Universidad de Huelva

Departamento de Química "Profesor José Carlos Vílchez Martín"

Características de calidad de fresas cultivadas en sistema sin suelo : estudio metabolómico preliminar

Memoria para optar al grado de doctor
presentada por:

Ikram Akhatou

Fecha de lectura: 27 de enero de 2016

Bajo la dirección de la doctora:

María Ángeles Fernández Recamales

Huelva, 2016

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UNIVERSIDAD DE HUELVA
FACULTAD DE CIENCIAS EXPERIMENTALES
DEPARTAMENTO DE QUÍMICA Y CIENCIA DE LOS MATERIALES
"PROFESOR JOSÉ CARLOS VÍLCHEZ MARTÍN"

CARACTERÍSTICAS DE CALIDAD DE FRESAS CULTIVADAS EN SISTEMA SIN SUELO. ESTUDIO METABOLÓMICO PRELIMINAR

PROGRAMA DE DOCTORADO:
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RESUMEN

Resumen

La relación directa entre la calidad de la fresa y su contenido en metabolitos determina la necesidad de estudiar su composición química utilizando metodología analítica que permita la identificación y cuantificación exacta y reproducible del mayor número posible de compuestos relacionados con sus cualidades organolépticas, nutricionales y nutracéuticas.

La importancia económica de la fresa en la provincia de Huelva y la competitividad existente dentro del sector hace necesario el desarrollo de nuevos métodos de producción, que basados en la manipulación de los recursos naturales (luz, agua y nutrientes), mejoren la resistencia de la planta, la productividad, la calidad y propiedades nutracéuticas. En este sentido, los cultivos sin suelo parecen una alternativa prometedora para incrementar la sostenibilidad y competitividad del cultivo de la fresa. Asimismo, la introducción de variedades precoces, que permiten la producción fuera de temporada, es otro de los retos que se plantea el sector.

La calidad de fruta, incluyendo las características nutricionales y nutracéuticas, está influenciada por la variedad, las prácticas de cultivo, el clima y la región de producción así como por el tiempo de muestreo, el grado de madurez y los estresantes bióticos y abióticos. Por lo tanto, muchas variables pueden afectar considerablemente los valores dietéticos de la fruta y numerosos estudios se han centrado en explicar las relaciones entre la calidad de la fruta y estos factores.[1,2] Es por ello que en este trabajo de Tesis Doctoral, se han estudiado y analizado varias variedades de fresa cultivadas en sistema de cultivo sin suelo en diferentes condiciones agronómicas, y en sistema convencional con el fin de evaluar el efecto de estas condiciones agronómicas y ambientales sobre la calidad de fresa.

Los resultados de este trabajo se han desglosado en tres capítulos. En el primer de ellos, se proporciona un conocimiento más profundo de la composición orgánica e inorgánica de la fresa para evaluar el efecto de los métodos de producción sobre la calidad de la misma. Para ello, se utilizaron dos grupos de muestras: el grupo I formado por seis variedades de fresas, Camarosa, Candonga, Chiflón, BG 269, Tamar y Diamante cultivadas en un sistema sin suelo usando fibra de coco como sustrato, y el grupo II integrado por cinco variedades, Camarosa, Candonga, Medina, Marina y Ventana cultivadas en sistema convencional. Con el fin de evaluar la calidad de la fresa se determinaron varios parámetros químicos (sólidos solubles, azúcares, ácidos orgánicos y elementos minerales) en distintas variedades de fresa. Además, se

investigaron las diferencias entre las fresas cultivadas en el sistema sin suelo frente al sistema de cultivo convencional aplicando técnicas estadísticas multivariantes. Los análisis se llevaron a cabo mediante cromatografía líquida (HPLC-DAD-RID) para la determinación de los azúcares y los ácidos orgánicos, y espectroscopía de emisión óptica de plasma acoplado inductivamente (ICP-OES) para la determinación del contenido mineral. Se observaron diferencias significativas entre las variedades basadas en la composición mineral y la concentración de glucosa y fructosa en ambos sistemas de cultivo. Sin embargo, no se detectaron diferencias significativas en la acidez. Tamar y Camarosa fueron las variedades más dulces entre todas las variedades cultivadas en el sistema sin suelo y el sistema convencional, respectivamente. Ambas variedades también presentaron la mayor proporción azúcares totales en relación al contenido en ácidos totales. Cuando se compararon ambos sistemas de cultivo también se observaron diferencias significativas en el contenido de minerales, azúcares y parámetros relacionados.

En el segundo capítