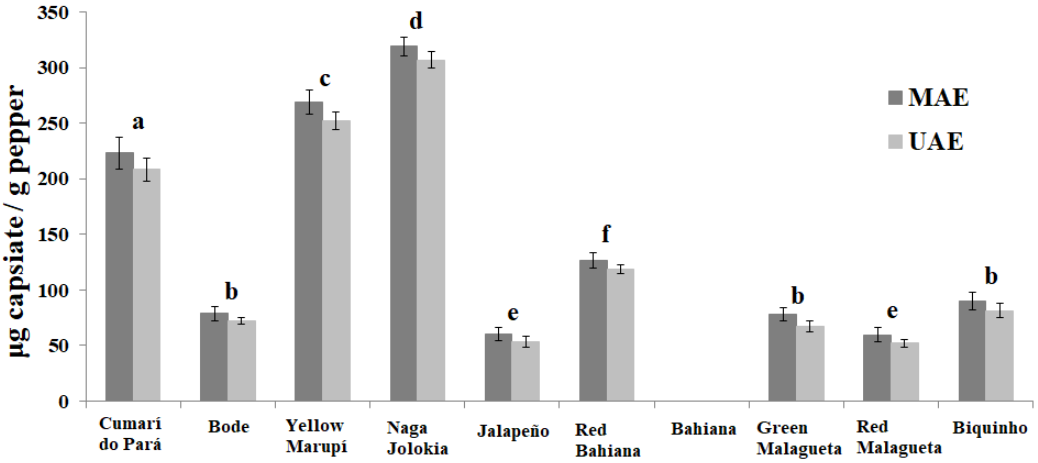

Figure 10. Capsiate extraction from real pepper samples (n = 3). The use of different letters indicates that there is a significant difference according to Tukey's test (p < 0.05).

3.5. Comparison between the Two Extraction Methods

Once all the experiments had been completed and their results collected, a comparison of both methods was performed (Table 5).

Table 5. Comparison of optimal extraction conditions of both developed methods.

	Optimal solvent	Most Influential Factor	Optimal Time	Average Amount of Capsiate Extracted (*n* = 3)
UAE	42% metanol + 58% ethyl acetate	Ratio	2 min	1323.68 ± 32.30 μg g^{-1}
MAE	100% methanol	-	5 min	1403.98 ± 39.94 μg g^{-1}

In relation to the optimum solvent, it should be mentioned that for the UAE method, although the extraction time was lower, the already centrifuged extract had to be submitted to a process of solvent evaporation and to the subsequent redissolution of the compound of interest, since ethyl acetate cannot be introduced into the chromatographic equipment used. On the contrary, the MAE method did not require this additional procedure, which meant a considerable reduction in the overall time. However, in this technique, not only the extraction time must be taken into account, but also the time necessary to reach the desired temperature and cooling to room temperature. Nevertheless, it should be noted that a number from 8 to 40 samples can be introduced in the carousel and all of them can be extracted simultaneously, while in UAE, they have to be extracted individually.

None of the variables had a significant influence on the response in either method. Nevertheless, similar trends to extreme values were registered on determining their optimal values for both methods; i.e., minimum temperature and time, and maximum solvent volume and pH.

Finally, it was observed that, on average, a greater amount of capsiate was extracted by means of the MAE method, specifically 5.72% more, although its error margin and variability was also greater. On the other hand, the UAE method is easier to implement, requires a smaller initial investment on facilities and equipment and is more commonly available in most laboratories [51].

In conclusion, two effective methods for the extraction of capsinoids in peppers have been developed, since there were no specific methods for the study and extraction of this type of compounds. However, the extraction of other antioxidant compounds presents in peppers, such as capsaicinoids, phenolic compounds or carotenoids by both extraction techniques have been recently carried out [52–54].

A comparative study of capsinoids extraction between the methods reported in the literature and the proposed one has also been carried out (Table 6).

Table 6. Comparative summary between the methods reported in the literature and the proposed one.

Extraction Method	**Capsiate Concentration (µg/g)**	**Solvent Used**	**References**
Maceration	11-369	Acetonitrile	[34]
Sonication	540-1310	Acetonitrile	[55]
Shaking	1.21-544.59	Ethyl acetate	[11]
UAE	150-250	Ethanol, acetone, hexane, metanol	[44]
UAE MAE	1323.68 1403.98	Methanol + ethyl acetate Methanol	This work

It can be observed that the values obtained by the proposed methods are in the same range as the ones found in literature. It is noteworthy to highlight that in this work a variety of peppers with a high content of capsiate has been used, as it can be seen in the results obtained (in the upper limit of the intervals).

3.6. Recovery study

To demoonstrate the efficiency of both extraction methods, a recovery study was perfomed. Firstly, extractions in the optimum conditions, both for UAE and MAE, were carried out in triplicate to obtain the capsiate and dihydrocapsiate concentration in the sample. Subsequently, after the addition of different amounts levels of both kind of compounds respectively (0.02 – 0.10 mg), new extractions were performed.

Finally, the amount of the capsiate and dihydrocapsiate recovered in each of these additions was analyzed. The results obtained are shown in Figure 11.

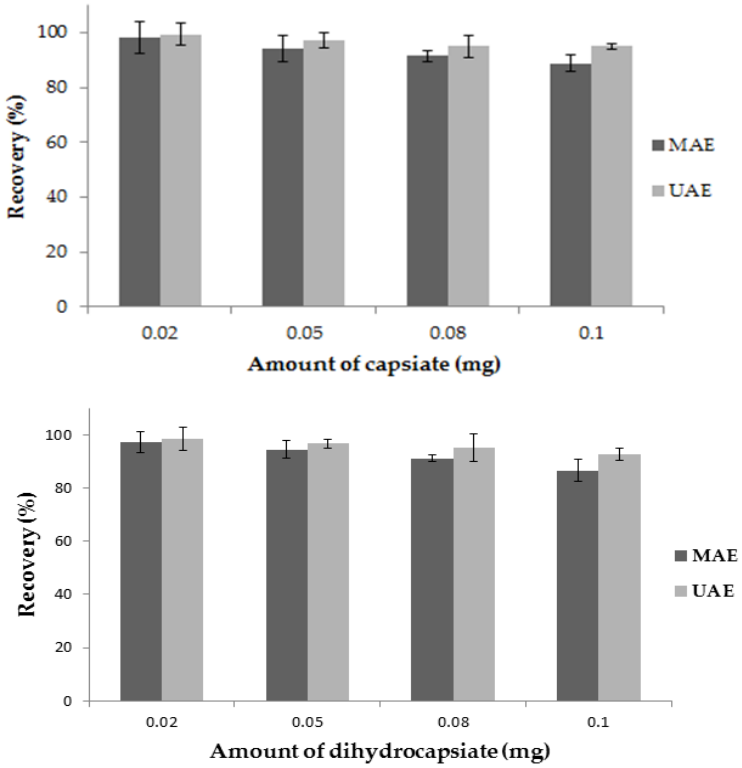

Figure 11. Recovery study for ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) (n = 3). (A) Capsiate extraction; (B) Dihydrocapsiate extraction.

It can be seen that there are no significant differences between ultrasound-assisted extraction and microwave-assisted extraction, being very close to 100%.

4. Conclusions

Based on the results obtained from the experiments that have been carried out, the mixture design of solvents and the surface response methodology have proven to be very important tools. It could be demonstrated that the two proposed extraction methods, UAE and MAE, are adequate, quick and effective for the extraction of capsinoids from peppers.

The optimal conditions for UAE were as follows: 5 min extraction time, 5.5 ºC temperature, pH 8, and 0.2:14.5 g:mL of "ratio", while in the case of MAE, the following best conditions were established: 5 min extraction time, 60 ºC temperature, pH 8, and 0.2:15 g:mL "ratio". Even though the studied variables (time, temperature, pH and ratio) do not have a statistically significant influence on the response, the same optimal conditions have been determined for both methods: minimum temperatura and time, and maximum ratio and pH.

Both methods have exhibited a high precision level with coefficients of variation under 5%. Although no significant differences in the extraction yields of capsinoids from peppers were registered, slightly higher amount of the compounds of interest could be visually noticed when MAE was used. Finally, the developed methods were successfully applied to different varieties of peppers, both sweet and spicy, and a quantitative extraction of capsinoids was achieved.

Peppers are currently considered in the food processing industries due to their antimicrobial or antioxidant activities, contributed in part by capsinoids. In the future, these developed methods could be used to analyze which varieties of peppers have a greater amount of these chemical compounds and focus on their cultivation to achieve genetic improvements. In addition, these bioactive compounds come from natural ingredients which could enhance the health properties of the cosmetic and pharmaceutical products.

Supplementary Materials: Figure S1. UV–Vis chromatogram (λ = 280 nm) of the 2 major capsinoids present in peppers. Peak assignment: (1) Capsiate (CTO); (2) Dihydrocapsiate (DHCTO); Figure S2. Previous study to obtain the extraction conditions in the selection of the optimal extraction solvents; Table S1. Statistical Mixture Design of Simple Centroid Extended Model for ultrasound-assisted extraction; Table S2. Statistical Mixture Design of Simple Centroid Extended Model for microwave-assisted extraction; Table S3. Box–Behnken experimental design matrix with coded variables; Table S4. Study of the precision of the methods developed in

terms of repeatability (1–10) and intermediate precision (1–30) for ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE).

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

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5.3. Estudio de los cambios en la concentración de capsaicinoides y capsinoides durante el desarrollo de los frutos de pimientos

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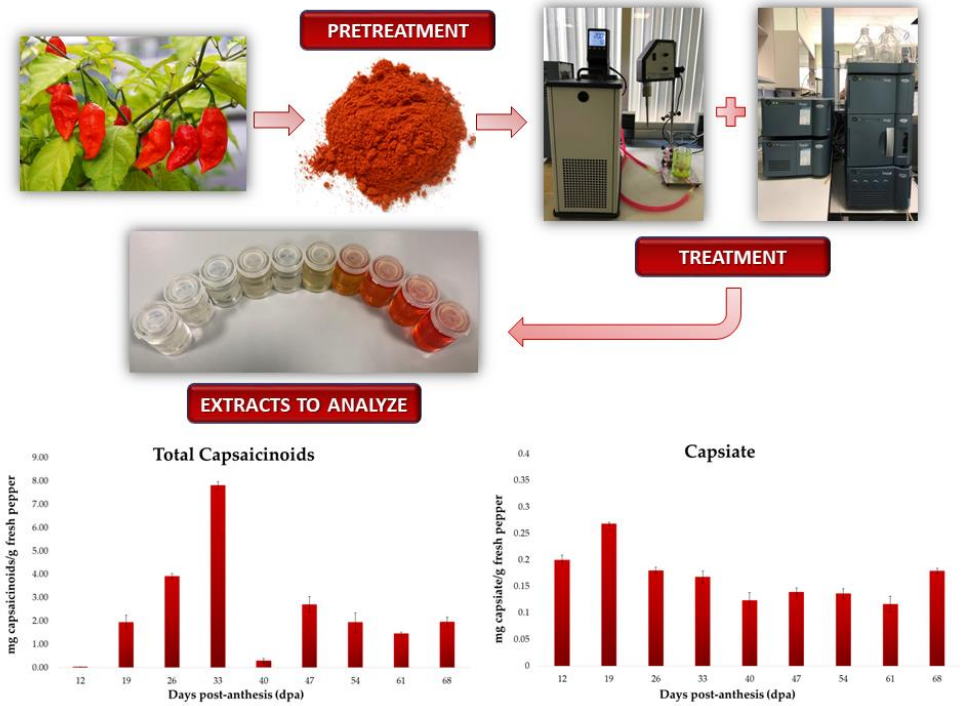
Vázquez-Espinosa, M.; Fayos, O.; González-de-Peredo, A.V.; Espada-Bellido, E.; Ferreiro-González, M.; Palma, M.; Garcés-Claver, A.; Barbero, G.F. Changes in Capsiate Content in Four Chili Pepper Genotypes (*Capsicum* spp.) at Different Ripening Stages. *Agronomy* **2020**, *10*, 1337. doi.org/10.3390/agronomy10091337

Vázquez-Espinosa, M.; Fayos, O.; González-de-Peredo, A.V.; Espada-Bellido, E.; Ferreiro-González, M.; Palma, M.; Garcés-Claver, A.; Barbero, G.F. Content of Capsaicinoids and Capsiate in "Filius" Pepper Varieties as Affected by Ripening. *Plants* **2020**, *9*, 1222. doi.org/10.3390/plants9091222



- **Influence of Fruit Ripening on the Total and Individual Capsaicinoids and Capsiate Content in Naga Jolokia Peppers (*Capsicum chinense* Jacq.)**

Abstract

"Naga Jolokia" (*Capsicum chinense* Jacq.) is a hot pepper variety native to India which has received the attention of the global scientific community due to its high capsaicinoid concentration. The present study evaluated the influence of fruit ripening on the total and individual capsaicinoids, as well as capsiate content. The aim was to determine the optimal moment to harvest the peppers depending on their pungent properties. Ultrasound-assisted extraction (UAE) using methanol as the extraction solvent and reverse-phase ultra-high-performance liquid chromatography (UHPLC-photodiode array (PDA)) were employed. Capsaicinoids gradually accumulated in the peppers from the moment they started growing until they reached a maximum concentration (7.99 ± 0.11 mg g^{-1} of fresh weight (FW)) at 33 days postanthesis (dpa).

For this reason, based on its content of pungent compounds, as it is one of the main attributes of this variety, the optimal time for collection would be on day 33. From then on, there was a sharp decrease (96.35% of the total concentration) due to the peroxidase enzymes. The evolution of the principal capsaicinoids in "Naga Jolokia" peppers had a different behavior with respect to literature reports. After this investigation, these changes in content can be attributed to each pepper genotype. Capsiate content reached it maximum value at 19 dpa (0.27 ± 0.01 mg g^{-1} of FW). Then, there was a gradual drop due to the activities of different peroxidases. Given the important biological activity of capsaicinoids and capsinoids, the information described here allows for determining the ideal time to harvest "Naga Jolokia" peppers.

1. Introduction

For several decades, the association between nutrition and health has been gaining popularity, and therefore, increased importance has been given to diets based on antioxidant-rich vegetables and fruits [1]. Pepper (*Capsicum* spp.) is one of the most valued vegetables because of its rich content in bioactive compounds, vitamins, and also for its high antioxidant properties. The genus *Capsicum* belongs to the Solanaceae family and it is native to tropical and humid areas in Central and South America. It is widely used worldwide as a culinary condiment for its flavor, aroma, and color, and it is also commercialized as a fresh product, dry crushed pepper, paprika oleoresin, or pepper paste [2,3]. The consumption of red peppers (chili peppers) is generally associated with spicy, burning, or pungent sensations, colloquially referred to as "hot flavor". The pungency of this vegetable is caused by two groups of chemical compounds known as capsaicinoids and capsinoids [4].

Capsaicinoids are nonvolatile alkaloids, which are chemically acid amides of C_9–C_{11} branched-chain fatty acids and vanillylamines. The main compounds are capsaicin (C) (*trans*-8-methyl-N-vanillyl-6-nonenamide) and dihydrocapsaicin (DHC) (8-methyl-N-vanillylnonanamide), which generally represent around 77%–98% of the total capsaicinoid content. Some other related compounds, such as nordihydrocapsaicin (n-DHC), homocapsaicin (h-C), or homodihydrocapsaicin (h-DHC), are also present in

minor amounts among the over 20 other reported compounds [5,6]. These compounds have exhibited numerous biológical properties of pharmacological relevance, such as antioxidant [7], anti-inflammatory [8], analgesic [9], antimicrobial [10], and anticarcinogenic [11] activities. Moreover, they are related to an increase in body energy and a decrease in both fat and cholesterol accumulation, which leads to a reduction in cardiovascular diseases, diabetes, or strokes [12]. One of its negative aspects is that high doses or long-term exposure have a detrimental effect on the gastric mucosa and, ultimately, on health. Epidemiological studies have revealed that the consumption of chili peppers in large amounts causes an increased risk of gastric cancer. In addition, some capsaicin metabolites may attack the DNA and trigger mutagenicity and malignant transformation [13].

Capsinoids were identified in the late 1980s in the low-pungent cultivar CH-19 Sweet (*Capsicum annuum* L.) [14]. Capsinoids exhibit similar health-promoting properties as capsaicinoids, although they are less irritating, nonspicy, and contain tastier compounds (its pungency is assessed to be about 1000 times lower than that of capsaicinoids), so they can be used at higher concentrations in agrifood applications [15]. The differences in the perception of pungency are related to the receptor vanilloid type-1 (TRPV1). This receptor is responsible for the burning sensation. Capsaicinoids actívate TRPV1 receptors on the consumer's tongue, while capsinoids have the capacity to activate them in the gut with a similar physiological effect. This difference in the activation site allows the absence of burning sensation in the capsinoids [16]. They have a very similar chemical structure, with the exception of their central bond, being vanillic alcohol esters with fatty acid chains similar to those of capsaicinoids. This structural difference could be the reason for the lower stability of capsinoids. To date, three different capsinoids have been isolated from the fruits of several pepper varieties (capsiate (CTE), dihydrocapsiate (DHCTE), and nordihydrocapsiate (n-DHCTE)) [17].

Capsaicinoids are naturally synthesized in the placenta of peppers by enzymatic condensation of vanillylamine with different length fatty acid chains C_9–C_{11} [18].

Capsinoids are natural compounds found in different varieties of sweet peppers. Their maximum interest lies in the demonstrated biological activity that they exhibit. That is the reason why the study of synthesis procedures of both natural and synthetic capsinoids, with similar properties to natural ones, is of great interest because of the difficulty in isolating these compounds. Therefore, the procedure for the chemical synthesis of this type of compound has been patented and published. It consists of four high-performance selective reactions: protection of the vanillin hydroxyl group, carbonyl reduction and subsequent esterification, and deprotection of the protected capsinoids [19,20].

Peppers are harvested and consumed at different ripening stages, from immature green to fully ripe. Throughout their maturation, numerous biochemical, physiological, and structural changes take place. The changes that occur during the maturation stage do not only have well-known agronomical implications (e.g., taste, color, size, postharvesting properties, etc.) but are also relevant to determine the harvested fruit application and quality [1]. For this reason, the accumulation of antioxidant compounds at different stages of fruit development is essential. The production and concentration of these compounds is influenced by both genetic and environmental factors. To mention some of them, the species and cultivars of *Capsicum*, the growth conditions and the cultivation techniques [21,22], the availability of water [23], the contribution of mineral supplements to the crop, light conditions, high temperatures, or plant infections [24,25] also may contribute to the concentration of capsaicinoids. Several studies have been carried out to elucidate the process of synthesis and accumulation of capsaicinoids over the maturation period [26,27]. It has been observed that these compounds begin to accumulate from the early stages of fruit development and continue to increase their content during ripening until a maximum concentration is reached, which is usually after 40–60 days postanthesis (dpa). Beyond this point there is a rapid turnaround in the trend due to their degradation by the action of some specific enzymes called peroxidases. The first report of capsaicin oxidation by a peroxidase enzyme was performed by Boersch et al. [28]. After that, Bernal et al. reported the first oxidation data of C and DHC by a pepper peroxidase [29].

Classical secretory plant peroxidases (class III Prx) are heme-containing glycoproteins able to oxidize different substrates using hydrogen peroxide as an electron donor. These are a type of basic peroxidases. Peroxidases may be directly related to capsaicinoid metabolism since the vanillyl moiety of capsaicin is easily oxidized by this enzyme. The oxidation of capsaicinoids by *Capsicum* peroxidase is strictly dependent on the presence of H_2O_2. Lema et al. proved that the basic peroxides of peppers are also responsible for the degradation of capsinoids [30].

The present study focused on the pepper variety known as "Naga Jolokia". It is native to northeastern regions of India and is mainly cultivated in Bangladesh and the Indian States of Assam, Nagaland, and Manipur [31]. This pepper is also of great commercial importance in Brazil. "Naga Jolokia" has received the attention of the global scientific community because of its extremely high pungency and unique aroma. In 2010, it was recognized by the Guinness Book of Records as the hottest chili in the world, reaching more than 1 million Scoville Heat Units (SHUs) [32]. It is used as a spice in both fresh and dried form or eaten raw along with the staple food. Because of its refreshing aroma, palatability, and medicinal properties, people have been using it for pickle preparation; to flavor curries; or as a popular remedy for different ailments such as gastritis, arthritis, or chronic indigestion problems. It has also been used to tone up body muscles after heavy workout sessions, whereas hot infusions are used for toothache or muscle pain [33].

Ultrasound-assisted extraction (UAE), a simple and inexpensive method, was used since it improves extraction efficiency by disrupting cell walls, reducing particle size, and improving mass transfer thanks to the cavitation effect [34]. Ultra-high-performance liquid chromatography working in reverse-phase (rp-UHPLC) was employed for its separation and quantification. The rp-HPLC technique is the most commonly used for the analysis of these compounds in fresh pepper [26]. However, rp-UHPLC has recently been proved to be more effective and faster, which makes it a feasible alternative for the analysis of such compounds of interest [35,36].

In order to minimize production costs by achieving the desirable high levels of pungency in the peppers, it is necessary to determine the optimum moment to harvest the peppers so that they present their maximum concentration of capsaicinoids and capsinoids, thus giving greater added value to the product. Therefore, the final objective of this work was to evaluate the accumulation of total and individual capsaicinoids and capsinoids during the ripening stages of "Naga Jolokia" peppers in order to determine the optimum moment to harvest them.

2. Materials and Methods

2.1. Chemicals

The methanol (99.9%) from Panreac Química S.L.U. (Castellar del Vallés, Barcelona, Spain) used for both extraction and chromatographic identification, the acetonitrile (99.9%) from Panreac Química S.L.U. (Castellar del Vallés, Barcelona, Spain) used for chromatographic separation, and the glacial acetic acid (99%) from Merck (Darmstadt, Germany) were HPLC grade. The water was obtained from a Milli-Q water deionization system (Millipore, Bedford, MA, USA). The reference standards of capsaicin (97%) and dihydrocapsaicin (90%) were acquired from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Capsiate standard was synthesized following the method described by Barbero et al. [19].

2.2. Plant Material

"Naga Jolokia" (*Capsicum chinense* Jacq.) seeds were supplied by the AgriFood Research and Technology Center of Aragon (CITA), located in Zaragoza, Spain. The seeds were germinated in Petri dishes until the cotyledons were developed. Then, each plantlet was placed in a Jiffy-7 pot (Clause-Tezeir Iberica, Almería, Spain). Once the plants had three true leaves (approximately 6 weeks after sowing), each Jiffy pot was transplanted into a black plastic pot (one plant per pot) of 17 cm in diameter. Each pot contained a substrate mixture made of Humin Substrat (Klasman-Deilmann, Geeste, Germany) (1:1:1:1, v/v) peat, sand, clay-loam, and soil, enriched with 2 g of a slow- release fertilizer (Osmocote 16N-4P-9K, Scotts, Tarragona, Spain), and watering

by a drip irrigation system. Twenty plants of cultivar “Naga Jolokia” were grown during the spring–summer seasons (April–September 2016) under controlled conditions in a climatized glasshouse located at CITA. The average day/night temperatures over the period of the study were 24/14 ºC in the spring and 27/19 ºC in the summer.

Blooming started in mid-July until the end of September. The monitoring of the fruit development was performed by labeling and dating the flowers at anthesis to determine the fruit stage of development. “Naga Jolokia” fruits were harvested at nine stages of development: 12, 19, 26, 33, 40, 47, 54, 61, and 68 dpa.

Once the samples were harvested, peppers were grouped according to their developmental stage (by dpa). While the stem and seeds were discarded, the pericarp and placenta were ground together in an Ultra-Turrax blender to obtain a completely homogeneous sample. Finally, they were frozen at -20 ºC until further analysis.

2.3. Ultrasound-Assisted Extraction of Capsaicinoids and Capsiate

The extracts from the pepper samples at the different maturation stages were obtained using a UAE technique. To apply the ultrasounds, a Sonoplus probe (BANDELIN ELECTRONIC, Heinrichstraβe, Berlín, Germany), which allows the control and modification of the amplitude and cycle, was used. This probe was coupled to a thermostatic bath with temperature control by means of a 7 L refrigerated circulator (PolyScience, Niles, IL, USA) and was submerged into a double-walled vessel that allowed the temperature of the liquid inside to be maintained. The capsaicinoids were extracted by applying a previously developed method [37], in which 1 g of fresh pepper sample was put in contact with 25 mL of methanol for 10 min at 50 ºC, using 80% of the maximum power (200 W) and a cycle of 0.5 s. The extraction of the capsinoids was performed by means of our previously developed method [38], in which 15 mL of solvent was added to 0.5 g of peppers, and the sample was subjected to UAE for 2 min at a temperature of 5.5 ºC, using 80% of the maximum power (200 W) and a cycle of 0.5 s. The extracts were filtered through a 0.22 µm nylon syringe filter (Membrane Solution, Dallas, TX, USA) prior to their chromatographic analysis.

The extraction process was carried out in triplicate for each homogeneous sample obtained on each different ripening days. The sample was considered homogeneous since it was composed of all the peppers obtained at each ripening state. Then, the quantification of the compounds in each replicate was performed by means of UHPLC. The final result obtained would be the average of these three values.

2.4. Capsaicinoids and Capsiate Identification

The five principal capsaicinoids as well as the major capsinoid present in "Naga Jolokia" pepper were identified by ultra-high-performance liquid chromatography coupled to a quadrupole-time-of-flight mass spectrometer (UHPLC-Q-ToF-MS) (Xevo G2 QToF, Waters Corporation, Milford, MA, USA). This equipment consisted of a self-sampler, a quaternary and binary solvent manager, a photodiode array (PDA) detector, and an rp-C18 analytical column (Acquity UPLC BEH C-18, Waters, MA, USA, 2.1 x 100 mm and 1.7 µm particle size). A gradient method, using wáter as solvent A and methanol as solvent B, both acidified by 0.1% formic acid, working at a flow of 0.4 mL min^{-1} was used. The elution gradient employed was as follows (time, % solvent B): 0 min, 0%; 0.85 min, 55%; 1.60 min, 55%; 1.95 min, 60%; 2.45 min, 63%; 2.80 min, 70%; 3.00 min, 70%; 6.00 min, 100%; 8 min, 100%. The injection volume was set to 3 µL and the column temperature was adjusted at 50 ºC.

The analytes were determined by an electrospray source operating in positive ionization mode under the following conditions: desolvation gas flow = 850 L h^{-1}; desolvation temperature = 500 ºC; cone gas flow = 10 L h^{-1}; source temperature = 150 ºC; capillary voltage = 0.7 eV; cone voltage = 20 V; and trap collision energy = 4 eV. Full-scan mode was used (*m/z* = 100–600). The molecular ions $[M + H]^+$ of the compounds identified had the following m/z ratios: nordihydrocapsaicin (n-DHC) 294, capsaicin (C) 306, dihydrocapsaicin (DHC) 308, homocapsaicin (h-C) 320, homodihydrocapsaicin (h-DHC) 322, and capsiate (CTE) 307. Additional information regarding the chromatographic and *m/z* parameters of these compounds for the UHPLC-QToF-MS method has been included in Table S1.

2.5. Capsaicinoids and Capsiate Analysis

Once the capsaicinoids and capsiate had been identified, the separation and quantification of these compounds were carried out by rp-UHPLC-PDA (Acquity Ultra Performance LC Class, Waters Corporation, Milford, MA, USA), since this is the technique available to our research group. This equipment consists of an ACQUITY UPLC H-Class Auto Sampler with temperature control adjusted at 15 ºC, an ACQUITY UPLC Quaternary Pump System, an ACQUITY UPLC PDA Detector, and a Waters ACQUITY UPLC BEH rp-C18 column at 50 ºC (100 x 2.1 mm, 1.7 µm particle size).

A gradient method using water as solvent A and acetonitrile from Panreac Química S.L.U. (Castellar del Vallés, Barcelona, Spain) as solvent B, both acidified by means of 0.1% acetic acid, running at a flow of 0.8 mL min^{-1} was used for the separation of capsaicinoids and capsiate. The elution gradient employed was as follows (time, % solvent B): 0 min, 0%; 0.50 min, 45%; 1.60 min, 45%; 1.95 min, 50%; 2.45 min, 55%; 2.80 min, 63%; 3.00 min, 63%; 4.00 min, 100%; 6.00 min, 100%. The injection volume was set at 3 µL and the wavelength for ultraviolet detection was 280 nm.

For the quantification, the calibration curves of C ($y = 1669.70x + 36.08$, $R^2 = 0.9997$) and DHC ($y = 1688.31x + 29.42$, $R^2 = 0.9998$), which are the two capsaicinoid standards that are commercially available, as well as another one of CTE ($y = 1682.50x - 164.74$, $R^2 = 0.9997$) were used. The detection limits (1.65 ng g^{-1} fresh weight (FW), 1.25 ng g^{-1} FW, and 3.60 ng g^{-1} FW) and quantification limits (5.50 ng g^{-1} FW, 4.17 ng g^{-1} FW, and 12.00 ng g^{-1} FW) for C, DHC, and CTE, respectively, were determined as the analytic concentration that corresponds to the standard deviation from the blank signal values ($n = 10$) plus 3 or 10 times, respectively, divided by the slope of the linear regression. Commercial standards of n-DHC, h-C, and h-DHC were not available and these compounds had to be quantified based on the calibration curve of DHC (n-DHC and h-DHC) and the calibration curve of C (h-C) given the structural similarities between these molecules and taking into account their molecular weights. Additional information regarding the chromatographic parameters of capsaicinoids and capsiate for the UHPLC-PDA method has been included in Table S2.

2.6. Statistical Analysis

The statistical significance of the model was evaluated by means of a Tukey's test using Statgraphic Centurion Version XVII (The Plains, Fauquier, WV, USA). MassLynx version 4.1 for identification (UHPLC-Q-ToF-MS) and Empower 3 for separation and quantification (UHPLC-PDA) software, both fromWaters Corporation (Milford, MA, USA), were used to control the equipment and for the acquisition and treatment of the data.

3. Results and Discussion

3.1. Evolution of the Total Capsaicinoid Content

The "Naga Jolokia" plants began to produce peppers during the second week in July and they were harvested on September 30th. The evolution of capsaicinoid content was monitored during the maturation of the fruits from the 12th dpa. The visual state of the peppers at the time of harvesting is shown in Table 1.

Table 1. Code and visual state of "Naga Jolokia" pepper fruits at different stages of fruit development (days post anthesis, dpa).

Code	Fruit Sprouting	Dpa	Visual State
M-1	18/09	12	Green color
M-2	11/09	19	Green color
M-3	04/09	26	Green color
M-4	28/08	33	Green color
M-5	21/08	40	Yellow color
M-6	14/08	47	Orange color
M-7	07/08	54	Red color
M-8	31/07	61	Red color
M-9	24/07	68	Red color/over-ripeness

The first step was to perform the sample extraction in each of the maturation stages by ultrasound-assisted extraction, using the conditions mentioned above with both developed methods. Subsequently, the quantification of the capsaicinoids was carried out by UHPLC-PDA to study the tendency of each of them throughout the ripening of the fruit.

Ananthan et al. [2] reported the content of capsaicinoids in different components of the cultivar "Naga Jolokia" peppers during the green, yellow, and red stages, but they did not perform a complete study over the ripening stage. Several authors have reported that the highest level of capsaicinoids in peppers is at 40 dpa, followed by a gradual degradation of these compounds due to the action of peroxidase enzymes [24,25,39]. However, during this study, it was found that "Naga Jolokia" peppers reached their maximum concentration before 40 dpa (specifically at 33 dpa), and a subsequent drastic drop of about 96% took place, which has not ever been reported for any other pepper variety [3]. These differences could be attributed to genotype, growing conditions, or environmental factors.

Figure 1 shows that the total capsaicinoid content increased from 12 dpa until the maximum level was reached at 33 dpa with a concentration of 7.99 ± 0.11 mg g^{-1} in fresh pepper (FW). This value is similar to other *C. chinense* varieties, such as "Habanero" pepper [40,41], and is quite similar to other studies on "Bhut Jolokia" peppers [42]. Then, between 33 and 40 dpa, a very sharp decrease took place down to 0.41 ± 0.10 mg g^{-1} of FW, which corresponded to a 96.35% reduction in capsaicinoid content. This drastic reduction could be attributed to the action of basic peroxidases, which may have degraded the capsaicin and dihydrocapsaicin molecules [43]. This degradation of the capsaicinoids by the action of the peroxidases coincided with the change of green to red color that took place with the ripening of the peppers.

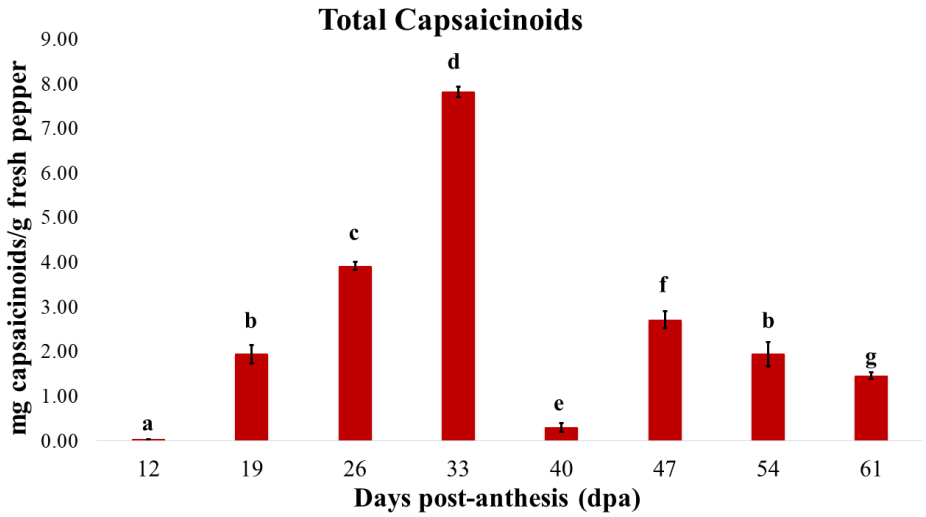


Figure 1. Evolution of total capsaicinoid content (mg g^{-1} of fresh weight (FW)) during "Naga Jolokia" pepper fruit development ($n = 3$). According to the Tukey's test, results with a *p*-value less tan 0.05 were considered to be statistically different. Taking this into account, the use of different letters in this figure indicates that there is a significant difference between results depending on Tukey's test.

It should be noted that this sharp decline has not been previously observed in other pepper cultivars. Several studies have shown a considerably smaller reduction in the total amount of capsaicinoids, ranging from 30% to 65% in different pepper cultivars such as "Habanero", "Piquín", "Chile de Árbol", or "Padrón" [25,44]. Meghvansi et al. [31] presented a comparison between different locations and suggested that weather and climatic conditions may affect the pungency intensity of "Naga Jolokia" peppers. However, Olguín-Rojas et al. applied similar growing conditions to other varieties of pepper that were grown simultaneously and did not observe any sharp reduction in the total amount of capsaicinoids [45]. This may suggest that changes in content could be attributed to each pepper genotype. After the sudden drop in the total concentration of capsaicinoids, an increase took place until 47 dpa. From then on, the concentration of capsaicinoids decreased slightly and then remained practically constant until the end of the ripening process. Subsequent days of ripening were not treated as a result of the over-ripening observed in the fruit, which caused water loss and wrinkling, among other changes.

3.2. Evolution of the Individual Content of Capsaicinoids

The five capsaicinoids identified in "Naga Jolokia" peppers, using the UHPLC-Q-ToF-MS equipment with a PDA detector and rp-C18 analytical column, were n-DHC, C, DHC, h-C, and h-DHC. Once identified, each of them was quantified by the UHPLC equipment. Their individual concentrations (mg of capsaicinoid g^{-1} of FW) throughout the ripening of the fruit are represented in Figure 2. It can be seen that, like other studies [22,46], C was the major capsaicinoid over the entire maturation of the fruit, followed by DHC, n-DHC, h-C, and finally h-DHC in smaller amounts. This is concordant with the results from similar studies on *C. chinense*, since capsaicin is the capsaicinoid responsible for the high pungency of the fruit, followed by dihydrocapsaicin [40,47].

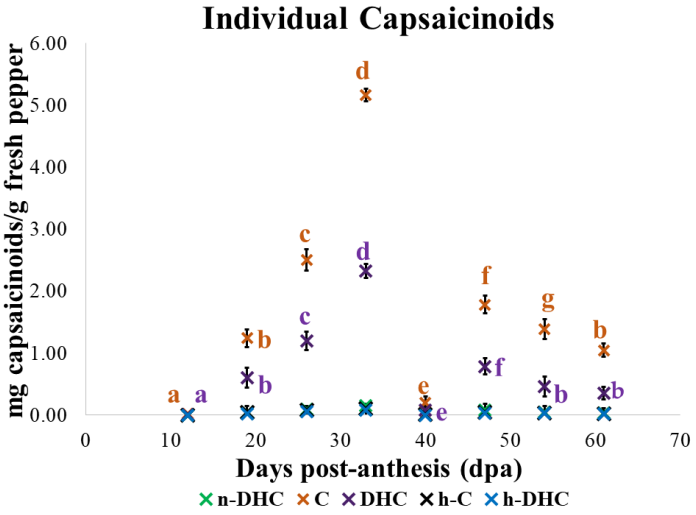


Figure 2. Evolution of individual capsaicinoid content (mg g^{-1} of FW) during "Naga Jolokia" pepper fruit development (n = 3). According to the Tukey's test, results with a p-value less than 0.05 were considered to be statistically different. Taking this into account, the use of different letters in this figure indicates that there is a significant difference between results depending on Tukey's test. In turn, the letters of each color refer to their respective capsaicinoid, orange for capsaicin (C) and purple for dihydrocapsaicin (DHC). Only the existence or not of a significant difference in the two major compounds has been indicated in Figure 2 for a better viewing. The results obtained for the three minor capsaicinoids (nordihydrocapsaicin (n-DHC),

homocapsaicin (h-C), and homodihydrocapsaicin (h-DHC)) are shown in detail in Figure S1 with a different scale.

The evolution of total capsaicinoids was similar to the evolution of each individual capsaicinoid. In this way, the five individual capsaicinoids increased their concentration until they reached their maximum at 33 dpa. Then, the concentration of capsaicinoids drastically fell at 40 dpa. As explained above, there was a new increase until 47 dpa and then a gradual decrease between 47 and 61 dpa.

Figure 3 shows the percentage patterns of the five major capsaicinoids during the ripening process. It can be observed that the increments in the percentage of capsaicin corresponded to decreases in the percentage of dihydrocapsaicin and vice versa. In addition, it can also be noticed that capsaicin slightly increased its concentration throughout maturation, while dihydrocapsaicin's concentration decreased slightly. These two major capsaicinoids may represent between 93% and 96% of the total capsaicinoid content (approximately 70% and 25% of capsaicin and dihydrocapsaicin, respectively). Their concentration depended on the fruit ripening stage, being therefore the two main capsaicinoids found in this pepper variety. The other three minor capsaicinoids (n-DHC, h-C, and h-DHC) had a similar behavior, since they were present in similar percentages that ranged between 0% and 3% for each of them and also varied according to the ripeness of the fruit.

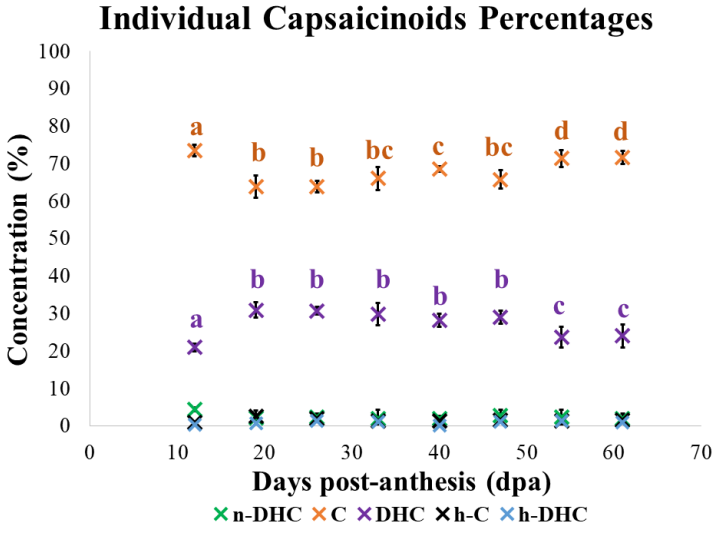


Figure 3. Evolution of individual capsaicinoid percentages during "Naga Jolokia" pepper fruit development (n = 3). According to the Tukey's test, results with a p-value less than 0.05 were considered to be statistically different. Taking this into account, the use of different letters in this figure indicates that there is a significant difference between results depending on Tukey's test. In turn, the letters of each color refer to their respective compound, orange for C and purple for DHC. Only the existence or not of a significant difference in the two major compounds has been indicated in Figure 3 for a better viewing. The results obtained for the three minor capsaicinoids (n-DHC, h-C, and h-DHC) are shown in detail in Figure S2 with a different scale.

3.3. Evolution of the Standardized Values of Capsaicinoids

The standardized evolution of the main "Naga Jolokia" pepper capsaicinoids can be observed in Table 2. These values were normalized with respect to the day of the greatest concentration of each compound; that is, all of them refer to 100% of their maximum content, which coincided in all of them at 33 dpa. It was observed that the evolution of the relative percentage throughout maturation followed the same above-explained trend.

Table 2. Relative percentages (%) of individual capsaicinoids during "Naga Jolokia" pepper fruit development (n = 3).

Dpa	n-DHC	C	DHC	h-C	h-DHC
12	1.03	0.48	0.31	0.29	0.12
19	27.59	24.04	25.83	46.03	16.88
26	62.01	48.44	51.38	71.82	59.82
33	100.00	100.00	100.00	100.00	100.00
40	3.65	3.90	3.56	3.83	0.00
47	50.96	34.55	33.71	37.83	37.97
54	30.34	26.89	19.79	25.32	31.55
61	19.46	20.25	15.02	20.22	16.92

It is noteworthy that all the capsaicinoids followed the same pattern throughout maturation. However, this did not occur in other pepper cultivars reported in the literature. For example, in "Cayenne" pepper, the relative percentages of n-DHC, DHC, h-C, and h-DHC followed the same pattern. They increased until 40 dpa, when they reached their maximum concentration in the fruit development process. Then, there was a gradual decrease until 80 dpa with a minimum level between 42% and 52% of maximum content. However, C presented a different behavior. Thus, the máximum relative percentage was reached on 20 dpa, much earlier than the others [23]. In the case of "Peter" pepper, C, h-DHC, and DHC showed the same linear pattern, while h-C and n-DHC presented a different trend [25].

In this sense, it would be necessary to monitor every detail of the cultivation conditions of each crop, as well as the rest of the environmental factors, since they may greatly influence the final product and its composition [48]. If these parameters are perfectly controlled and described, the results should be reproducible and comparable to those obtained by other researchers.

3.4. Evolution of Capsiate Content

As explained before for capsaicinoids, capsiate was identified with the UHPLC-Q-ToF-MS system and quantified with the rp-UHPLC-PDA system. Figure 4 shows the evolution of capsiate during the maturation of the peppers. Capsiate accumulation reached its maximum value at 19 dpa. Subsequently, the capsiate content decreased significantly, corresponding to approximately 70% of its máximum content. Such reduction could be associated with a decrease in the expression of the biosynthetic structural genes of capsinoids or, alternatively, to the activities of different peroxidases, as described for capsaicinoids [49]. Two main peroxidase isoenzyme groups can be distinguished in *Capsicum* by their individual isoelectric points. The first main group is composed of acidic isoelectric point peroxidase isoenzymes called APrx, and the second group corresponds to basic isoelectric point peroxidase isoenzymes (BPrx) [29].

Basic peroxidases are found in cell walls and vacuoles, which are the hypothetical places for capsiate accumulation. The use of different inhibitors allowed for confirmation of the nature of this peroxidase for the detected activity. These results strongly support the role of the basic peroxidases of *C. annuum* as being responsible for capsiate oxidation [50]. Over several subsequent days, the capsiate content remained practically constant. Tanaka et al. [51] claimed that "CH-19 Sweet" content increased between 10 and 30 dpa and then decreased to around 58% of its máximum level. This decrease in content took place slightly later than did in our study.

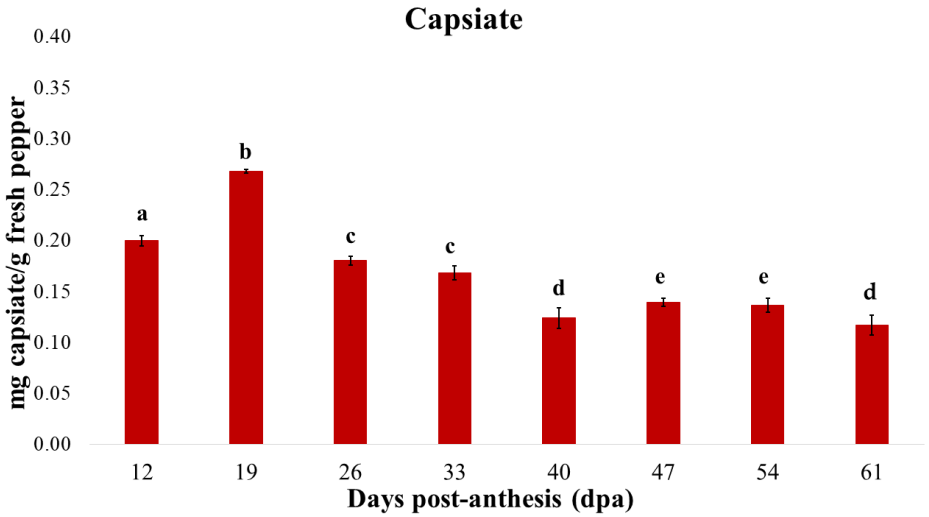


Figure 4. Evolution of capsiate content (mg g^{-1} of FW) during "Naga Jolokia" pepper fruit development (n = 3). According to the Tukey's test, results with a p-value less than 0.05 were considered to be statistically different. Taking this into account, the use of different letters in this figure indicates that there is a significant difference between results depending on Tukey's test.

Capsinoids, which are esters of fatty acid and vanillyl alcohol, are stable in nonpolar solvents such as ethyl acetate but decompose easily in polar solvents, such as water, methanol, and so forth [52]. This instability in water can also be responsible for the rapid decrease of capsinoid contents in pepper fruits. Capsinoids are also unstable in water and at high temperatures, which is why their content may decrease during fruit ripening. Therefore, fruits containing capsinoids should be consumed raw and before full maturation [53].

In order to obtain a high level of capsinoids, mature green fruits should be collected approximately at 19 dpa, when the capsiate content reaches it maximum level. Similar trends to accumulate capsinoids during fruit ripening have been observed in several *Capsicum* cultivars at different levels of pungency [54,55].

4. Conclusions

The behavior of capsaicinoids present in "Naga Jolokia" peppers during the ripening process differed from previous reports regarding other *Capsicum* cultivars. Capsaicin is the major capsaicinoid and its proportion with respect to the rest of the individual capsaicinoids did not vary with the fruit ripening state. Once the capsaicinoids reached their maximum concentration at 33 dpa, it dropped drastically (by 96.35%). Such a decrease is somewhat advanced with respect to what has been described in the literature and could be attributed to this variety's own genetic factors or to specific growing conditions. This study intends to contribute to establishing appropriate harvesting techniques to obtain pungent peppers. It has been proved that the ripening stage is essential to determine the ideal time for harvesting, since drastic changes in capsaicinoid content have been observed over the ripening period. This is of great interest since one of the most sought-after attributes in peppers, and particularly in the cultivar "Naga Jolokia", is the content of pungent compounds that confer to it its highly spicy character. Given the important biological activity of capsaicinoids and capsinoids, the information described here allows the harvesting of "Naga Jolokia" peppers at the moment when their content is at its máximum value. The optimal time for the collection of peppers, depending on their pungent characteristics, would be when they have their highest capsaicinoid content, due to the aforementioned excellent biological properties of these compounds. Based on the results obtained in this research, the optimal time would be at 33 dpa. Furthermore, harvesting should be carried out before the over-ripening stages of the fruit have been visibly reached. In any case, choosing the right moment to harvest this fruit should always be taken into account while bearing in mind that substantial variations in their total capsaicinoid content depending on the harvesting time are to be expected.

With respect to the capsiate content, the maximum is reached in the first weeks of maturation, after which a moderate drop in its concentration is observed.

Supplementary Materials: Table S1. Chromatographic and *m/z* parameters of capsaicinoids and capsiate for the UHPLC-QToF-MS method. Table S2. Chromatographic parameters of capsaicinoids and capsiate for the UHPLC-DAD method. Figure S1. Individual Capsaicinoids. Figure S2. Individual Capsaicinoids Percentages.

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Abbreviations

C	Capsaicin
CITA	Agri-Food Research and Technology Center
CTE	Capsiate
DHC	Dihydrocapsaicin
DHCTE	Dihydrocapsiate
Dpa	Days postanthesis
FW	Fresh weight
h-C	Homocapsaicin
h-DHC	Homodihydrocapsaicin
n-DHC	Nordihydrocapsaicin
n-DHCTE	Nordihydrocapsiate
PDA	Photodiode array detector
QToF-MS	Quadrupole-time-of-flight mass spectrometry
rp-HPLC	Reserve-phase high-performance liquid chromatography
rp-UHPLC	Reserve-phase ultra-high-performance liquid chromatography
SHUs	Scoville Heat Units
UAE	Ultrasound-assisted extraction

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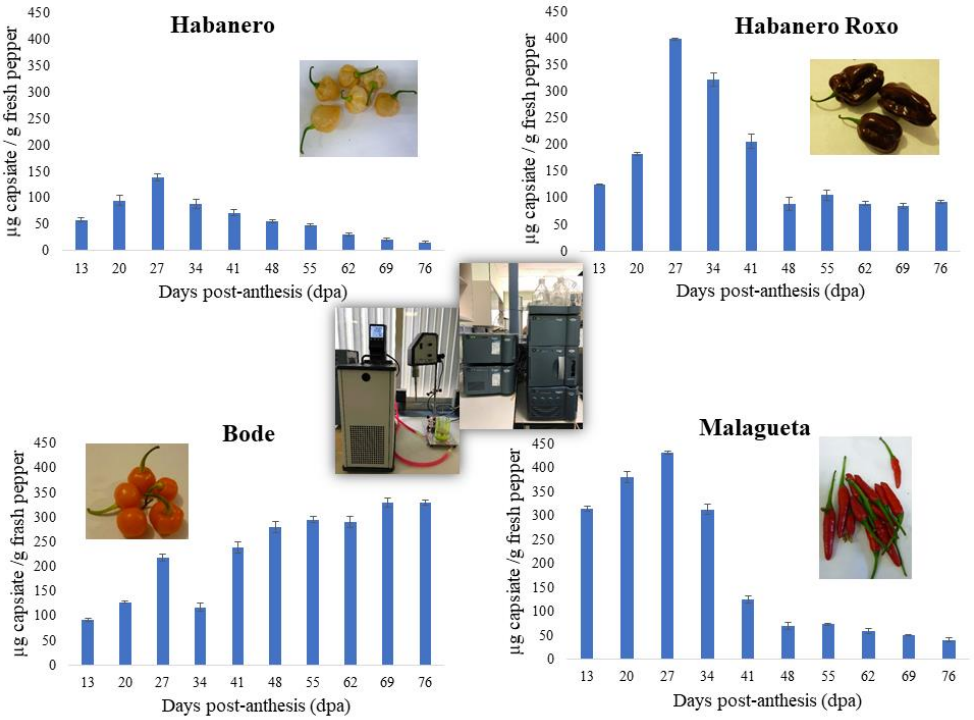

- **Changes in Capsiate Content in Four Chili Pepper Genotypes (*Capsicum* spp.) at Different Ripening Stages**

Abstract

Interest in the consumption of the fruits of pepper (*Capsicum* spp.) is not only due to its organoleptic characteristics, but also due to its bioactive compounds content, which are reported to provide essential benefits to human health. However, the amount and diversity of these compounds in each fruit specimen depend on its genotype and on a number of environmental factors. This work describes the quantitative ultra-high-performance liquid chromatography coupled to photodiode-array (UHPLC-PDA) analysis of the capsinoids content in four varieties of pepper (‘Habanero’, ‘Habanero Roxo’, ‘Bode’, and ‘Malagueta’) grown until different development stages in a greenhouse under controlled conditions. In all the varieties analyzed, capsiate was the only capsinoid found. The accumulation of capsiate, in all the pepper varieties, started from the 10th to the 20th day post-anthesis (dpa), and increased during the first days

(between the 20th and the 27th dpa). From that moment a drastic reduction took place until the end of the ripening stage, except for 'Bode' peppers, where the capsiate content increased from the first harvest point on the 20th dpa up to the 76th dpa. The capsiate accumulation patterns over the development of the fruit has been related to the capsaicionoids accumulation patterns in the same samples of the four varieties of pepper. According to our results, the content evolution of both families of compounds will vary depending on each fruit's genotype, as well as on environmental conditions. No clear trends have been established and, therefore, an in-depth analysis under controlled conditions should be carried out.

1. Introduction

Pepper belongs to the genus *Capsicum* and the Solanaceae family, original from tropical areas in America. From the 35 described, only five species have been domesticated: *Capsicum chinense* Jacq., *C. frutescens* L., *C. annuum* L., *C. baccatum* L., and *C. pubescens* Ruiz & Pav., with significant economic and social impact worldwide [1]. *Capsicum* fruits vary in size (thick or thin), shape (round, elongated, etc.), color (green, purple, chocolate, yellow, orange, or red, depending on pepper variety and maturation stage), flavor, and pungency (from the non-pungent varieties to the hottest species) [2]. Due to the vast quantity and the diverse varieties consumed, pepper is among the most valued and commonly cultivated produce because of their color, flavor, and taste sensory attributes. The food industry is the principal user of pepper fruits. It is often used as a coloring and flavoring agent in sauce, soup, processed meat, snacks, candies, soft drinks, and alcoholic beverages [3]. In addition to their sensory features, oleoresin is extracted from pepper fruits and used as an ingredient in numerous comercial products such as insect repellent or even self-defense sprays; and peppers can be also employed in medicinal applications, since they are an important source of the kind of bioactive compounds that provide health benefits to consumers [4]. Among such bioactive compounds two families should be noted: capsaicinoids and capsinoids, exclusive to the genus *Capsicum* and responsible for pepper pungency [5].

Both are widely known for their pharmacological properties, such as anti-inflammatory, anti-carcinogenic, neurological, antimicrobial, and antioxidant. They also contribute to weight loss treatments, relieve pain, and provide gastrointestinal and cardiovascular benefits when ingested regularly [6–10].

Capsaicinoid and capsinoid biosynthesis takes place in the placenta between the 10th and the 20th days post anthesis (dpa), but they can also be detected in some of the fruit tissues, such as sedes or pericarp, due to the fact that they are eventually excreted [11]. Capsaicinoids, and also probably capsinoids, are ultimately produced by capsaicin synthase through the condensation of an aromatic moiety, derived from vanillin, with fatty acid branched-chains of 9 – 11 carbon atoms [12]. In addition, their fundamental chemical structures are rather similar, with the exception of their central bond. Thus, while capsinoids have an ester group, capsaicinoids have an amide group. This difference in their structure seems to be responsible for the lower pungency of capsinoids, roughly determined as 1000 times lower compared to that of capsaicinoids. Therefore, the employment of capsinoids is particularly attractive as a food additive or medicinal product, since they do not present the side effects of capsaicinoids such as irritation or burning sensations [13,14].

Capsinoids were first reported by Yazawa et al., in a non-pungent pepper cultivar CH-19 Sweet [15]. To date, three capsinoids, capsiate, dihydrocapsiate, and nordihydrocapsiate, have been described in pepper fruits, capsiate being the major one. Later on, these compounds were detected in other varieties of non-pungent and low pungent peppers, as well as in hot and super-hot pepper cultivars, although in considerably lower concentrations than capsaicinoids. In pungent peppers, vanillin is converted to both vanillylamine and vanillyl alcohol, which in turns gives place to the production of capsaicinoids and capsinoids, respectively [16]. The putative aminotransferase (p-AMT) gene encodes the aminotransferase enzyme (p-AMT) that catalyzes the formation of vanillylamine from vanillin in the capsaicinoid biosynthetic pathway. Different mutations in the p-AMT gene have been described as always leading to a loss-of-function with the subsequent increment in the production of vanillyl

alcohol. Consequently, pepper genotypes carrying a pamt allele are non or low-pungent due to the high production of capsinoids over that of capsaicinoids [17,18]. Therefore p-AMT allele could be considered as a useful gene to control the content of capsaicinoids and capsinoids in pepper breeding programs [19,20].

The content of capsaicinoid and capsinoid compounds in peppers can be affected by different factors, including water availability (there is a significant reduction in fruit yield when a reduced amount of water is applied during the periods of vegetative growth, flowering, and fruiting) [21], light (it regulates morphological characteristics and acts as a source of energy for the primary metabolism and photosynthetic processes) [22], temperature, climatic conditions, genotype, cultivation techniques, mineral supply, growing conditions, and maturity stage (during the fruit ripening stage, several biochemical, physiological, and structural changes take place, and those changes govern the characteristics of the final fruit) [23,24]. Sampling and storage conditions need to be closely controlled to produce high-quality plant material for its characterization and further use [25].

The present study has focused on four of the main pepper varieties consumed in Brazil: ‘Habanero’, ‘Habanero Roxo’, ‘Bode’, and ‘Malagueta’. ‘Habanero’ peppers are from the *C. chinense* family [26]. This intensely aromatic fruit is claimed to be one of the hottest varieties in the world, with pungency values between 100.000 and 300.000 Scoville Heat Units (SHUs). This chili pepper is dark green changing to orange, orange-red, red, or even chocolate (‘Habanero Roxo’) when fully ripe. Pod size normally varies from 2.9 to 6.0 cm in length, 2.5 to 4.6 cm in width, and 7 to 12 g in weight, and it is mainly used in sauces, chutneys, and marinades for seafood or pickles [27,28]. Its unique aroma, pungency and color are its most attractive properties and a quality reference for consumers. Brazil is considered a center of diversity for some *Capsicum species* (domesticated and wild) [29]. However, ‘Habanero’ pepper holds an enormous social and commercial relevance in other American countries, such as Mexico. The main production zones of ‘Habanero’ chili pepper in México are located in the states of Yucatan, Campeche, and Quintana Roo [3]. There is a current great

interest in exporting this crop as a whole dehydrated product to the USA and Europe, where it is becoming an important source of extractable oleoresin. Pepper fruits and its derivatives are also commercialized worldwide as condiments, additives, and as the lachrymatory agent in pepper sprays; as well as a fungicidal and cytotoxic agent [30].

The 'Bode' pepper variety also belongs to the *C. chinense* family and is native to Recife. It is widely cultivated throughout the northern and northwestern regions in Brazil [31]. Its fruits, of an intermediate pungency (15.000 – 30.000 SHU), are round and small, and their coloration varies between yellow, orange, and red when fully mature. This variety is highly valued in the kitchen for its smoky and fruity flavor and for its aroma. It is mostly consumed as pickles [32].

'Malagueta' pepper is a variety of the *C. frutescens* species; mostly cultivated and consumed in Brazil, and particularly in the states of Minas Gerais, São Paulo, Bahia, and Goiás. It is widely used in the production of sauces and also in preserves, jams, and pastes [33]. Its color changes rapidly from green (unripe fruit) into red (ripe fruit) and, in some cases, it may present a light red color intermediate stage. As for the size of the fruit, it varies from 1 to 3 cm long and 0.4 to 0.5 cm wide, and they are conical with very thin walls [34].

Capsaicinoid accumulation patterns for the four species above mentioned have been previously studied [35,36]. However, no assessment of the capsinoid accumulation patterns over the different fruit development stages, as well as a description of the correlation between capsinoid and capsaicinoid contents throughout those fruit development stages have been reported. For the purposes of this study, ultrasound-assisted extraction techniques will be used. Thus, high frequency ultrasonic waves, capable of causing cavitation due to the expansion and contraction cycles that the material goes through, will be applied. Such expansion and contraction cycles disrupt the cell walls in the vegetable matrix to favor the penetration of a solvent and, in turn, the mass transfer, which results in increasing extraction rates and yields [37].

This paper intends to cast some light on two aspects that have been scarcely studied in relation to pepper cultivation: capsinoids accumulation at the different ripening stages of pepper fruits; and the potential correlation between capsinoid and capsaicinoid accumulation patterns in the varieties studied over their fruit development. The conclusions that may be reached with regards to these two aspects should help pepper breeders to determine the optimum harvesting moment that allow them to obtain the maximum added value from their crops.

2. Materials and Methods

2.1. Plant Material

Chili pepper seeds of the var. 'Habanero' (*C. chinense*), 'Habanero Roxo' (*C. chinense*), 'Bode' (*C. chinense*), and 'Malagueta' (*C. frutescens*) were supplied by the Vegetable Germplasm Bank in Zaragoza at the CITA of Aragón (Zaragoza, Spain). The seeds of the four varieties were germinated in Petri dishes, and then 10 plants per genotype were grown in a random distribution inside an acclimatized greenhouse in 17 cm diameter black plastic pots (one plant per pot), filled with a substrate mixture formed by peat, sand, and clay-loam soil as well as Humin Substrat (Klasman-Deilmann, Geeste, Germany) (1:1:1:1, v/v). Two grams of a slow-release fertilizer (Osmocote 16N-4P-9K, Scotts, Tarragona, Spain) were used as a topdressing for each pot. The plants were also watered daily by a drip irrigation system to maintain their optimum humidity levels for growth. Temperature levels were controlled of the whole process with values between 12 – 24 ºC in the spring and 20 – 28 ºC in the summer.

The flowers were labeled at the onset of their anthesis, so as to allow the fruit stage of development to be determined and hence each pepper's age at the time of harvesting. The peppers were harvested during the last week of September, since the plants stopped producing new peppers (around 6-month-old plants). A total pepper weight varying between 232 and 346 g was harvested from all the plants at different stages of development in order to avoid particular effects from individual pepper plants. The maturation stages of the peppers at the time of harvest varied between immature green and senescent.

Once the samples were harvested, all the fruits from all the plants of each variety were grouped together according to their dpa. The stem and seeds of the peppers were discarded before their analysis, while their pericarp and placenta were ground together in an Ultra-Turrax blender (IKA, Staufen, Germany) to produce a fully homogeneous sample that was then frozen at -20 ºC until analysis.

2.2. Chemicals and Reagents

The analytical standards of the two major capsinoids, capsiate (CTE) (4-hydroxy-3-methoxybenzyl (E)-8-methyl-6-nonenoate) and dihydrocapsiate (DHCTE) (4-hydroxy-3-methoxybenzyl 8-methylnonanoate), were synthesized in the Department of Organic Chemistry at the University of Cadiz by Barbero et al. [38]. All of the samples were prepared in a mixture of HPLC grade methanol and ethyl acetate (99.9%) from PanreacQuímica S.L.U. (Castellar del Vallés, Barcelona, Spain), and Milli-Q water provided by a deionization system (Millipore, Bedford, MA, USA). For the chromatographic separation, HPLC grade acetonitrile (99.99%) from Panreac Química S.L.U. (Castellar del Vallés, Barcelona, Spain), glacial acetic acid (99%) from Merck (Darmstadt, Germany), and Milli-Q water were employed.

2.3. Fresh Pepper Extraction Procedure

The capsinoid extraction process from peppers at the different maturation stages was performed following a method previously developed by our research team [39]. The ultrasounds were applied by means of a Sonoplus probe (BANDELIN ELECTRONIC, Heinrichstraβe, Berlín, Germany) coupled to a 7 L refrigerated circulator for temperature control (PolyScience, Niles, IL, USA). The sample was immersed into a temperature-insulated double-walled vessel. Approximately 0.5 g of chili pepper from each different ripening stages were placed in a 50 mL plastic holder, followed by the addition of 15 mL of extraction solvent (which was composed by 42% methanol + 58% ethyl acetate). The sample was sonicated for 2 min at 5.5 ºC, under 80% of the maximum allowed power (70 W) and applying duty cycles of 0.5 s. The extraction process was carried out in duplicate for each group of homogeneous samples.

The average of the two values obtained would be considered as the final results. The extracts were centrifuged twice for 5 min at 7500 rpm (orbital radius 9.5 cm) and the supernatants were transferred to a 25 mL volumetric flask, which was made up to the mark with the same extraction solvent. The samples were filtered using a 0.22 µm nylon syringe filter (Membrane Solution, Dallas, TX, USA) and analyzed by means of a ultra-high-performance liquid chromatography coupled to photodiode-array (UHPLC-PDA) to confirm the presence of capsinoids.

2.4. UHPLC-Q-ToF-MS Identification of Capsinoids

In order to identify the capsinoids present in the pepper samples, a UHPLC system (Waters Corporation, Milford, MA, USA) with a 2.1 x 100 mm, 1.7 µm particle size rp-C18 analytical column (Acquity UPLC BEH C-18, Waters, MA, USA) was used. The UHPLC system was coupled to a quadrupole time-of-flight mass spectrometer (Q-ToF-MS) equipped with an electrospray ionization source (ESI) interface (Xevo G2 QToF Waters Corporation, Milford, MA, USA) operating in positive ion mode. For the control of the equipment, its integration, and the subsequent data analysis, Masslynx software version 4.1 was employed. The UHPLC variables, as well as the operating conditions of the mass spectrometer were performed according to the method described by Vázquez-Espinosa et al. [40]. Spectra were acquired in the full-scan mode (*m/z* = 100–600). The molecular ions $[M + H]^+$ and $[M + Na]^+$ monitored for their identification were: CTE (*m/z* 307 and *m/z* 329), and DCHTE (*m/z* 309 and *m/z* 330), respectively. However, CTE was the only capsinoid detected and, therefore, quantified in the different varieties of pepper analyzed. Its chemical structure is shown in Figure 1. Furthermore, the mass spectrum showing the characteristic fragments that allow their identification can be found in supplementary Figure S1.

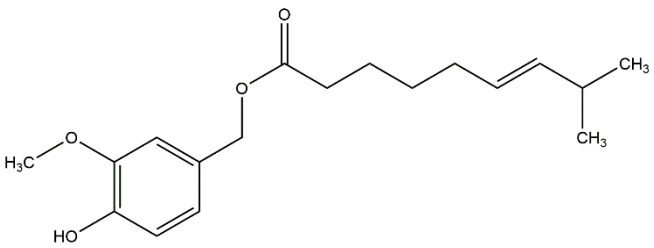

Figure 1. Chemical structure of capsiate (CTE).

2.5. UHPLC-PDA Analysis of Capsinoids

After identifying the only capsinoid present in these pepper samples (CTE), the extracts were subjected to UHPLC-PDA using an Acquity Ultra Performance LC Class system (Waters Corporation, Milford, MA, USA) equipped with an autosampler operated at 15 ºC, a Quaternary Pump System, and a Photodiode Array Detector (PDA) set to a wavelength of 280 nm for the detection and subsequent quantification of the compound present in the different pepper varieties. A Waters ACQUITY UPLC BEH rp-C18 100 x 2.1 mm column with 1.7 µm particle size, maintained at 50 ºC, was used as the analytical column. Empower 3 software (Waters Corp., Milford, MA, USA) was used for the data treatment and equipment control. The UHPLC equipment variables were the same as in the method previously described by Vazquez-Espinosa et al. [40]. CTE, the major capsinoid, was the only one found in all the four pepper varieties that were analyzed. A calibration curve (y = 1682.50x + 164.74) was used for CTE quantification. Its regression equation and correlation coefficients (R^2 = 0.9997), limit of detection (LOD = 3.60 ng g^{-1} of fresh weight (FW)) and quantification (LOQ = 12.00 ng g^{-1} of FW) were all determined. The quantitative data were obtained based on the integration of the UHPLC peak areas corresponding to three injections of the CTE analytical standard. A chromatogram of each variety obtained by UHPLC-PDA (280 nm), at the time of maximum capsiate concentration for each variety, has been included in supplementary material (Figure S2).

2.6. Statistical Analysis

A one-way analysis of variance (ANOVA), followed by a Tukey's test, were performed to determine any significant differences (p-value < 0.05) in CTE contents depending on ripening stage. The results were expressed as the mean ± standard deviation (SD) for duplicate analysis. All of the data obtained from the analyses were dealt with by means of Statgraphic Centurion Version XVII (Statgraphics Technologies, Inc., The Plains, VA, USA).

3. Results and Discussion

3.1. Evolution of the Total Capsinoids Content

As mentioned above, capsinoids have excellent pharmacological effects on human health. In addition, they are considerably less spicy than capsaicinoids, which makes them more attractive and favorable for a regular daily intake, so that they provide all their benefits without the pungency side-effects. This is what makes any study on the correlation between fruit development stage and capsinoids content so interesting, so that harvesting can take place when highest capsinoid concentration is to be expected.

The number of reports that can be found in the literature on the evolution of capsinoids content in the different varieties of peppers is very low. Fayos et al., analyzed capsinoids content in the varieties 'Chiltepín', 'Tampiqueño 740, and 'Bhut Jolokia', but only at four specific moments over the fruit ripening period, specifically on the 10th, 20th, 40th, and 60th dpa [41]. According to their study, similar trends could be observed in the three genotypes, with the accumulation of capsinoids beginning between the 10th and the 20th dpa and then increasing up to their maximum concentration on the 40th dpa (with values reaching 276.39 μg g^{-1} of FW, 69.31 μg g^{-1} of FW, and 122.62 μg g^{-1} of FW, respectively). Finally, the content gradually decreased over the last stages of development [41]. Jang et al., also studied the evolution of these compounds at four different moments during the ripening process of four different varieties of pepper that ranged between spicy and slightly spicy ones. Similarly, they registered the largest content of capsinoids at the intermediate stages of the fruit development, i.e., between the 30th and the 40th dpa (with values around 603.66 μg g^{-1} of DW for 'Habanero' and around 300 – 400 μg g^{-1} for the other varieties) [42]. Finally, Jarret et al. carried out the same study on the variety C. annuum '509-45-10. Immature green, mature green, turning, and mature red stages were considered in that occasion. As expected, capsinoid concentrations in the fruits increased rapidly on the 10th dpa and reached their maximum value over the mature green stage (at 1013 μg g^{-1} of DW), followed by a fall in capsinoid concentration levels [43].

The aim of the present work was to complete a much more detailed monitoring process by increasing the number of time lapses analyzed throughout the ripening of the fruit, so that a deeper knowledge of and more detailed information about pepper's beneficial compounds was attained, since previous studies had not reached such a thorough understanding of the process for any of the pepper varieties of interest. The ultimate objective is to precisely determine the moment of largest level of capsinoid concentration, and therefore, their optimal harvesting time. 'Habanero', 'Habanero Roxo', 'Bode', and 'Malagueta' are the pepper varieties selected for the study, and their content has been controlled at 10 different fruit developing stages (specifically on the 13th, 20th, 27th, 34th, 41st, 48th, 55th, 62nd, 69th, and 76th dpa). The visual appearance after the harvesting of the peppers analyzed at each one of the developing stages is shown in Figure 2. The samples were crushed before carrying out the extraction in order to increase the contact surface and facilitate the penetration of the solvent to favor a larger recovery [44].

Figure 2. Pepper fruits assayed for their capsiate accumulation patterns during development and maturation. Fruits of '*Habanero*' (A), '*Habanero Roxo*' (B), '*Bode*' (C), and '*Malagueta*' (D) at 13, 20, 27, 34, 41, 48, 55, 62, 69, and 76 dpa from left-to-right.

Capsiate, the major capsinoid, was the olnly one to be found in all of the pepper varieties analyzed. The evolution with regards to capsiate content (µg g^{-1} of FW) over the ripening process of the fruits is represented in Figure 3.

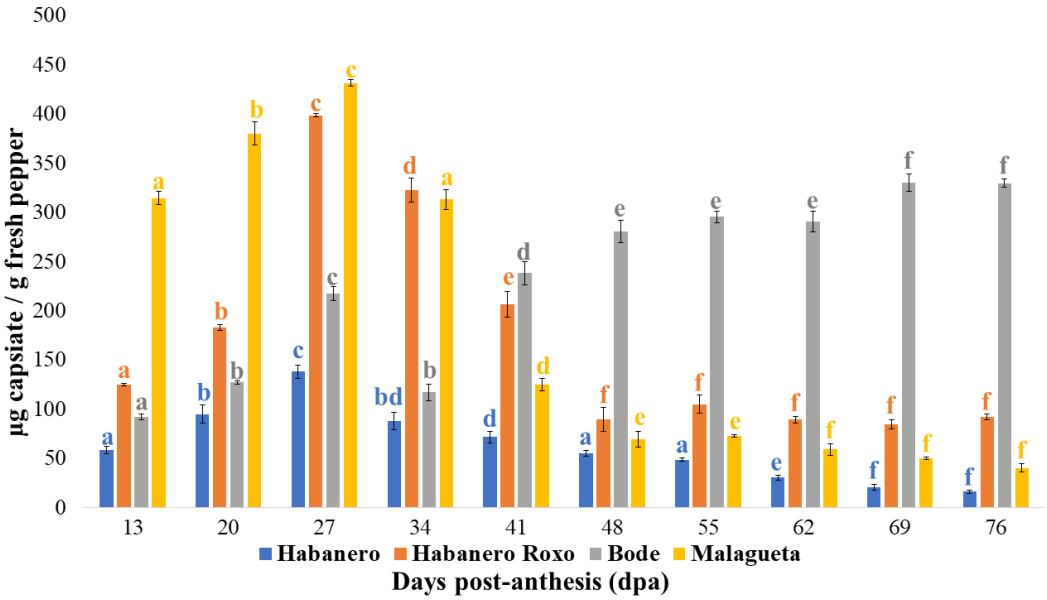

Figure 3. Evolution of total capsiate content (µg g^{-1} of FW) during the development of pepper fruits ($n = 2$). According to the Tuckey's test, results with a *p*-value less tan 0.05 wereconsidered to be statistically different. Taking this into account, the use of different letters in this figure indicates that there is a significant difference between results depending on Tuckey's test. In turn, the letters of each color refer to their respective variety, that would be from left-to-right, blue for '*Habanero*', orange for '*Habanero Roxo*', grey for '*Bode*', and yellow for '*Malagueta*'.

Similar behavior was observed in the 'Habanero', 'Habanero Roxo', and 'Malagueta' peppers, where the maximum CTE content was registered on the 27th dpa (with 137.84 µg g^{-1} of FW, 398.28 µg g^{-1} of FW, and 431.10 µg g^{-1} of FW, respectively). After that, 'Habanero Roxo' and 'Malagueta' presented drastic reductions in CTE content between the 27th and 48th days, corresponding to 77.66% and 83.96% of their maximum registered concentration, respectively. After the 48th day, their CTE concentration remained practically stable until the end of the ripening process. In the case of 'Habanero', after reaching its maximum level of CTE content,

there was a substantial reduction (88.47% decrease) over this last period until the day 76th. All of these results are in agreement with those obtained by Jarret et al., for *C. annuum* '509-45-10 pepper, as above explained. This evolution has also been reported by other researchers in relation to other *Capsicum* spp. such as 'Chiltepín', 'Tampiqueño 740 or 'Bhut Jolokia', although in their case, they reached their maximum concentration a few days later; this difference could be attributed to growing conditions or genotype reasons [41].

Generally, the short number of studies that have been conducted on the accumulation of capsinoids in *Capsicum* fruits have shown that the concentration of these compounds increase during the early stages of the fruit development, and this trend goes on during the first stages of the ripening process until a maximum value is reached, usually between the 20th and 40th dpa [41–43]. After that time, there is an inversion of the trend and a marked reduction in capsinoids content is observed. Such reduction in capsinoids content over the last stages of the fruit development, could be associated with a reduction in the biosynthesis of capsinoids inside the pepper according to the specific cultivation conditions in the greenhouse [45] or, alternatively, to the effect of the peroxidases that can be found in peppers. Hot pepper peroxidases, especially peroxidase isoenzyme 6, oxidizes the phenolic precursors involved in capsaicin biosynthesis. Basic peroxidase isoenzyme 6 is located in the placental epidermal cells and in the vacuoles, where capsaicinoids are synthesized, and their capacity to degrade these compounds is due to the strong capsaicin-oxidizing activity of this isoenzyme [46,47]. Cell walls and vacuoles are also the places where capsiate is accumulated. Based on this fact, Lema et al., have suggested that the same chili peroxidases that oxidize capsaicinoids vanillyl residues were also capable of oxidizing the vanillyl residues from capsinoids. The use of different inhibitors allowed to confirm that this peroxidase actually has the capacity to perform such oxidation. These results strongly support the assumption that the basic peroxidases that can be found in *C. annuum* could be responsible for the oxidation of their own CTE content [48].

Conversely, it can be seen from Figure 3 that, in a global context, the total capsiate content in 'Bode' peppers raised from the first point of harvest on the 13th dpa until the 76th dpa, at which point there was a concentration of 329.46 µg g^{-1} of FW, which corresponds to 350% increment compared to the initial content. And this was so, despite two perceptible CTE content falls that were registered during the maturation period: specifically, a decrease of 46.15% between the 27th dpa and the 34th dpa, and a slight reduction by just 1.63% between the 55th and the 62nd dpa. These falls in CTE content could be attributed to the action of the peroxidases in the peppers and to the subsequent reduction in the synthesis of their capsinoid content. In contrast to what is generally reported, a significant increase in the content of CTE was observed over the first ripening days, specifically from the first point of harvest on the 13th dpa (91.96 µg g^{-1} of FW) up to the 27th dpa, where it reached a concentration of 217.03 µg g^{-1} of FW. This was followed by an increment in CTE content between the 34th and 55th dpa. The final increase in the total content of CTE that took place in the last stage of the maturation process could be due to the loss of water suffered by overripen peppers. Nevertheless, these facts will not be considered for the object of this study, since such overripe peppers are not suitable for commercialization due to inadequate organoleptic attributes.

Most of the studies conducted on the different pepper varieties have reported that capsinoid content decreased rapidly as the fruits matured and changed color. This substantial reduction in capsinoid content as pepper ripens supports the need to perform sampling of the fruit at the appropriate development stages. In addition to all the factors that affect capsinoid content and that been already mentioned, including the decreased gene expression or peroxidase action, the instability of these compounds in different solvents should also be taken into account. Capsinoids are esters of fatty acid and vanillyl alcohol, so they are stable in non-polar solvents such as ethyl acetate, but they decompose easily in polar solvents such as water, methanol, and so on [49]. This is why, if the highest concentration of these compounds of interest and their health benefits are to be attained, green peppers should be eaten raw.

Since these compounds are unstable in water, and also when subjected to high temperatures, cooking should be avoided in order to keep the largest possible content of capsinoids. Another factor to keep in mind is that as these fruits mature and turn from green into red color, their present a smaller capsinoid content [50].

Although it has been seen that, in general, the accumulation pattern of CTE content in pepper fruit during its ripening stages followed similar trends, it is also true that, depending on the pepper's genotype, as well as on the growing conditions or environmental factors, such content may vary and result differently accordingly. In this sense, it would be necessary to monitor every detail of each crop's cultivation conditions, including all the possible environmental factors, since they may greatly influence the final product and its composition [51,52]. For this reason, it would be necessary to complete deeper studies where a greater number of varieties and under a wider range of different conditions would be analyzed. The present work intends to be a preliminary study that can be used as a starting point to demonstrate that there is no fixed pattern with regards to the variations in capsinoid content during the fruit ripening, so that each variety reaches a maximum concentration of these beneficial compounds at different stages of development.

3.2. Comparison of Capsiate and Capsaicinoids Contents

A comparison between the results obtained in this work with respect to the capsaicinoid accumulation patterns previously reported has been carried out in order to determine any similarities or differences in both compound families (capsinoids and capsaicinoids), both with similar pharmacological properties but different pungent capacities. It should be noted that the fruits have been grown in acclimatized greenhouses and under the same conditions (they are the same samples as the ones used for our previous work on capsaicinoids [35,36]), which should allow a more reliable comparison. It should also be noted that both families of compounds share part of their biosynthetic pathway.

3.2.1. '*Habanero*' pepper

The maximum capsaicinoid content in 'Habanero' peppers was obtained on the 33th dpa (≈1400 μg g^{-1} of DW) [35]. A slight reduction in their content (8.5%) was observed from the 34th until the 48th dpa, which was associated to the effect of the peroxidases. After that, the capsaicinoid content remained practically constant over the rest of the ripening process. The evolution of the CTE content followed a similar trend. However, it reached its maximum concentration (137.84 μg g^{-1} of FW) a few days earlier, specifically on the 27th dpa, possibly as a consequence of the greater degradability and instability of this compound [53]. Furthermore, it was notable that such reduction was substantially more drastic (88.47%) than that in capsaicinoids and continued decreasing until the end of the maturation process. This correlation between the accumulation patterns from each compound family seems to indicate that the environmental factors have had similar effects on both biosynthetic pathways.

3.2.2. '*Habanero Roxo*' pepper

As can be seen in our previous work [35], each family of compounds present distinctive evolution patterns, even when capsaicinoid and capsinoid contents have been determined for the same plant that had been grown under the same environmental conditions. It can then be said that the differences in content between the two families seem to be due to genetic factors inherent to this variety. The máximum capsaicinoid content was registered on the 41st dpa, which coincided, in this case, with a change of color in the peppers from green to violet. From that moment, there was a period over which the concentration remained practically stable until the 55th dpa. After that, the capsaicinoid concentration increased substantially until the over-ripening stage was reached. On the contrary, the maximum CTE content was registered on the 27th dpa, and then a drastic reduction by 77.66% took place between the 27th and 48th dpa. From then on, the CTE concentration remained practically stable until the end of the fruit's ripening process.

3.2.3. '*Bode*' pepper

In our previous studies on this pepper variety [35], a similar behavior was observed with regards to the accumulation pattern of the two families of compounds. Both the total capsaicinoid and CTE content raised from the first point of harvest until the end of the ripening period. A substantial increase in the content of these compounds was observed in the early stages of the fruit development up to the 33rd dpa for capsaicinoids and to the 20th dpa for CTE. At the end of the fruit's maturation, two more moderate increments were registered (the former took place between the 48th and the 69th dpa, and the latter between the 76th and 83rd dpa), which could be reasonably associated with genetic factors since the plant had been cultivated under the same environmental conditions and, therefore, we should assume that both families of compounds had been equally affected. The specific growing conditions that were controlled in the greenhouse were temperature, humidity, irrigation, and fertilization, and these controlled conditions resulted in some increment in the amount of both compounds. It was also observed that halfway through the development of the fruit, a decrease in the amount of these compounds took place as a result of the action of the peroxidase enzymes in the peppers [48].

3.2.4. '*Malagueta*' pepper

The fruit accumulation pattern for capsaicinoids during the ripening period of this variety followed the same trend as the 'Bode' variety [36]. Thus, in general, there was a concentration increment over the ripening, even if, as previously mentioned, there was a series of increases and decreases throughout the process. Nevertheless, CTE evolution followed a particular evolution pattern where the máximum concentration was reached on the 27th dpa, after which a drastic reduction in the content (83.96%) took place. This was followed by a content stability period until the end of the maturation process.

Firstly, it should be noted that for all of the pungent varieties that have been analyzed, capsaicinoids were found in peppers in substantially larger concentrations that capsinoids. The main capsaicinoids found in peppers are capsaicin and dihydrocapsaicin, both with considerably larger concentrations than that of CTE, the only capsinoid detected. Nevertheless, the concentration of CTE in the varieties that have been analyzed was slightly above the concentration levels registered for other capsaicinoids (nordihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin). Moreover, no similar concentration pattern or trend has been encountered that could equally be applied to the different varieties under study. This indicates, as mentioned above, that the genetic factors that are inherent to each variety play a significant role with regards to accumulation patterns or compound contents. Furthermore, since environmental factors seem to have a considerable influence on such contents and patterns, every possible detail with regards to growing conditions should be closely monitored in order to determine their influence on the fruit final composition [50,51]. Thus, and considering the greater degradability of capsinoids, growing conditions should be carefully contemplated and implemented. Future studies that intend to deepen not only in the study of capsinoid accumulation patterns in a greater number of varieties, but also in their comparison with those of capsaicinoids in the same varieties should be covered.

4. Conclusions

The current work has demonstrated, for a number of pepper varieties, that the bioactive content of their fruits with regards to the bioactive compounds responsible for pepper pungency, capsaicinoids, and capsinoids, may vary widely depending on their genotype, the fruit developmental stage, and the specific growing conditions. Since drastic changes in CTE content have been observed over the ripening period, determining how maturity stages may affect the composition of the peppers with regards to such biologically interesting bioactive compounds is of the utmost interest.

This study intends to determine the optimal harvesting moment based on the moment of the greatest CTE content, which would be on the 27th dpa for '*Habanero*', '*Habanero Roxo*', and '*Malagueta*' peppers, and on the 55th dpa for '*Bode*' peppers (the later increase has not been taken into account since they are overripe peppers that are not suitable for consumption due to their organoleptic properties). This study also has deepened knowledge of the accumulation patterns for CTE content over the fruit development and their correlation with pepper capsaicinoid content. For the four varieties under study, different accumulation patterns of the two families of compounds of interest have been determined. Since no definitely clear pattern has been established, an in-depth study with a greater number of varieties and fruit-development monitoring points would be necessary. The variations that our study has registered with regards to bioactive compound contents, and that can be attributed to genetic factors, constitute a practical foundation for the improvement of the nutritional qualities of pepper products.

Supplementary Materials: Figure S1. Mass spectrum of capsiate obtained by UHPLC-QToF-MS. Figure S2. Representative chromatograms for the four varieties of peppers studied ((A) '*Habanero*'; (B) '*Habanero Roxo*'; (C) '*Bode*'; (D) '*Malagueta*') obtained by UHPLC-PDA (280 nm) at the point of maximum capsiate content. (1) Nor-dihydrocapsaicin (n-DHC); (2) Capsaicin (C); (3) Dihydrocapsaicin (DHC); (4) Homo-capsaicin (h-C); (5) Homo-dihydrocapsaicin (h-DHC); (6) Capsiate (CTE).

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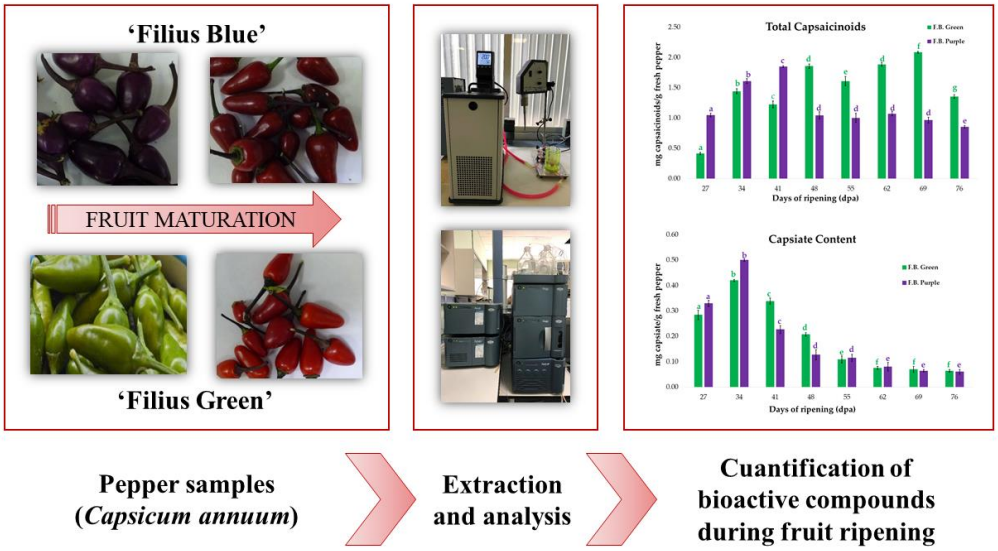

- **Content of Capsaicinoids and Capsiate in "Filius" Pepper Varieties as Affected by Ripening**

Abstract

Peppers are fruits with wide genetic variability and multiple ways of being consumed that hold a relevant position in the human diet. Nowadays, consumers are interested in new gastronomic experiences provided by pepper cultivars that present new shapes, colors, and flavors while preserving their bioactive compounds, such as their capsaicinoids and capsinoids. However, numerous changes take place during their development that may alter their biological properties. Therefore, this work evaluates the capsaicinoid and capsiate contents in two traditional varieties of ornamental peppers ("Filius Blue" and "Filius Green") during fruit maturation. The aim is to determine the ideal harvesting moment depending on the farmer's objective (e.g., achieving a specific color, shape, or flavor; achieving the maximum concentrations of bioactive compounds). The capsaicinoid contents followed different patterns in the two varieties analyzed. The "Filius Blue" variety exhibited increasing concentrations of capsaicinoids up to the 41st day post-anthesis (dpa), from which point on this trend was reversed. The concentrations in the "Filius Green" variety increased and decreased several times, reaching maximum concentrations on the 69th dpa.

Regarding capsiate contents, both varieties varied in the same way, reaching maximum concentrations on the 34th dpa and then decreasing.

1. Introduction

Peppers belong to the Solanaceae family from the genus *Capsicum*, which is native to Central and South America. They are cultivated in tropical and warm climate regions worldwide [1]. Peppers are fruits that hold a significant position in human diets because of their versatility, which allows them to be consumed fresh in salads, fried, boiled, in their dehydrated form as a seasoning, or even as a sauce or jam [2]. Peppers have ample genetic diversity and comprise a substantial number of varieties that differ in plant size (from short, compact plants to plants as tall as three to four feet), color (green, purple, yellow, chocolate, orange, or red, depending on the pepper variety and maturation stage), flavor (from the non-pungent varieties to the hottest species), shape (round, elongated, wide, narrow, as well as special shapes such as bells), and pepper size (from small to full-size fruits) [3]. This diversity is the reason for the remarkable potential for peppers to be used in the agri-food industry, either as coloring or flavoring agents or in forms that utilize their sensory characteristics [4].

Sometimes, when we eat a food, we seek a certain sensation; however, a colorful dish can also be more appetizing. For this reason, in recent years new consumption trends have increased considerably, since current consumers seek new culinary experiences, resulting in the demand for novel cultivars with new morphologies, colors, textures, flavors, and fragrances. In this sense, there has been an increase in the commercialization and consumption of new hot pepper varieties that meet all of these premises, which have gained consumers' interest [5]. One of these new pepper varieties, known as "Filius" (*Capsicum annuum* L.), stands out amongst the rest, since it satisfies this new consumption trend and therefore has considerable marketing potential. This variety is characterized by its small, ovate shape and its numerous spicy fruits that grow vertically on the plant.

The “Filius” variety can be found in blue and green cultivar forms, according to the color of their immature fruit, which are blue-purple and green, respectively. Both cultivars of “Filius” peppers mature to a red color [6].

Additionally, consumers appreciate the valuable health properties that are attributed to this food group. In fact, peppers are known to have also been used as medicinal plants because of their substantial contents of bioactive compounds, which provide health benefits to consumers [7,8]. Capsaicinoids and capsinoids, which are among those bioactive compounds of interest, are also responsible for the pungency of chili peppers, which is one of their most important comercial traits [9,10]. Therefore, both capsaicinoids and capsinoids have received great attention from consumers because of their extensive pharmacological and physiological effects, i.e., antitumor [11], antioxidant [12], antiobesity [13], anti-inflammatory [14], and analgesic [15] effects. The basic chemical structure of capsaicinoids is formed by a combination of vanillylamine and a branched-chain fatty acid via an amide moiety; capsaicin and dihydrocapsaicin are the most significant capsaicinoids. Capsinoids have a similar structure except for their central bond, which is a combination of vanillyl alcohol with a branched-chain fatty acid via an ester group [16,17]. Capsinoids were first isolated from a low-pungengy variety known as *C. annuum* cv. CH-19 Sweet, with capsiate and dihydrocapsiate being the major capsinoids in nature [18]. Although their main difference lies with the fact that they present either amide or ester bonds, capsinoids are not spicy; that is, they do not produce an intense burning sensation. The pungency of capsinoids has been estimated at approximately 1/1000 compared to that of capsaicinoids. This makes them more propitious and appealing for consumption, which in turn supports their inclusion in both food and dietary supplements, as well as in other products with medicinal purposes [19]. The receptor vanilloid type-1 (TRPV1) is the one responsible for the burning sensation. Due to their slightly different structure, capsinoids do not stimulate the pungency receptors in the mouth; however, their effect in the intestines results in similar physiological activities [20].

The biosynthesis of capsaicinoids and capsinoids begins in the placenta in peppers, although they can then be excreted and detected in the seeds or pericarp [21]. The presence of capsaicinoids and capsinoids in peppers is controlled by the single dominant gene Pun1 [22]. However, the accumulation of one of the two compound types over the other seems to be due to the action of the putative aminotransferase (pAMT) gene, which encodes an aminotransferase (pAMT) involved in the production of vanillylamine from vanillin. The mutations that have been described in the pAMT gene result in a loss-of-function of pAMT, which in turn increases the production of vanillyl alcohol over that of vanillylamine, and consequently capsinoid accumulation dominates over capsaicinoid production [23]. While the genotype clearly plays a significant role with regards to the content and diversity of these bioactive compounds in peppers, the environmental conditions and agronomic practices can interact with their genotypes and have an influence on the bioactive properties of the final food [24]. It seems clear that numerous changes—such as the degradation and synthesis of fruit metabolites—may take place over the different stages of the ripening process of pepper fruits [25]. The characterization of the phytochemical changes that take place over the peppers' ripening process is particularly interesting from dietary and nutritional perspectives, since these changes may affect antioxidant activities, aroma, taste, quality, and ultimately consumers' preferences. Peppers are harvested and consumed at different ripening stages, from immature to fully ripe. It is, therefore, very important to be able to determine their optimal harvesting time during the ripening process. For this purpose, it should be kept in mind that the optimum color stage for a culinary application may not coincide with the moment of maximum bioactive compounds concentration, since each characteristic follows different development patterns [26]. Therefore, and depending on the attributes that are sought by producers (color, pungency, etc.), the fruit optimal harvesting moment will vary [7,27].

Nowadays, peppers are mainly used for cooking; however, when they were first brought over to Europe in the 15th century, they were highly appreciated as ornamental plants, rather tan food. Ornamental plants are those plants that are grown for their beauty and generally used in gardening, interior decoration, and landscaping [28].

Therefore, they should exhibit some remarkable morphological characteristics that support their aesthetic value, such as small, erect, and colorful fruits that present a color contrast with the foliage over the whole ripening process. Other positive features would include: vivid foliage, easiness to grow, long durability and the possibility of being grown in small pots [29–31]. The subsequent search for new shapes and colors as well as new flavors when used as food encouraged their cultivation and commercialization. In fact, the interest in these new pepper varieties has increased worldwide to eventually become a considerable financial source for growers [32].

The changes in both individual and total capsaicinoids concentrations, as well as capsiate contents, over the fruit ripening period, has already been analyzed in several pepper varieties, including some super-hot, pungent, and non-pungent varieties [33–35]. However, given the great variability between the different varieties of peppers and the growing conditions, it is necessary to deepen this type of study to have more precise knowledge about the evolution of these beneficial compounds throughout maturation. Moreover, other traditionally ornamental varieties, such as the one studied here, which have been recently incorporated to our diets despite their smaller fruits, have not yet been analyzed for capsaisinoids and capsiate contents. Only the Peter pepper variety has been analyzed just for capsaicinoids content [36]. These types of ornamental varieties can be used for food and dishes with a dual purpose (nutritional and decorative) due to the large amount of nutritious and healthy compounds and for aesthetic or decoration purposes, thanks to the variety of colors that they present during the ripening stages. Therefore, this work intends to apply Ultrasound-Assisted Extraction (UAE)—an extraction method that is well known to be efficient and easy to use [37]—plus Ultra-High-Performance Liquid Chromatography (UHPLC) to determine capsaicinoids and capsiate accumulation in two ‘Filius’ pepper cultivars over their ripening process. Additionally, fruit color changes over the ripening process have been registered, so that they can be used as a guidance to select their optimal harvesting moment depending on their final purpose.

2. Results and Discussion

2.1. Changes in Peppers' Total Capsaicinoids Content

The two cultivars under study used to perform the experiments were grown in an automated and acclimatized greenhouse under controlled conditions. They began to produce peppers during the second week of July and they were harvested 11 weeks later, specifically on 30 September. They were monitored during the development of the pepper fruits, from the 13th until the 76th day post anthesis (dpa). The visual appearance of the peppers at the different development stages was registered and is presented in Table 1 and Figure 1.

Once the peppers of both varieties were obtained in each of the maturation stages, the seeds and stems were discarded. The pericarp and placenta were ground together, forming the sample for analysis. These biological samples were totally homogenous and representative. A quantity between 232 – 346 g of peppers for each dpa were collected from 10 pepper plants of each genotype, cultivated in a greenhouse, to avoid any particular effect fromindividual pepper fruits [38]. Two technical replications were performed in each maturation stage; thus, the figures throughout the manuscript show the mean of both results. As can be seen in Figure 2, total capsaicinoids concentration varied significantly depending both on the particular cultivar and the development stage of its fruits. A substantial variation in capsaicinoids content could be attributed to the genotype, as well as to environmental factors and growing conditions [39]. However, it should be remembered that, in this case, the environmental and growing conditions were controlled, being the same for both varieties. Moreover, Olguín-Rojas et al. and Vázquez-Espinosa et al. grew their pepper cultivars under similar conditions to those in the present study and did not report the same trends for the total amount of capsaicinoids [34,40]. This may suggest that the changes in the contents could be attributed to each pepper genotype. In both varieties analyzed, capsaicinoids were not detected until the 13th dpa.

Table 1. Color of 'Filius' pepper fruits at the different stages of their development (dpa: days post-anthesis).

Start of Fruit Development	dpa	Color of the Fruits	
		'Filius Green'	**'Filius Blue'**
17/09	13	Green	Brown/Purple
10/09	20	Green	Purple
03/09	27	Green	Purple
27/08	34	Green/Red	Purple/Red
20/08	41	Green/Red	Red
13/08	48	Red	Red
06/08	55	Red	Red
30/07	62	Red	Red
23/07	69	Red	Red
16/07	76	Over-ripeness	Over-ripeness

Figure 1. Changes in the color of the fruits in both varieties over their maturation process. (A) Purple variety 'Filius Blue'; (B) green variety 'Filius Green'. The images of each variety were taken at 13, 34, 55 and 76 dpa from left-to-right, respectively. The respective UHPLC-PDA chromatograms (λ = 280 nm) are included in Figure S1 of Supplementary Material.

Most studies on the accumulation of capsaicinoids in *Capsicum* fruits have shown an increase in the concentration of these compounds in the early stages of the fruit development. This climbing trend remains during part of the ripening process until a maximum value is reached, approximately on the 40th dpa. Finally, a gradual degradation of these compounds is observed that ranges from 30% to 65% depending on the different pepper cultivars [41–44]. The purple variety 'Filius Blue' analyzed in

this work shows exactly that trend. The concentration of total capsaicinoids increases from the first days of maturation until a maximum value is reached on the 41st dpa (corresponding to 1.85 mg g^{-1} concentration). After that day, the trend is inverted and a marked 44% reduction in capsaicinoids content on the 48th dpa was registered. This change may be associated both with a considerable inhibition in the biosynthesis of these compounds [45], and with the action of peroxidases, which are enzymes capable of degrading capsaicin (C) and dihydrocapsaicin (DHC), as evidenced by the results of in vitro experiments performed by Bernal et al. [46,47]. Capsaicin is synthesized in the placenta and accumulates in the vacuoles of placental epidermal cells until it is metabolized [48]. The activity of basic peroxidases may be directly related to this catabolic reaction. The arguments that support a relevant role by peroxidase in the degradation of capsaicin are based on the unique location of peroxidase in the placental epidermal cells of hot pepper fruits. Furthermore, this evidence is supported by the strong oxidizing activity of capsaicin on basic peroxidase isoenzyme [49,50]. Finally, during the last stages of fruit ripening, the total capsaicinoid content remains practically constant until the end of their maturation.

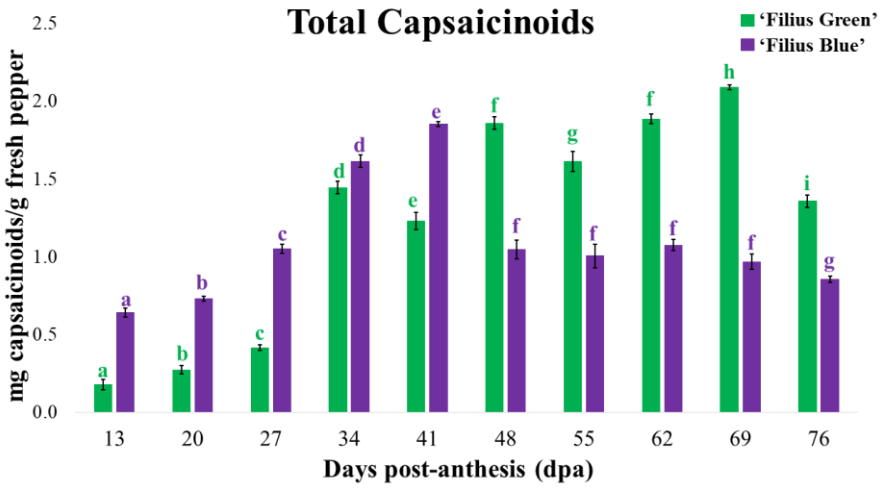

Figure 2. Total capsaicinoid concentration (mg g^{-1} fresh weight (FW)) during fruit ripening (n = 2). The same letter assigned to each variety indicates that there was no significant variation in capsaicinoids content according to Tuckey's test, that is, both values have a p-value > 0.05. Letter colors correspond to their respective variety, i.e., green for 'Filius Green' and purple for 'Filius Blue'.

Unlike what is generally reported in the literature, the green variety 'Filius Green' presented a significant increase in the content of capsaicinoids at the early stages of the peppers' development (between the 13th and the 34th dpa, when it reached 1.44 mg g^{-1}), followed by two more moderate increments around the end of the ripening process. In addition, two perceptible decreases were observed in the amount of capsaicinoids during the maturation period: a decrease of approximately 15% was observed between the 34th and the 41st dpa, and the second one by 13.5% was registered between the 48th and 55th dpa. Finally, over the last stages of maturation, a considerably greater drop of around 35% was observed in the total content of capsaicinoids. These decreases in capsaicinoids content could be due to both the action of the peroxidases and the reduced synthesis of capsaicinoids in the peppers due to the specific cultivation conditions in the greenhouse [49]. Comparable capsaicinoid accumulation patterns, with maximum capsaicinoid content during the last developmental stage, have been previously described for a number of cultivars such as Habanero, which exhibited máximum content levels on the 63th dpa [51], Habanero Roxo, with maximum levels on the 62nd dpa [34], and Malagueta on the 68th dpa [33]. It is believed that this behavior could be attributed to the particular growing conditions in the greenhouse, where temperature, humidity, irrigation, and fertilization were all controlled [24].

As mentioned above, nowadays, consumers look for new gastronomic experiences, including different colors of pepper fruits, but the desired color may not coincide with their máximum concentration of bioactive compounds.

If consumers are looking for less spicy sensations, they should collect the peppers during the early stages of the fruit development, when they present lower capsaicinoids concentrations and the varieties studied exhibit purple and green colors. After 1 month of maturation (around 27 – 34 dpa), the fruits maintain a purple or green color, respectively; however, their capsaicinoids content is considerably larger, so that consumers can experience sensations of greater pungency, despite the fact that their visual appearance remains unaltered.

On the other hand, capsaicinoids content in 'Filius Blue' variety is very similar on the 27th dpa, and between the 48th and the 69th dpa, which means that consumers can experience similar flavor sensations despite their different appearance, since in the first case the fruits present a green color, while during the second period they exhibit a red color. Finally, over the last stages of the fruit development, that is, when they look red, their capsaicinoids content is relatively high in all cases, so that their consumption is preferably associated to the seeking of pungent sensations.

2.2. Changes in the Content of Individual Capsaicinoids

Five capsaicinoids—nordihydrocapsaicin (n-DHC), capsaicin (C), dihydrocapsaicin (DHC), homocapsaicin (h-C), and homodihydrocapsaicin (h-DHC)—were identified in the two varieties of pepper. The content of each individual capsaicinoid in the pepper samples during the ripening of the fruit is displayed in Figure 3. The analyses were carried out in duplicate for each developmental stage (dpa).

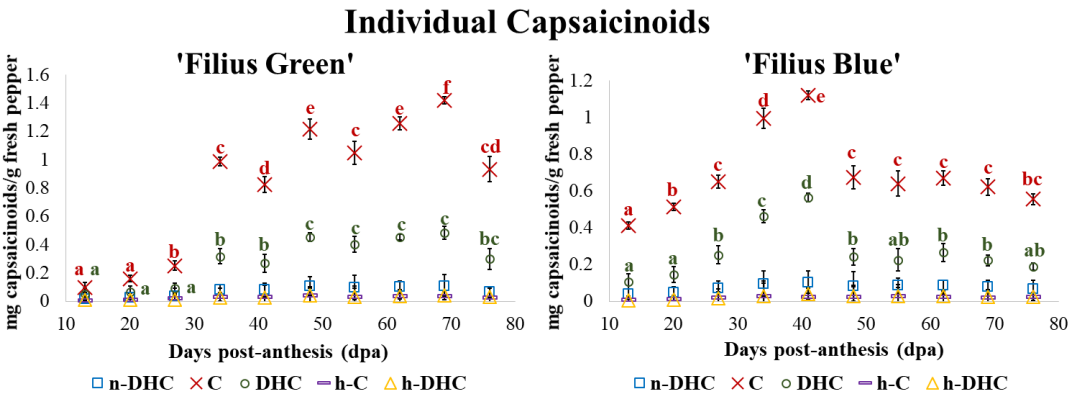

Figure 3. Individual capsaicinoids concentrations (mg g^{-1} fresh pepper (FW)) over the fruit ripening process (n = 2). The same letter indicates that no significant difference according to Tuckey's test were observed, that is, both values have a p-value > 0.05. In addition, a different letter color has been assigned to each specific capsaicinoid, i.e., blue for nordihydrocapsaicin (n-DHC), red for capsaicin (C), green for dihydrocapsaicin (DHC), purple for homocapsaicin (h-C), and yellow for homodihydrocapsaicin (h-DHC). Significant differences in the two major compounds have been highlighted to improve readability.

C was the one to present the highest content over the entire maturation period of the fruit in both varieties, followed by DHC, and then a small amount of n-DHC. Other capsaicinoids, such as, for instance, h-C and h-DHC, were detected in minimal amounts. The capsaicinoids content profile that was obtained is consistent with the results reported by similar studies [52,53]. C and DHC are considered the major capsaicinoids to contribute to the typical pungency of chili peppers that causes a heat sensation in the mouth, palate, throat and back tongue [54]. It was also observed that the changes registered for each individual capsaicinoid throughout the development of the fruits followed a similar pattern to that of the total capsaicinoids previously explained. The percentages of each specific capsaicinoid were also calculated during the maturation of the fruit and are represented in Figure 4.

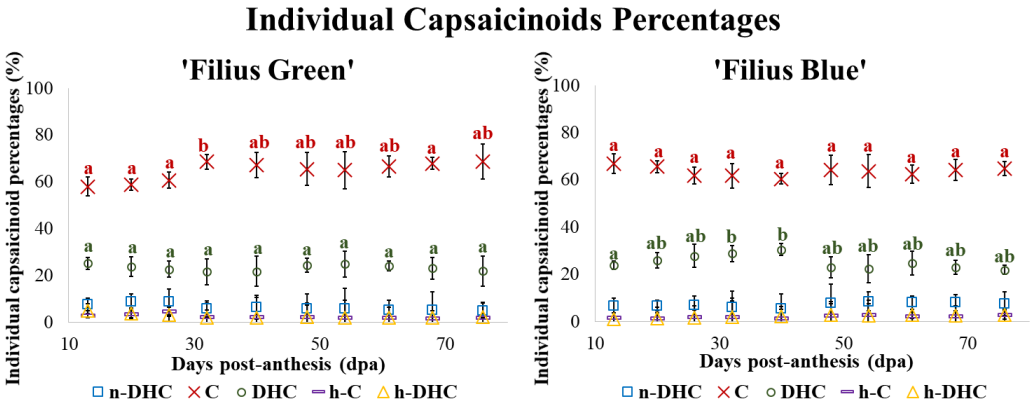

Figure 4. Individual capsaicinoid percentages (%) during the fruit ripening ($n = 2$). The same letter has been assigned in this figure to indicate that there are no significant differences according to Tuckey's test, that is, both values have a p-value > 0.05. In addition, a different letter color has been assigned to each specific capsaicinoid, i.e., blue for nordihydrocapsaicin (n-DHC), red for capsaicin (C), green for dihydrocapsaicin (DHC), purple for homocapsaicin (h-C), and yellow for homodihydrocapsaicin (h-DHC). Significant differences in the two major capsaicinoids have been highlighted to improve readability.

In both varieties, it was observed that the percentage of each individual capsaicinoid remained practically constant during the maturation period, with just a slight variation.

C content rated around 60–70% during fruit ripening. Similarly, DHC changed between 20 – 30%; and both together represent approximately 90% of the total capsaicinoids content. These results were similar to those reported in the literature for other varieties [53,55]. However, they differed from the description by Barbero et al. for the Peter pepper variety, where the percentage of C and DHC reversed during the maturation of the fruit. In the study of Barbero et al. DHC was the major capsaicinoid over the early stages and C over the final period [36]. In other pepper varieties, DHC was registered as the main capsaicinoid [56]. It can also be noticed that a decrease in the percentage of C content corresponds to an increment in the percentage of DHC and vice versa. n-DHC was the third major capsaicinoid at between 5% and 8% of the total capsaicinoid content depending on the fruits ripening stage. Lower percentages of around 1 – 3% were registered for the minor capsaicinoids, h-C and h-DHC.

2.3. Changes in the Standardized Value of Capsaicinoids

The standardized values of themain capsaicinoids were determined according to the following equation:

$$RP_i = \frac{Cx_i}{CT_i} x100$$

where RPi is the relative percentage of each capsaicinoid, Ci is the concentration of the selected capsaicinoid at “xi” dpa, and CTi is the maximum concentration of that particular capsaicinoid during the whole ripening process.

It can be seen from Table 2 that, in both varieties analyzed, the content of most individual capsaicinoids, with the exception of h-C, followed a similar pattern. In addition, they all follow the same trend as for total capsaicinoids content previously explained for each one of the two varieties under study.

With regards to the ‘Filius Green’ variety, all the capsaicinoids showed a fairly similar behavior and reached their maximum content level of n-DHC, C, DHC, and h-DHC on the 69th dpa. On the other hand, h-C reached it at an earlier stage of maturation, specifically on the 48th dpa, at which point the rest of the capsaicinoids were also very close to their maximum content level.

Table 2. Standardized values (%) of individual capsaicinoids during the fruit development of the two varieties analyzed.

(dpa)		13	20	27	34	41	48	55	62	69	76
'Filius Green'	n-DHC	12.49	25.66	33.77	76.11	73.54	98.58	89.03	94.43	100.00	63.49
	C	6.96	11.27	17.78	69.48	58.12	85.76	73.82	88.54	100.00	65.75
	DHC	10.33	13.43	19.58	65.83	55.50	93.78	83.52	94.20	100.00	61.76
	h-C	12.62	22.15	45.57	74.49	68.94	100.00	77.35	85.56	87.04	66.92
	h-DHC	20.83	24.34	30.84	62.77	61.29	95.43	77.14	84.74	100.00	69.98
'Filius Blue'	n-DHC	37.41	47.41	69.05	92.43	100.00	77.56	82.14	83.57	74.46	62.53
	C	36.81	45.91	58.07	88.85	100.00	60.13	57.19	59.84	55.56	49.59
	DHC	19.12	25.77	44.81	82.24	100.00	42.80	39.81	46.94	39.47	33.25
	h-C	32.64	36.08	67.50	98.33	88.68	86.53	100.00	83.88	73.67	86.17
	h-DHC	13.40	24.78	45.22	75.21	100.00	70.37	66.47	72.12	62.67	59.93

Regarding the 'Filius Blue' variety, the relative percentages of n-DHC, C, DHC, and h-DHC increased until the 41th dpa, when they reached their maximum concentration during the fruit development process. From that moment on, there was a gradual decrease in their concentration, then there is a slight increase on day 62th dpa, to finally decrease again until the end of the fruit maturation. Contrarily, h-C reached its máximum concentration some days later, so that its relative percentage increased until the 34th dpa, when it reached levels close to its maximum concentration. Then, it went down slightly and increased again to reach its maximum value on the 55th dpa. A gradual concentration drop took place over the last stages and finally a new increment that could be attributed to the effect of fruit dehydration. Most of the pepper varieties described in the literature present a standardized evolution pattern that is specific for each one of the main capsaicinoids present in their fruits. In fact, not all the particular capsaicinoids that can be found in a particular variety exhibit the same trend for their normalized values and, therefore, each particular capsaicinoid reach its maximum concentration at a specific moment during the maturation process [33,36,41]. However, Vázquez-Espinosa et al. reported that in the 'Naga Jolokia' pepper variety, all the capsaicinoids followed the same pattern throughout the ripening process [40].

In this work, the variation of bioactive compounds in each variety are mainly due to their genotype, since they have been grown under the same controlled conditions. However, the same variety may be cultivated under different environmental conditions, which would also affect its compounds content. In those cases, cultivation parameters should be closely controlled so that the resulting measurements are reproducible and comparable to those obtained by other researchers.

2.4. Changes in Capsiate Content during Fruit Ripening

First of all, it should be noted that capsinoids, in both varieties, were detected in smaller amounts than capsaicinoids throughout the whole fruit ripening period. Specifically, capsiate (CTE) content represented around 5 – 30% with respect to the total amount of capsaicinoids, depending on the developmental stage. Regarding the CTE accumulation pattern over the peppers development (Figure 5), a similar trend was observed for the two pepper cultivars.

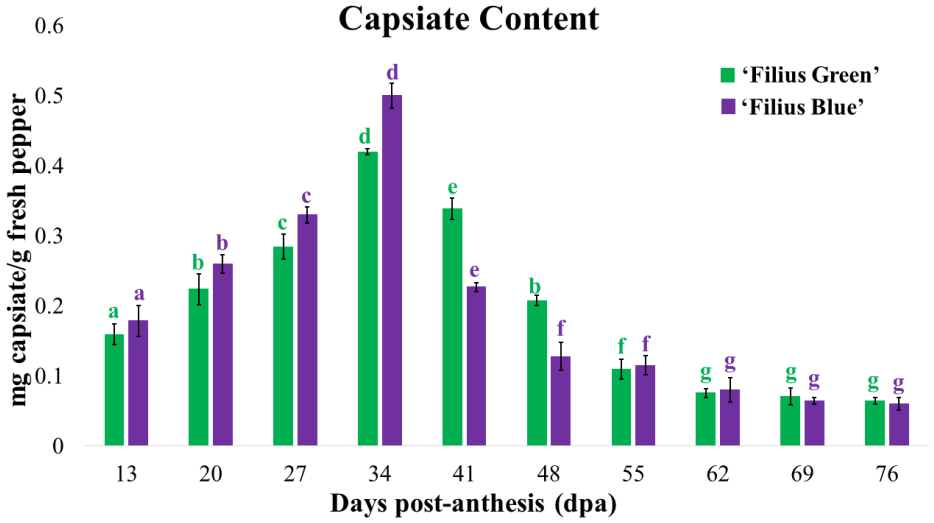

Figure 5. Capsiate concentration (mg g^{-1} FW) during fruit ripening (n = 2). The same letter assigned to each variety in this figure indicates that there were no significant differences in capsaicinoids content according to Tuckey's test, that is, both values have a p-value > 0.05. In addition, a different letter color has been assigned to each specific variety, i.e., green for 'Filius Green' and purple for 'Filius Blue'.

The CTE content increased until the 34th dpa, when it reached its maximum value (0.42 mg g^{-1} of FW for 'Filius Green' and 0.50 mg g^{-1} of FW for 'Filius Blue'), and, from then on, its concentration decreased drastically until the 62nd dpa by 82% and 84%, respectively. Such a drastic reduction in capsiate content after reaching its maximum value may be related either to a decrease in the expression of the biosynthetic structural genes of capsinoids [57] or to the action of the peroxidase enzymes present in peppers, similar to what has been previously explained for capsaicinoids [47]. Capsinoids also accumulate in cell walls and vacuoles [48]. In this sense, Lema et al. suggested that the same peroxidases that oxidize capsaicinoids are also capable of oxidizing capsinoids [58]. Finally, capsinoids content remained constant over the last stages until the end of the fruit maturation process.

These results are in agreement with those described for other *C. annuum* varieties with different pungency levels. According to Yazawa et al., CTE and dihydrocapsiate (DHCTE) reached their máximum content on the 30th dpa and then decreased [59]; Jang et al. also registered the highest capsinoid content between the 30th and the 40th day of the fruit ripening period [60]; Fayos et al. reported that capsinoids content in the varieties 'Chiltepín', 'Tampiqueño 740 and 'Bhut Jolokia' increased to its maximum value on the 40th dpa [35].

Another fact to keep in mind is that the CTE accumulation pattern in the green variety 'Filius Green' was different from that of capsaicinoids'. Whereas CTE content increased up to the 34th dpa and then decreased to a constant level, capsaicinoids content had a series of ups and downs throughout the whole maturation process of the fruit and reached its maximum value on the 69th dpa. These differences in trends could be attributed to the presence of a number of factors that would act as regulators of the accumulation of capsinoids and capsaicinoids in the biosynthesis pathway of this cultivar [61]. However, with regards to the purple variety 'Filius Blue' the accumulation pattern for both capsaicinoids and CTE was the same, even if the maximum value of capsiate content was reached at an earlier stage of maturation. This could be due to the greater instability exhibited by capsinoids either in water or under

high temperatures with respect to that of capsaicinoids'. Other methodologies were not tested because capsinoid extraction has been previously optimized in our research group using Ultrasound-Assisted Extraction (UAE) and Microwave-Assisted Extraction (MAE) [62]. Our previous work demonstrated that quantitative capsinoid extractions were achieved using these methods. Since no significant differences were observed between both techniques, UAE was used for availability. Natural capsinoids are probably stable in non-polar solvents and labile in polar solvents and they probably tend to decompose in protic solvents such as alcohol or water [63]. Due to the nutraceutical contributions attributed to capsiate, that is, because of its attractive beneficial properties for human health, pepper fruits should be consumed when they present the largest concentration of this compound. According to the data resulting from this study, that moment would take place during the first stages of the fruit development, specifically between the 20th and 41st dpa for both varieties. During that period of time, the fruits exhibit green and purple hues, respectively.

3. Materials and Methods

3.1. Reagents

The methanol (99.9%) and acetonitrile (99.9%), of HPLC grade used for the extraction and chromatographic separation, respectively, were purchased from Panreac Química S.L.U. (Castellar del Vallés, Barcelona, Spain). The glacial acetic acid (99%), also of HPLC grade, was obtained from Merck (Darmstadt, Germany). The ultra-pure water was supplied by a Milli-Q water purification system manufactured by EMD Millipore Corporation (Bedford, MA, USA). The capsaicinoids reference standards, i.e., capsaicin (97%) and dihydrocapsaicin (90%) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The capsiate standard was synthesized following the method described by Barbero et al. [64] from the Department of Organic Chemistry at the University of Cadiz.

3.2. Pepper Cultivar and Growing Conditions

Ten plants of each genotype were grown in an automated greenhouse, under controlled conditions, at the Agrifood Research Centre of Aragón (CITA-Zaragoza, Zaragoza, Spain). The experiment was carried out over the spring-summer season (April–September 2016) with an average day/night temperature of 24/14 ºC during the spring and 27/19 ºC during the summer. The plants were distributed randomly within the greenhouse in 17 cm diameter and 18 cm height black plastic pots (one plant per pot). The pots were filled with a substrate mixture formed by peat, sand, and clay-loam soil as well as Humin Substrat (Klasman-Deilmann, Geeste, Germany) (1:1:1:1 v/v/v/v). They were enriched with two grams of a slow-release fertilizer (Oscomote 16N-4P-9K, Scotts, Tarragona, Spain), and watered by means of a drip irrigation system to maintain their optimum humidity levels for growth.

The plants began to bloom by mid-July and the flowers were marked, at the onset of their anthesis, in 7-day intervals. The pepper fruits were harvested during the last week of September at different ripening stages, from immature to senescent. The fruits from the 'Filius' variety were harvested and grouped according to ten different stages of development: 13, 20, 27, 34, 41, 48, 55, 62, 69, and 76 days post-anthesis (dpa), and between 232 and 346 g were collected for each pepper sample. The pepper seeds and stems were discarded prior to analysis. The pericarp and placenta were ground together to a fine pulp by means of an Ultra-Turrax blender until a fully homogenous mass was obtain. All the samples were stored at -20 ºC until analysis.

3.3. Ultrasound-Assisted Extraction of Fresh Pepper

The ultrasound equipment was mainly formed by three components: an ultrasound Sonoplus probe (BANDELIN ELECTRONIC, Heinrichstraβe, Berlín, Germany), a thermostatic bath with a 7 L refrigerated circulator (PolyScience, Niles, IL, USA), and a double-walled vessel (the double mantle allowed us to control the temperature of the medium by means of additional cooling/heating systems).

The methanolic pepper extracts at the different maturation stages were prepared according to the methodology that had been previously developed by our teamfor capsaicinoids [65] and capsinoids [62]. The pepper pulp from each one of the ripening stages was weighed into a plastic tube, which were filled up to the desired volume with the corresponding solvent. Then, the plastic tubes were placed in the ultrasound bath to start the extraction process. Ultrasonic power, extraction temperature and sonication time were controlled via the equipment panel. All the samples were extracted in duplicate, and then the extracts were centrifuged twice for 5 min at 7500 rpm (9.5 cm orbital radius). The supernatant from either of the centrifuged was collected into the same 25 mL volumetric flask, which were then made up to the mark with the same solvent. Finally, the extracts were maintained at -20 ºC until analysis.

The extraction methods used to carry out the analyses [62,65] had been previously developed and optimized in our research group and present a repeatability and intermediate precision lower tan 5%. Furthermore, both extraction methods are quantitative. For these reasons, we considered that it was not necessary to perform a greater number of technical replications since the methods have been proven to be efficient, accurate and adequate, and the samples employed were totally representative.

3.4. Capsaicinoids and Capsiate Content

The resulting extracts were filtered through a 0.22 µm nylon syringe filter (Membrane Solution, Dallas, TX, USA) and 3 µL of the extract was injected into an ultra-high-performance liquid chromatography (UHPLC) equipment in order to identify the capsaicinoids and the capsiate. This equipment was coupled to a Quadrupole-Time-of-Flight Mass Spectrometer (Q-ToF-MS) (Synapt G2, Waters Corp., Milford, MA, USA) fitted with an Electrospray Ionization Source (ESI). The spectra were acquired in the *m/z* = 100–600 and the compounds that were identified with their corresponding *m/z* ratios were as follows: nordihydrocapsaicin (n-DHC) 294, capsaicin (C) 306, dihydrocapsaicin (DHC) 308, homocapsaicin (h-C) 320, homodihydrocapsaicin (h-DHC) 322, and capsiate (CTE) 307. Masslynx software version 4.1 was used to control the equipment and for the acquisition, integration and

analysis of the data. The operating conditions, the parameters of the equipment components, and the variables used were the same as in one of our previously published works [39].

The chromatographic separations and quantifications were performed on an ACQUITY UPLC H-Class system (Waters Corp., Milford, MA, USA). This equipment comprised four main parts: A Quaternary Pump System, an Auto Sampler with temperature control, a Photodiode Array Detector (PDA), and a Waters ACQUITY UPLC BEH rp-C18 column (100 x 2.1 mm, 1.7 µm particle size). Empower 3 software (Waters Corp., Milford, MA, USA) was employed to control the equipment and for data analysis. The identified compounds were quantified based on their calibration curves. All the instrumental parameters, as well as the analytical characteristics, including the calibration curves followed the method previously developed by our team [39]. The chromatographic analyses were performed in duplicate, and the results were expressed in milligrams of compound per gram of fresh pepper (FW).

3.5. Statistical Analysis

The results were estimated as the mean ± standard deviation (SD) values of two replicates of each maturation stage. The resulting means were compared by Tukey's test to determine if the differences were significant for $p < 0.05$. All the data analyses were performed by means of Statgraphic Centurion Version XVII (Statgraphics Technologies, Inc., The Plains, VA, USA).

4. Conclusions

The current work has demonstrated that pepper fruits fromthe varietiesknownas 'Filius Green' and 'Filius Blue' may undergo significant variations with regards to their content in the bioactive compounds that are responsible for pepper pungency, i.e., capsaicinoids and capsinoids. Such differences should be attributed to genotype intrinsic characteristics, since both varieties were grown in a greenhouse under the same controlled conditions.

The ‘Filius’ varieties can be used for decorative and aesthetic purposes in foods and dishes due to their colorful fruits, for nutritional purposes and as a medicinal supplement because of their high content in capsaicinoids and capsinoids.

In this study, it has been seen that pepper fruit color experiences a number of changes over its ripening process, which have been analyzed in relation to the content of capsaicinoids and capsinoids throughout the fruit maturation. The possibility of eating peppers with similar hot sensations and different colors, as well as peppers of the same color with different pungency, which can be attractive to the consumer, has been observed. On the one hand, their optimal harvesting time should be determined by the fruit color in relation to the desired gastronomic application, which would be purple or green in the early stages of fruit development and red in the final stages. On the other hand, and considering their attractive biological activities exhibited by the compounds of interest that have been studied herein, when harvested for medical purposes, peppers should be collected at the moment when its bioactive content is the greatest or the pungent taste is the required. While both varieties analyzed follow the same trend for capsinoids accumulation and reach their maximum value on the 34th dpa, the content curve for capsaicinoids differs between the two varieties, so that the highest concentration levels were reached either on the 41st or the 69th dpa. C was the major capsaicinoid found in both varieties, followed by DHC, n-DHC, h-C, and h-DHC, and their content percentages hardly varied over the fruit maturation process. Based on the standardized values of each capsaicinoid, different patterns have been registered for h-C in comparison to the rest of the capsaicinoids.

Supplementary Materials: Figure S1: Representative chromatograms for the two varieties of pepper studied ((A) ‘Filius Blue’; (B) ‘Filius Green’) obtained by UHPLC-PDA (280 nm) at (1) 13 dpa; (2) 34 dpa; (3) 55 dpa; (4) 76 dpa. The identified compounds were: (1) Nordihydrocapsaicin (n-DHC); (2) Capsaicin (C); (3) Dihydrocapsaicin (DHC); (4) Homo-capsaicin (h-C); (5) Homo-dihydrocapsaicin (h-DHC); (6) Capsiate (CTE).

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6. Discusión conjunta de los resultados

En el presente apartado se realizará una discusión conjunta de los resultados obtenidos en cada uno de los capítulos de esta Tesis Doctoral. En primer lugar, se realizó la optimización y validación de un método UHPLC para la separación de los compuestos bioactivos de interés, por lo que en este apartado se compararán las mejoras y avances obtenidos con respecto a lo reportado en la bibliografía. A continuación, se llevará a cabo la comparación de los parámetros más importantes de los métodos de extracción desarrollados y optimizados. Finalmente, estos métodos se han empleado en la determinación y cuantificación de los metabolitos secundarios implicados en el carácter pungente del pimiento, es decir, capsaicinoides y capsinoides. En este caso, se estudiarán las similitudes y diferencias de los patrones de acumulación de estos compuestos de interés a lo largo del proceso de maduración de distintas variedades de pimientos, con el fin de encontrar el momento óptimo de recolección del fruto.

1. Optimización y validación de un método UHPLC para la separación de los compuestos bioactivos de interés.

En primer lugar, se hará un recorrido por los avances y resultados más relevantes encontrados tras una búsqueda bibliográfica sobre la separación de ambas familias de compuestos, capsaicinoides y capsinoides, empleando técnicas cromatográficas.

En 1979, Iwai y colaboradores [1] intentaron separar por primera vez los cinco capsaicinoides mayoritarios (n-DHC, C, DHC, h-C y h-DHC). Para ello, trabajaron en fase normal y modo isocrático, empleando una mezcla de isopropanol: n-hexano: metanol en relación 10:90:1 como fase móvil y una columna Zorbax SIL. Consiguieron determinar los cinco compuestos de interés en 8 minutos, pero sin buena resolución, debido a que la h-DHC se solapa con la DHC y n-DHC; y la h-C junto con la C.

A continuación, Reilly y colaboradores [2] y Karnka y colaboradores [3], trabajaron en fase reversa en lugar de fase normal. Para ello, emplearon como fase móvil mezclas de metanol y agua destilada y columnas C8. Con esto, consiguieron una gran mejora, separando con buena resolución vanillilamida, nonivamida, C y DHC en 16 minutos; y n-DHC, C y DHC en 11 minutos, respectivamente.

En 2005, Kozukue y colaboradores [4] intentaron separar una mayor cantidad de compuestos, en concreto 8, que serían C, DHC, h-C I, h-C II, h-DHC I, h-DHC II, n-DHC y nonivamida. Además, emplearon una columna C18, que se diferencia con respecto a la anterior en el número de átomos de carbono de la cadena alquilo de la fase estacionaria. Este tipo de columnas, tiende a retener más los analitos, por lo que se eluirán más lentos de la columna y permite una mejor separación. Sin embargo, consiguen separarlos en un tiempo bastante largo, de algo menos de 70 minutos.

Posteriormente, Barbero y colaboradores [5] emplearon una columna monolítica C18 trabajando en fase reversa y consiguieron la separación de los 5 capsaicinoides mayoritarios (n-DHC, C, DHC, h-C, h-DHC) en 7,5 minutos.

Ya en 2014, Sganzerla y colaboradores [6], emplearon por primera vez la UHPLC en lugar de HPLC, lo que supone un gran avance en la separación de esta familia de compuestos. Este equipamiento soporta una mayor presión, lo que permite trabajar a flujos mayores y con un menor tamaño de partícula en la columna, acortando así el tiempo de análisis. Consiguieron la separación de 7 capsaicinoides principales (nn-C, nn-DHC, n-DHC, C, n-C, DHC y h-C) en tan solo 4 minutos, con una columna C18 de 1,9 µm de tamaño de partícula.

En 2016, Barbero y colaboradores [7] consiguieron la separación de los cinco capsaicinoides mayoritarios (n-DHC, C, DHC, h-C y h-DHC) en algo menos de 3 minutos, haciendo uso también de la UHPLC y una columna BEH C18.

En 2017, Schmidt y colaboradores [8] emplearon también UHPLC y una columna BEH C18 para la separación exclusiva de C y DHC, pero lo consiguieron en tan solo 1,7 minutos.

Más recientemente, Stipcovich y colaboradores [9] emplearon la HPLC con una columna C18 fused-core que, como se explicó en la introducción, permiten separaciones muy rápidas debido no solo a su pequeño tamaño, sino a que están formadas por un núcleo sólido recubierto de una capa de sílice porosa, lo que hace que el analito tenga una ruta de difusión más corta, minimizando el ensanchamiento de los

picos cromatográficos. Gracias a esto, consiguieron la separación de nn-DHC, n-DHC, C, DHC, h-C y h-DHC en tan solo 3 minutos de tiempo de análisis.

También se han encontrado autores que, además de compuestos mayoritarios, han separado otros compuestos minoritarios pertenecientes a esta familia. En concreto, Schweiggert y colaboradores [10] consiguieron la separación de 23 compuestos, empleando la HPLC con una columna Phenomenex C18, en 20 minutos. Posteriormente, en 2016, Coutinho y colaboradores [11] separaron 17 capsaicinoides (de los cuales 2 estaban presentes en las distintas variedades de pimiento, C y DHC, y 15 habían sido sintetizados previamente), en tan solo 5 minutos, empleando también una columna C18, pero con un equipamiento cromatográfico de UHPLC, en lugar de HPLC.

Con respecto a los capsinoides, se han encontrado muy pocos autores que hayan llevado a cabo el desarrollo y optimización de un método para su separación. Singh y colaboradores [12] desarrollaron un método en el que separan únicamente los dos compuestos mayoritarios de esta familia, CTO y DHCTO, con una columna monolítica C18 y empleando un tiempo de análisis de 10 minutos. Posteriormente, Coutinho y colaboradores [13], logran la separación de 17 capsinoides (dos de ellos naturales presentes mayoritariamente en pimientos, CTO y DHCTO, y 15 sintéticos), en tan solo 4 minutos. Para ello, emplean la UHPLC en lugar de la HPLC empleada en el caso anterior y una columna BEH C18.

En 2019, Fayos y colaboradores [14] identificaron tentativamente dos capsinoides minoritarios presentes en los pimientos. Desarrollaron un método para la separación de dos capsinoides mayoritarios, CTO y DHCTO, y otros 2 minoritarios, llegando a la conclusión de que podrían atribuirse al ion $[M+Na]^+$ de cualquiera de los isómeros O8C y O9C, respectivamente. También incluía la separación de 10 capsinoides sintetizados previamente. No obstante, el tiempo de análisis fue demasiado largo, consiguiendo una separación efectiva en 55 minutos.

Sin embargo, se ha visto que todos los métodos reportados en la bibliografía optimizan la separación de capsaicinoides y capsinoides de forma independiente, por lo que actualmente no existe ningún método que permita la determinación de ambas familias de compuestos al mismo tiempo.

En esta Tesis Doctoral se ha desarrollado, optimizado y validado un método UHPLC-PDA que permite la separación simultánea, no solo de los cinco principales capsaicinoides (n-DHC, C, DHC, h-C y h-DHC), sino también de los dos capsinoides mayoritarios (CTO y DHCTO). Además, se ha conseguido en un tiempo de análisis de menos de 2 minutos, reduciendo el tiempo total reportado para la separación de cada grupo de compuestos por separado; y con una buena resolución de los picos cromatográficos.

2. Comparación de los métodos de extracción desarrollados y optimizados

En primer lugar, se ha realizado una búsqueda bibliográfica para analizar qué hay reportado sobre la extracción de capsaicinoides y capsinoides y así poder comparar y observar las mejoras conseguidas en esta Tesis Doctoral. En la Tabla 1 se muestra lo que hay reportado sobre extracción de capsaicinoides mediante extracción asistida por ultrasonidos y microondas.

Tabla 1: *Resumen de las condiciones óptimas para la extracción de capsaicinoides por UAE y MAE.*

Técnica extracción	Disolvente	Tiempo	Temperatura	Relación muestra:disolvente (g:mL)	Ref.
UAE	100% etanol	3 horas	45 ºC	1:5	[15]
UAE	100% metanol	10 minutos	50 ºC	1:25	[16]
MAE	100% acetona	7 minutos	-	2:20	[17]
MAE	100% etanol	5 minutos	125 ºC	0,5:25	[18]

Por otro lado, la extracción de capsinoides ha sido realizada mediante extracción Sohhlet convencional, empleando 200 mL de una solución de MeOH durante 24 horas [19] y, más recientemente, se ha optimizado su extracción con técnicas a alta presión utilizando CO_2, mediante fluidos supercríticos (SFE) [20] y líquidos presurizados (PLE) [21]. En la primera de ellas se empleó acetona como disolvente de extracción debido a su menor polaridad con respecto al metanol o etanol, una temperatura de 60 ºC, una presión de 15 MPa y un tiempo de extracción de 90 minutos. Finalmente, la relación de masa entre disolvente y la alimentación se mantuvo constante en 420 ± 20 kg CO_2 / kg de alimentación. En el caso de la extracción con líquidos presurizados se empleó etanol al 75% como disolvente a un caudal de 3,52 mL min^{-1}, resultando en un caudal másico de 3,0 g/min. La presión se mantuvo en 10,0 ± 0,5 MPa, a una temperatura de 65 ºC y durante un tiempo de extracción de 60 minutos. Sin embargo, nunca se ha desarrollado un método para la extracción de esta familia de compuestos mediante ultrasonidos o microondas. Por este motivo, en esta Tesis Doctoral se ha realizado el desarrollo y optimización de un método asistido por ultrasonidos y otro por microondas para la extracción de capsinoides presentes en pimiento. Además, se compararán los resultados obtenidos con respecto a lo reportado en la bibliografía para los capsaicinoides.

Una de las principales diferencias es que, en este caso, en lugar de analizar cada una de las variables que influyen en el proceso de extracción de manera independiente, se ha realizado un diseño de experimentos, que permite no solo reducir el número de experiencias a realizar, sino que, además, permite estudiar las interacciones que pueden ocurrir entre los distintos factores del proceso. Esto conlleva un ahorro de tiempo y trabajo de laboratorio y un menor consumo de disolventes y energía, por lo que sería un proceso más económico y amigable con el medio ambiente.

Se ha utilizado un diseño estadístico de mezcla y un diseño de experimentos de Box-Behnken (BBD) con metodología de superficie de respuesta para evaluar el efecto de las variables independientes, así como sus interacciones, sobre la eficacia de la extracción de capsinoides, con el fin de determinar las condiciones óptimas.

Para llevar a cabo la optimización de los métodos de extracción, se ha empleado la variedad de pimiento Biquinho, suministrada por el banco de germoplasma de Zaragoza, debido a que es la que presentó una mayor cantidad de capsiato en los estudios preliminares.

En la Tabla 2 se muestran las condiciones óptimas para cada uno de los métodos de extracción desarrollados. Basándonos en la experiencia del grupo de investigación para la extracción de compuestos similares a los que aquí son objeto de estudio y en la bibliografía, la potencia fue fijada a un 80% del total (200 W) para la UAE y en 800 W para la MAE.

Tabla 2: *Condiciones óptimas de extracción de los dos métodos desarrollados*.

FACTOR	**UAE**	**MAE**
Disolvente de extracción	42% metanol + 58% acetato de etilo	100% metanol
Tiempo (minutos)	2	5
Temperatura (ºC)	5,5	60
Potencia (W)	160	800
pH	8	8
Relación muestra:disolvente (g:mL)	*0,2:14,5*	0,2:15
Cantidad de capsiato extraída (µg g^{-1})	1323,68 ± 32,30	1403,98 ± 39,94

*En cursiva se muestra la única variable influyente para la extracción de capsiato.

Los métodos desarrollados para la extracción de capsinoides presentan condiciones muy similares. Aunque no hubo una gran significancia de las variables analizadas, ya que la única influyente fue la relación muestra: disolvente en el caso de UAE, en ambos se observan tiempo y temperaturas mínimas y pH y ratio máximos. Esto pone de manifiesto que se trata de métodos bastante robustos y eficaces, que permiten la extracción de la mayor cantidad de capsiato independientemente de la variación de cualquiera de las variables del sistema.

Al observar el disolvente óptimo de extracción, se obtiene un resultado ligeramente diferente en los dos métodos desarrollados, debido a que presentan diferentes propiedades, condiciones y procedimiento de operación. En el caso de la UAE, las ondas sonoras viajan a través del material con el que se encuentra en contacto, provocando el fenómeno de cavitación y calentando toda la muestra de manera uniforme durante el proceso de extracción [22]. Por el contrario, en el caso de la MAE, el calor se genera por la interacción entre la radiación y las moléculas, ya que éstas intentan alinearse con las ondas del campo electromagnético. La radiación excita principalmente el enlace OH, presente en metanol y etanol. Al absorber energía, pasa rápidamente del estado fundamental al excitado, lo que eleva la energía de las moléculas y su temperatura, permitiendo la extracción de los compuestos de interés de la matriz vegetal [23]. Se ha visto que para la extracción de capsaicinoides, el disolvente óptimo es 100% de metanol, etanol o acetona. Debido a la menor polaridad de los capsinoides en comparación con los capsaicinoides [24], cabe esperar que el disolvente o mezcla de disolventes óptima tenga una polaridad inferior para facilitar el proceso de extracción. Efectivamente, después de la optimización y tal y como se observa en la Tabla 2, el disolvente óptimo en el caso de UAE es una combinación de metanol y acetato de etilo, que hace que tenga una polaridad más similar a esta familia de compuestos. En el caso de MAE, no hubo diferencias significativas, influenciado también porque la radiación microondas excita principalmente el enlace OH, presente en metanol y etanol, pero no en acetato de etilo. Por este motivo, se seleccionó 100% de metanol como disolvente óptimo al ser más compatible con los métodos cromatográficos.

Con respecto al tiempo y la temperatura de extracción se obtienen valores muy próximos al mínimo del intervalo utilizado para ambos métodos. Esto es debido a la elevada inestabilidad y degradabilidad de esta familia de compuestos cuando se somete durante mucho tiempo a la irradiación de ultrasonidos y microondas; así como a una temperatura moderadamente elevada [25].

Al compararlo con la extracción de capsaicinoides se puede observar que el tiempo necesario para conseguir una extracción cuantitativa es inferior en todos los casos. Si observamos la temperatura, ésta es también bastante inferior debido, como ya se ha mencionado, a la mayor degradabilidad de estos compuestos frente a los capsaicinoides, lo que puede deberse a la diferencia en el enlace central de ambas estructuras.

Centrándonos en la relación muestra: disolvente, se puede ver que, cuanto mayor es la cantidad de disolvente, mayor será la cantidad de capsiato extraída. Esto concuerda con los principios de transferencia de masa, ya que una mayor relación implica un mayor gradiente de concentración entre el sólido y el líquido, lo que resulta en una mayor fuerza impulsora para la difusión de los compuestos de interés al disolvente, pero hasta un cierto valor, donde el extracto estaría demasiado diluido, lo que impediría realizar su cuantificación [26]. Gracias a los avances en la tecnología y desarrollo de métodos, se puede observar que tanto la cantidad de muestra como de disolvente empleado es bastante inferior a la reportada para los capsaicinoides. Esto supone un gran ahorro en materia prima y disolventes orgánicos empleados, los cuales son tóxicos para el medio ambiente, lo que conlleva una gran ventaja.

Finalmente, se puede observar que, aunque no hay diferencia significativa, de media, se extrae una mayor cantidad de capsiato mediante el método de MAE, concretamente un 5,72% más, aunque su margen de error y variabilidad también es algo mayor.

3. Estudio de los patrones de acumulación de capsaicinoides y capsinoides en distintas variedades de pimientos durante el desarrollo del fruto.

En primer lugar, se comparará la evolución de los capsaicinoides totales en las distintas variedades analizadas (Figura 1) y, posteriormente, la evolución del capsiato (Figura 2).

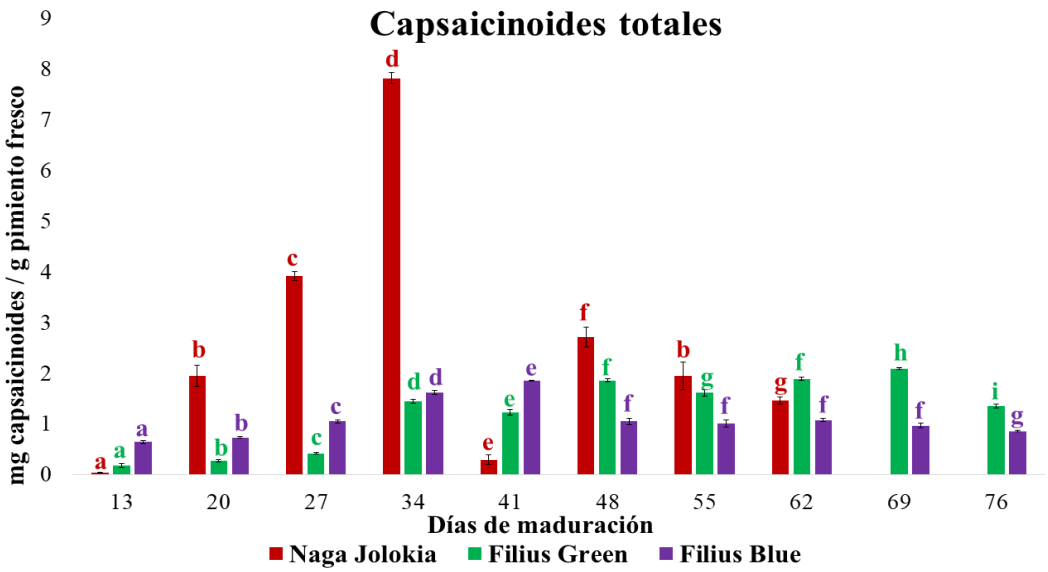

Figura 1: *Evolución del contenido de capsaicinoides totales durante el proceso de maduración (n = 3). El uso de la misma letra en cada variedad indica que no hay diferencias significativas en el contenido de capsaicinoides de acuerdo al test de Tuckey, es decir, que presentan un p-valor inferior al 5%. A su vez el color de las letras corresponde a las distintas variedades, rojo para 'Naga Jolokia', verde para 'Filius Green' y morado para 'Filius Blue'.*

Primeramente, se puede observar que la variedad 'Naga Jolokia' es la que presenta, con mucha diferencia, la máxima concentración de capsaicinoides totales, compuestos responsables del sabor picante de los pimientos. Esto tiene sentido, ya que se trata de una variedad muy picante, recibiendo en 2010 el récord Guinness por ser reconocida como la variedad más picante del mundo [27].

También se puede ver que cada variedad sigue un comportamiento completamente diferente, alcanzando su máxima concentración en días diferentes de maduración. Hay que tener en cuenta que todas las variedades de pimiento fueron suministradas por el Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA). Allí, fueron cultivadas en invernaderos bajo las mismas condiciones controladas de temperatura y riego, por lo que las condiciones ambientales y prácticas agronómicas son idénticas. Por tanto, las diferencias entre ellas se deben exclusivamente al genotipo intrínseco de cada variedad.

Según lo reportado en la bibliografía, la máxima concentración de capsaicinoides se alcanza en torno al día 40 de maduración, seguido de una reducción gradual de su contenido [28,29]. Este es el comportamiento que presenta la variedad 'Filius Blue', alcanzando su valor de concentración máxima el día 41, seguido de una disminución de en torno el 44%.

En el caso de la variedad 'Naga Jolokia', alcanza su máxima concentración varios días antes, concretamente el día 33 de maduración. Además, a continuación, se produce una reducción muchísimo más marcada, superior incluso al 96%, valores no reportados previamente, ni coincidiendo con ninguna otra variedad analizada en este proyecto de tesis.

Finalmente, la variedad 'Filius Green' presenta su máximo contenido de capsaicinoides en las últimas etapas del proceso de maduración, en concreto el día 69. Aunque no siga la misma tendencia que las otras variedades analizadas, este comportamiento si ha sido descrito previamente en la bibliografía para otras variedades como Habanero [30] o Malagueta [31]. Sin embargo, se puede observar un máximo en el día 34 de maduración, seguido de una disminución gradual. El incremento posterior podría ser debido a una pérdida de agua como consecuencia de la sobremaduración del fruto, lo que provoca un aumento de la concentración de sus compuestos.

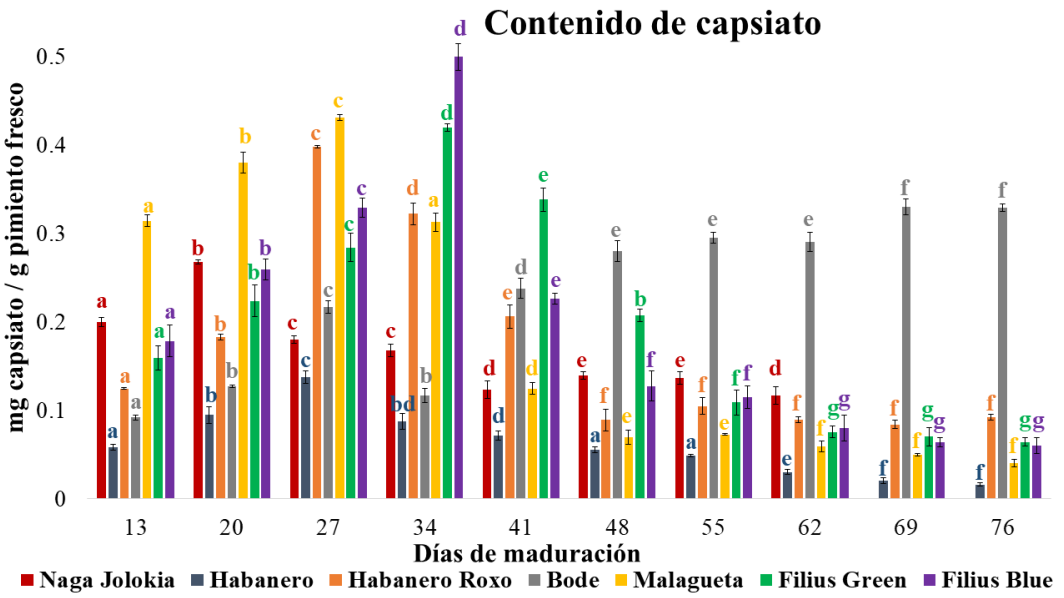

Figura 2: *Evolución del contenido de capsiato durante el proceso de maduración (n = 3). El uso de la misma letra en cada variedad indica que no hay diferencias significativas en el contenido de capsiato de acuerdo al test de Tuckey, es decir, que presentan un p-valor inferior al 5%. A su vez el color de las letras corresponde a las distintas variedades, rojo para 'Naga Jolokia', azul para 'Habanero', naranja para 'Habanero Roxo', gris para 'Bode', amarillo para 'Malagueta', verde para 'Filius Green' y morado para 'Filius Blue'.*

Al igual que ocurría anteriormente, todas las variedades de pimiento fueron suministradas por el Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA). Allí, fueron cultivadas en invernaderos bajo las mismas condiciones controladas de temperatura y riego, por lo que las condiciones ambientales y prácticas agronómicas son idénticas. Por tanto, las diferencias entre ellas se deben exclusivamente al genotipo intrínseco de cada variedad. Pero, a diferencia de los capsaicinoides donde cada variedad seguía un patrón de acumulación completamente diferente, en el caso del capsiato se puede observar una tendencia común en la mayoría de las variedades excepto en 'Bode'. No obstante, aunque la tendencia sea la misma, no coinciden en cuanto a sus días de maduración, lo que se explicará a continuación.

En todas las variedades estudiadas excepto ‘Bode’, la concentración de capsiato se incrementa hasta alcanzar un valor máximo y luego disminuye drásticamente, para permanecer constante en las últimas etapas del desarrollo del fruto. La diferencia entre una y otra es que, aunque la tendencia que siguen sea la misma, el máximo de concentración se alcanza en un día diferente de maduración y la reducción drástica se produce hasta una etapa diferente. Esta reducción en el contenido de capsinoides podría estar asociada con una reducción en la biosíntesis de estos compuestos en el interior del pimiento según las condiciones específicas de cultivo en invernadero [32] o, alternativamente, al efecto de las peroxidasas que se pueden encontrar en los pimientos [33]. En concreto, en la variedad ‘Naga Jolokia’, el máximo contenido de capsiato se alcanza el día 19 de maduración y luego disminuye un 55,6% hasta el día 40. En el caso de las variedades ‘Habanero’, ‘Habanero Roxo’ y ‘Malagueta’, la máxima concentración se alcanza el día 27 de maduración; y posteriormente, se reduce un 86,2% hasta el día 62 y un 77,7% y 83,9% hasta el día 48, respectivamente. Finalmente, las variedades ‘Filius Green’ y ‘Filius Blue’ presentaron su máximo contenido de capsiato el día 34 de maduración, seguido de una reducción del 82% y 84%, respectivamente, también hasta el día 62.

Además, se puede observar que, en todas las variedades, la máxima concentración de capsiato se alcanza en etapas de maduración más tempranas con respecto a la de los capsaicinoides.

Todos estos resultados coinciden con los reportados en la bibliografía. Fayos y colaboradores [34], observaron tendencias similares en los tres genotipos de pimientos analizados, ‘Chitlepín’, ‘Tampiqueño 74’ y ‘Bhut Jolokia’. La acumulación de capsinoides comenzó entre el día 10 y 20 de maduración, aumentando hasta su concentración máxima el día 40 y disminuyendo finalmente su contenido en las últimas etapas de desarrollo. La diferencia en los días en que se producen estos cambios de concentración con respecto a las variedades analizadas en esta Tesis Doctoral podría atribuirse a las condiciones de crecimiento o al propio genotipo de cada variedad.

Estos resultados también concuerdan con los obtenidos por Jarret y colaboradores [35] para la variedad de pimiento *C. annuum* '509-45-1', cuya concentración de capsinoides en los frutos aumentaron rápidamente a los 10 días de maduración y alcanzaron su valor máximo durante la etapa intermedia, seguido de una caída en las últimas etapas del proceso.

Finalmente, se puede observar que la variedad 'Bode' presenta un comportamiento completamente diferente al resto de las variedades analizadas. En este caso, la concentración de capsiato aumenta desde el principio del proceso de maduración hasta el final del mismo. No obstante, el aumento final del contenido de capsiato podría deberse a la pérdida de agua que sufren los pimientos con la sobremaduración.

Se ha visto que, por lo general, el patrón de acumulación del contenido de capsiato en frutos de pimiento durante su maduración sigue tendencias similares. Aunque, dependiendo del genotipo del pimiento, así como de las condiciones de cultivo o factores ambientales, dicho contenido puede variar ligeramente. De ahí, la importancia de realizar estos estudios de maduración donde se analizan un gran número de variedades para estudiar el perfil de composición de estos compuestos de interés.

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7. Conclusions

The results obtained in this Doctoral Thesis on the development and optimization of fast and efficient extraction and analysis methodologies for the determination of capsaicinoids and capsinoids in different varieties of peppers, have allowed reaching the following conclusions:

Regarding the publication:

Vázquez-Espinosa, M.; González-de-Peredo, A.V.; Espada-Bellido, E.; Ferreiro-González, M.; Barbero, G.F.; Palma, M. Simultaneous determination by UHPLC-PDA of major capsaicinoids and capsinoids in peppers. *Food Chem*. **2021**, *356*, 129688. doi.org/10.1016/j.foochem.2021.129688.

- The rp-UHPLC-PDA method developed is a rapid and reproducible analytical tool for the first simultaneous determination of the main capsaicinoids and capsinoids present in peppers.

- The separation of all the compounds of interest was achieved with high resolution in less than 2 minutes. This means a significant improvement and a substantial reduction in the analysis time, solvents and associated costs compared to the methods reported in the literature.

- The optimal gradient selected was the following: 0 min, 0% B; 0.1 min, 45% B; 0.4 min, 45% B; 0.7 min, 50% B; 0.9 min, 55% B; 1.3 min, 75% B; 2.4 min, 100% B; 4.4 min, 100% B; 4.6 min, 0% B. The optimal chromatographic variables were: water and acetonitrile, both acidified at 0.1% with formic acid as mobile phase, 65 ºC column temperature, 0.95 mL min^{-1} flow rate and 3 µL injection volume.

- The developed method has exhibited high precision in terms of repeatability and intermediate precision (with coefficients of variation less than 5%); as well as excellent validation results in terms of sensitivity, linearity, detection and quantification limits and robustness.

- The proposed method was successfully applied for the determination and quantification of major capsaicinoids and capsinoids present in peppers. In addition, it would be suitable for the determination of other products that may contain *Capsicum* extracts.

- It can be concluded that the combination of UHPLC under carefully optimized chromatographic conditions results in a considerable performance improvement when compared to other conventional methods.

Regarding the publication:

Vázquez-Espinosa, M.; González-de-Peredo, A.V.; Ferreiro-González, M.; Barroso, C.G.; Palma, M.; Barbero, G.F.; Espada-Bellido, E. Optimizing and Comparing Ultrasound- and Microwave-Assisted Extraction Methods Applied to the Extraction of Antioxidants Capsinoids in Peppers. *Agronomy* **2019**, *9*, 633. doi.org/10.3390/agronomy9100633.

- The statistical mixture design and the Box-Behnken design of experiments with response surface methodology have been proven to be effective in the optimizing capsinoid extraction in peppers.

- The optimal conditions were: 2 minutes of extraction time, 5.5 ºC temperature, pH 8, and a sample-solvent ratio of 0.2:14.5 g:mL for the UAE; and 5 minutes of extraction time, 60 ºC temperature, pH 8 and a sample-solvent ratio of 0.2:15 g:mL for MAE. Moreover, both optimized methods exhibited a high precision level in terms of repeatability and intermediate precision, with coefficients of variation values below 5%.

- The studied variables did not have a statistically significant influence on the response, but the same optimal conditions have been determined for both methods: minimum temperature and time, and maximum sample-solvent relationship and pH.

- Although no significant differences in the extraction yields were registered, slightly higher amount of the compounds of interest could be visually noticed when MAE was used.

- The developed methods were successfully applied to different varieties of peppers, both sweet and spicy, and a quantitative extraction of capsinoids was achieved.

- It can be concluded that the two proposed extraction methods are adequate, fast, economical, and effective to obtain pepper extracts with high capsinoids yields.

Regarding the publication:

Vázquez-Espinosa, M.; Olguín-Rojas, J.A.; Fayos, O.; González-de-Peredo, A.V.; Espada-Bellido, E.; Ferreiro-González, M.; Barroso, C.G.; Barbero, G.F.; Garcés-Claver, A.; Palma, M. Influence of Fruit Ripening on the Total and Individual Capsaicinoids and Capsiate Content in Naga Jolokia Peppers (*Capsicum chinense* Jacq.). *Agronomy* **2020**, *10*, 252. doi.org/10.3390/agronomy10020252.

- Capsaicin was the major capsaicinoid and its proportion with respect to the rest of the individual capsaicinoids did not vary with the fruit ripening stage.

- The maximum capsaicinoids concentration was reached on 33 rd dpa and then it dropped drastically (by 96.35%). This decrease takes place somewhat advanced with respect to what has been described in the literature and could be attributed to this variety's own genetic factors or to specific growing conditions.

- Based on the results obtained in this research, the optimal time for harvesting peppers based on their spicy characteristics would be at 33 rd dpa, that is, when they have their highest content of capsaicinoids, due to the excellent biological properties of these compounds. Furthermore, harvesting should be carried out before the over-ripening stages of the fruit have been visibly reached.

- With respect to the capsiate content, the maximum is reached in the first weeks of maturation, specifically on 19 th dpa, after which a moderate drop in its concentration was observed until the end of the maturation process.

- It has been proved that the ripening stage is essential to determine the ideal time for harvesting, since drastic changes in the capsaicinoid and capsinoid content have been observed during the ripening period. This is of great interest since one of the most sought-after attributes in peppers, and particularly in the "Naga Jolokia" cultivar, is the content of pungent compounds that confer to it its highly spicy character.

Regarding the publication:

Vázquez-Espinosa, M.; Fayos, O.; González-de-Peredo, A.V.; Espada-Bellido, E.; Ferreiro-González, M.; Palma, M.; Garcés-Claver, A.; Barbero, G.F. Changes in Capsiate Content in Four Chili Pepper Genotypes (*Capsicum* spp.) at Different Ripening Stages. *Agronomy* **2020**, *10*, 1337. doi.org/10.3390/agronomy10091337.

- The current work has demonstrated, for different pepper varieties, that the bioactive content of their fruit with regards to the bioactive compounds responsible for pepper pungency, capsaicinoids and capsinoids, may vary widely depending on their genotype, the fruits developmental stage, and the specific growing conditions.

- Drastic changes in capsiate content have been observed during the ripening period. In addition, it has been seen that the 'Habanero', 'Habanero Roxo' and 'Malagueta' varieties follow the same trend; while the 'Bode' variety follows a different trend. For this reason, it is of great interest to determine how maturity stages may affect the composition of the peppers with regard to such biologically interesting bioactive compounds.

- This study intends to determine the optimal harvesting time based on the moment of the greatest capsiate content, which would be on the 27 th dpa for 'Habanero', 'Habanero Roxo' and 'Malagueta' peppers, and on the 55 th dpa for

'Bode' peppers (the later increase has not been taken into account since they are overripe peppers whose organoleptic properties are not suitable for consumption).

- A comparison of the accumulation patterns for capsaicinoids and capsiate content for the different pepper varieties has been performed. In the case of 'Habanero' and 'Bode', both families of compounds follow the same trend; with the only difference that the maximum content of capsiate is reached a few days earlier, which may be due to the greater degradability and instability of this compound. In contrast, 'Habanero Roxo' and 'Malagueta' showed a completely different trend.

- Since no definitely clear pattern has been established, an in-depth study with a greater number of varieties and fruit-development monitoring points would be necessary. This would constitute a practical foundation for the improvement of the nutritional qualities of pepper products.

Regarding the publication:

Vázquez-Espinosa, M.; Fayos, O.; González-de-Peredo, A.V.; Espada-Bellido, E.; Ferreiro-González, M.; Palma, M.; Garcés-Claver, A.; Barbero, G.F. Content of Capsaicinoids and Capsiate in "Filius" Pepper Varieties as Affected by Ripening. *Plants* **2020**, *9*, 1222. doi.org/10.3390/plants9091222.

- The current work has demonstrated that pepper fruits from the varieties known as 'Filius Green' and 'Filius Blue' may undergo significant variations with regard to their content in the bioactive compounds that are responsible for pepper pungency, capsaicinoids and capsinoids, during the ripening process.

  Such differences should be attributed to genotype intrinsic characteristics, since both varieties were grown in a greenhouse under the same controlled conditions.

In addition, it must be taken into account that the optimum harvest time depends on the interest and objective of the farmer. This is because these varieties can be used both for decorative and aesthetic purposes in foods and dishes due to their colorful fruits, as well as for nutritional purposes and medicinal supplements due to their high content of bioactive compounds.

- Focusing on its gastronomic application, the optimum harvest time should be determined by the fruit color, which would be purple or green in the early stages of its development and red in the final stages.

- When harvested for medicinal purposes, peppers should be harvested at the moment when its bioactive is the highest, the attractive biological activities or the pungent taste is the required. In relation to capsaicinoids, the trend differs between the two varieties, reaching the maximum level on 41st and 69 th dpa for the 'Filius Blue' and 'Filius Green' varieties, respectively. With regard to capsinoids, both varieties follow the same accumulation trend and reach their maximum value on the 34 th dpa.

- C was the major capsaicinoid found in both varieties, followed by DHC, n-DHC, h-C and h-DHC, and their content percentages hardly varied over the fruit maturation process.

- Based on the standardized values of each capsaicinoid, different patterns have been registered for h-C in comparison to the rest of the capsaicinoids analyzed.

- Finally, the possibility of eating peppers with similar hot sensations and different colors, as well as peppers of the same color with different pungent, which can be very attractive to the consumer, has been observed.

8. Anexos

Anexo 1

Vázquez-Espinosa, M.; González-de-Peredo, A.V.; Espada-Bellido, E.; Ferreiro-González, M.; Barbero, G.F.; Palma, M. Simultaneous determination by UHPLC-PDA of major capsaicinoids and capsinoids in peppers. *Food Chem*. **2021**, *356*, 129688. doi.org/10.1016/j.foochem.2021.129688.

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Simultaneous determination by UHPLC-PDA of major capsaicinoids and capsinoids contents in peppers

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Nordihydrocapsaicin, *N*-[(4-hydroxy-3-methoxyphenyl)methyl]-7-methyloctanamide (PubChem CID: 168836)
Capsaicin, *trans*-8-methyl-*N*-vanillyl-6-nonenamide (PubChem CID: 1548943)
Dihydrocapsaicin, *N*-(4-hydroxy-3-methoxybenzyl)-8-methylnonanamide (PubChem CID: 107982)
Homocapsaicin, (*E*)-*N*-[(4-hydroxy-3-methoxyphenyl)methyl]-9-methyldec-7-enamide (PubChem CID: 6442566)
Homodihydrocapsaicin, *N*-[(4-hydroxy-3-methoxyphenyl)methyl]-9-methyldecanamide (PubChem CID: 3084336)
Capsiate, (4-hydroxy-3-methoxyphenyl)methyl (*E*)-8-methylnon-6-enoate (PubChem CID: 98395199)
Dihydrocapsiate, (4-hydroxy-3-methoxyphenyl)methyl 8-methylnonanoate (PubChem CID: 9873754)

ABSTRACT

Capsaicinoids and capsinoids compounds have been a focus of special attention for their health benefits. An effective and rapid Ultra-High-Performance Liquid Chromatography (UHPLC-PDA) method has been developed and validated for the simultaneous separation and quantitative determination of the major capsaicinoids and capsinoids present in peppers. The separation of all the compounds of interest was achieved in less than 2 min by means of an ACQUITY UPLC BEH rp-C18 column (100 mm × 2.1 mm i.d., 1.7 μm particle size). The variables that have been optimized are the mobile phase (water as solvent A and acetonitrile as solvent B, both acidified by adding 0.1% acetic acid), separation gradient, column temperature (35–70 °C), flow rate (0.6–0.95 mL min^{-1}), and injection volume (2.5–3.5 μL). The evaluation of the chromatographic performance revealed excellent resolution, retention factor, and selectivity. The method was satisfactorily validated in terms of linearity, detection and quantification limits, precision, and robustness.

1. Introduction

Peppers are fruits produced by plants in the *Capsicum* genus from the Solanaceae family. Its first use dates from the year 7000 BCE, and at that time its main benefits came from its organoleptic characteristics and physiological effects (da Silva Antonio, Moreira Wiedmann & da Veiga Junior, 2019). They have been for centuries and are still a very popular spice in our food industry for culinary applications. Peppers are an efficient way to eliminate insipidity and to add flavor and color to many foods. In addition to their organoleptic value, peppers also have

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Además, declaran que Dª María de las Mercedes Vázquez Espinosa ha realizado las siguientes aportaciones a los mencionados artículos:

- La totalidad de los estudios experimentales incluidos en el artículo, así como la contribución a la recopilación de la bibliografía y su análisis.
- La colaboración de manera intensiva en la interpretación y análisis de los datos extraídos de la investigación, así como también en la escritura de dicha publicación y en la preparación de figuras y tablas.

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Anexo 2

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Article

Optimizing and Comparing Ultrasound- and Microwave-Assisted Extraction Methods Applied to the Extraction of Antioxidant Capsinoids in Peppers

Mercedes Vázquez-Espinosa, Ana V. González de Peredo, Marta Ferreiro-González, Carmelo G. Barroso, Miguel Palma, Gerardo F. Barbero * and Estrella Espada-Bellido

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Abstract: Capsinoids are very similar antioxidant compounds to capsaicinoids, but less irritating, non-pungent and more palatable, and can thus be used in greater concentrations for food applications. To date, three capsinoids (capsiate, dihydrocapsiate, and nordihydrocapsiate) have been isolated from the pepper fruits. Due to its substantial commercial importance, it would be convenient to determine which pepper varieties have a richer content. Ultrasound- (UAE) and microwave- (MAE) assisted extraction have been implemented and analyzed using multivariate statistical methods. Firstly, different solvents were tested individually. The three best solvents were used in a set mixture design, where 42% methanol and 58% ethyl acetate were determined as the optimum combination for UAE, and 100% methanol for MAE. Subsequently, a Box–Behnken experimental design with four variables for both UAE and MAE (time, temperature, pH and sample mass:solvent volume "ratio") was performed. The sample mass:solvent volume was the most influential variable in UAE; while for MAE no variable was any more influential than the others. Finally, both optimized extraction methods were successfully applied to different varieties of peppers. Besides, to demonstrate the efficiency of both extraction methods, a recovery study was performed. The results prove the potential of both techniques as highly adequate methods for the extraction of capsinoids from peppers.

Keywords: agri-food analysis; antioxidant compounds; capsaicinoids; capsinoids; microwave-assisted extraction (MAE); multivariate analysis; peppers; ultrasound-assisted extraction (UAE)

1. Introduction

Peppers (*Capsicum spp*.) are plants from the *Solanaceae* family, originating in Central and South American tropical and rainy areas [1]. Nowadays, the pepper is a well-known fruit due to its high content in bioactive compounds and its strong antioxidant capacity. It is among the most popular fresh vegetables worldwide due to its combination of aroma, color, flavor, and nutritional value [2]. Peppers are not only valued for their sensory attributes, but also have a significant role in medical and pharmaceutical applications [3]. This fruit is a great source of nutraceutical compounds such as capsaicinoids and capsinoids, bioactive components that support a healthy diet [4].

One of the main features of red peppers is their pungency, which is caused by a specific type of chemical compounds known as capsaicinoids [5]. They have three clearly differentiated sections, the vanillyl group, the carboxamide group and the aliphatic chain [6]. These compounds have

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Además, declaran que Dª María de las Mercedes Vázquez Espinosa ha realizado las siguientes aportaciones a los mencionados artículos:

- La totalidad de los estudios experimentales incluidos en el artículo, así como la contribución a la recopilación de la bibliografía y su análisis.
- La colaboración de manera intensiva en la interpretación y análisis de los datos extraídos de la investigación, así como también en la escritura de dicha publicación y en la preparación de figuras y tablas.

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Anexo 3

Vázquez-Espinosa, M.; Olguín-Rojas, J.A.; Fayos, O.; González-de-Peredo, A.V.; Espada-Bellido, E.; Ferreiro-González, M.; Barroso, C.G.; Barbero, G.F.; Garcés-Claver, A.; Palma, M. Influence of Fruit Ripening on the Total and Individual Capsaicinoids and Capsiate Content in Naga Jolokia Peppers (*Capsicum chinense* Jacq.). *Agronomy* **2020**, *10*, 252. doi.org/10.3390/agronomy10020252.

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Article

Influence of Fruit Ripening on the Total and Individual Capsaicinoids and Capsiate Content in Naga Jolokia Peppers (*Capsicum chinense* Jacq.)

Mercedes Vázquez-Espinosa [1], José Arturo Olguín-Rojas [1,2], Oreto Fayos [3], Ana V. González-de-Peredo [1], Estrella Espada-Bellido [1], Marta Ferreiro-González [1], Carmelo G. Barroso [1], Gerardo F. Barbero [1,*], Ana Garcés-Claver [3] and Miguel Palma [1]

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Abstract: "Naga Jolokia" (*Capsicum chinense* Jacq.) is a hot pepper variety native to India which has received the attention of the global scientific community due to its high capsaicinoid concentration. The present study evaluated the influence of fruit ripening on the total and individual capsaicinoids, as well as capsiate content. The aim was to determine the optimal moment to harvest the peppers depending on their pungent properties. Ultrasound-assisted extraction (UAE) using methanol as the extraction solvent and reverse-phase ultra-high-performance liquid chromatography (UHPLC-photodiode array (PDA)) were employed. Capsaicinoids gradually accumulated in the peppers from the moment they started growing until they reached a maximum concentration (7.99 ± 0.11 mg g^{-1} of fresh weight (FW)) at 33 days postanthesis (dpa). For this reason, based on its content of pungent compounds, as it is one of the main attributes of this variety, the optimal time for collection would be on day 33. From then on, there was a sharp decrease (96.35% of the total concentration) due to the peroxidase enzymes. The evolution of the principal capsaicinoids in "Naga Jolokia" peppers had a different behavior with respect to literature reports. After this investigation, these changes in content can be attributed to each pepper genotype. Capsiate content reached it maximum value at 19 dpa (0.27 ± 0.01 mg g^{-1} of FW). Then, there was a gradual drop due to the activities of different peroxidases. Given the important biological activity of capsaicinoids and capsinoids, the information described here allows for determining the ideal time to harvest "Naga Jolokia" peppers.

Keywords: Naga Jolokia; *Capsicum chinense*; capsaicinoids; capsiate; pepper fruit development; ultrasound-assisted extraction

1. Introduction

For several decades, the association between nutrition and health has been gaining popularity, and therefore, increased importance has been given to diets based on antioxidant-rich vegetables and fruits [1]. Pepper (*Capsicum* spp.) is one of the most valued vegetables because of its rich content

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- La colaboración de manera intensiva en la interpretación y análisis de los datos extraídos de la investigación, así como también en la escritura de dicha publicación y en la preparación de figuras y tablas.

En Puerto Real, a 22 de febrero de 2022

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Anexo 4

Vázquez-Espinosa, M.; Fayos, O.; González-de-Peredo, A.V.; Espada-Bellido, E.; Ferreiro-González, M.; Palma, M.; Garcés-Claver, A.; Barbero, G.F. Changes in Capsiate Content in Four Chili Pepper Genotypes (*Capsicum* spp.) at Different Ripening Stages. *Agronomy* **2020**, *10*, 1337. doi.org/10.3390/agronomy10091337.

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Article

Changes in Capsiate Content in Four Chili Pepper Genotypes (*Capsicum* spp.) at Different Ripening Stages

Mercedes Vázquez-Espinosa [1], Oreto Fayos [2], Ana V. González-de-Peredo [1], Estrella Espada-Bellido [1], Marta Ferreiro-González [1], Miguel Palma [1], Ana Garcés-Claver [2] and Gerardo F. Barbero [1,*]

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Abstract: Interest in the consumption of the fruits of pepper (*Capsicum* spp.) is not only due to its organoleptic characteristics, but also due to its bioactive compounds content, which are reported to provide essential benefits to human health. However, the amount and diversity of these compounds in each fruit specimen depend on its genotype and on a number of environmental factors. This work describes the quantitative ultra-high-performance liquid chromatography coupled to photodiode-array (UHPLC-PDA) analysis of the capsinoids content in four varieties of pepper ('*Habanero*', '*Habanero* Roxo', 'Bode', and '*Malagueta*') grown until different development stages in a greenhouse under controlled conditions. In all the varieties analyzed, capsiate was the only capsinoid found. The accumulation of capsiate, in all the pepper varieties, started from the 10th to the 20th day post-anthesis (dpa), and increased during the first days (between the 20th and the 27th dpa). From that moment a drastic reduction took place until the end of the ripening stage, except for 'Bode' peppers, where the capsiate content increased from the first harvest point on the 20th dpa up to the 76th dpa. The capsiate accumulation patterns over the development of the fruit has been related to the capsaicionoids accumulation patterns in the same samples of the four varieties of pepper. According to our results, the content evolution of both families of compounds will vary depending on each fruit's genotype, as well as on environmental conditions. No clear trends have been established and, therefore, an in-depth analysis under controlled conditions should be carried out.

Keywords: 'Bode' pepper; capsiate content; *Capsicum* spp.; capsinoids; fruit ripening; '*Habanero*' pepper; '*Habanero* Roxo' pepper; '*Malagueta*' pepper; UHPLC

1. Introduction

Pepper belongs to the genus *Capsicum* and the Solanaceae family, original from tropical areas in America. From the 35 described, only five species have been domesticated: *Capsicum chinense* Jacq., *C. frutescens* L., *C. annuum* L., *C. baccatum* L., and *C. pubescens* Ruiz & Pav., with significant economic and social impact worldwide [1]. *Capsicum* fruits vary in size (thick or thin), shape (round, elongated, etc.), color (green, purple, chocolate, yellow, orange, or red, depending on pepper variety and maturation stage.), flavor, and pungency (from the non-pungent varieties to the hottest species) [2]. Due to the

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Modelo de consentimiento de coautores para inclusión de trabajos en la modalidad de tesis por compendio de publicaciones

Los investigadores/as:
Dª. Oreto Fayos, Dª. Ana Velasco González de Peredo, Dª. Estrella Espada Bellido, Dª. Marta Ferreiro González, D. Miguel Palma Lovillo, Dª. Ana Garcés Claver y D. Gerardo Fernández Barbero como coautores/as del artículo:

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Manifiestan su conformidad para la presentación de las citadas publicaciones como parte de la tesis doctoral de Dª María de las Mercedes Vázquez Espinosa, titulada "Aplicación de herramientas metabolómicas para el estudio del carácter pungente en pimientos" y **expresan su renuncia** a presentar la citada publicación como parte de otra tesis doctoral en cualquier otra universidad.

Además, declaran que Dª María de las Mercedes Vázquez Espinosa ha realizado las siguientes aportaciones a los mencionados artículos:

- La totalidad de los estudios experimentales incluidos en el artículo, así como la contribución a la recopilación de la bibliografía y su análisis.
- La colaboración de manera intensiva en la interpretación y análisis de los datos extraídos de la investigación, así como también en la escritura de dicha publicación y en la preparación de figuras y tablas.

En Puerto Real, a 22 de febrero de 2022

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Anexo 5

Vázquez-Espinosa, M.; Fayos, O.; González-de-Peredo, A.V.; Espada-Bellido, E.; Ferreiro-González, M.; Palma, M.; Garcés-Claver, A.; Barbero, G.F. Content of Capsaicinoids and Capsiate in "Filius" Pepper Varieties as Affected by Ripening. *Plants* **2020**, *9*, 1222. doi.org/10.3390/plants9091222.

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Content of Capsaicinoids and Capsiate in "Filius" Pepper Varieties as Affected by Ripening

Mercedes Vázquez-Espinosa [1], Oreto Fayos [2], Ana V. González-de-Peredo [1], Estrella Espada-Bellido [1], Marta Ferreiro-González [1], Miguel Palma [1], Ana Garcés-Claver [2] and Gerardo F. Barbero [1,*]

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Abstract: Peppers are fruits with wide genetic variability and multiple ways of being consumed that hold a relevant position in the human diet. Nowadays, consumers are interested in new gastronomic experiences provided by pepper cultivars that present new shapes, colors, and flavors while preserving their bioactive compounds, such as their capsaicinoids and capsinoids. However, numerous changes take place during their development that may alter their biological properties. Therefore, this work evaluates the capsaicinoid and capsiate contents in two traditional varieties of ornamental peppers ("Filius Blue" and "Filius Green'") during fruit maturation. The aim is to determine the ideal harvesting moment depending on the farmer's objective (e.g., achieving a specific color, shape, or flavor; achieving the maximum concentrations of bioactive compounds). The capsaicinoid contents followed different patterns in the two varieties analyzed. The "Filius Blue" variety exhibited increasing concentrations of capsaicinoids up to the 41st day post-anthesis (dpa), from which point on this trend was reversed. The concentrations in the "Filius Green" variety increased and decreased several times, reaching maximum concentrations on the 69th dpa. Regarding capsiate contents, both varieties varied in the same way, reaching maximum concentrations on the 34th dpa and then decreasing.

Keywords: capsaicinoids; capsiate; *Capsicum* spp.; Filius variety; fruit ripening; UHPLC

1. Introduction

Peppers belong to the Solanaceae family from the genus *Capsicum*, which is native to Central and South America. They are cultivated in tropical and warm climate regions worldwide [1]. Peppers are fruits that hold a significant position in human diets because of their versatility, which allows them to be consumed fresh in salads, fried, boiled, in their dehydrated form as a seasoning, or even as a sauce or jam [2]. Peppers have ample genetic diversity and comprise a substantial number of varieties that differ in plant size (from short, compact plants to plants as tall as three to four feet), color (green, purple, yellow, chocolate, orange, or red, depending on the pepper variety and maturation stage), flavor (from the non-pungent varieties to the hottest species), shape (round, elongated, wide, narrow, as well as special shapes such as bells), and pepper size (from small to full-size fruits) [3]. This diversity is the reason for the remarkable potential for peppers to be used in the agri-food industry, either as coloring or flavoring agents or in forms that utilize their sensory characteristics [4].

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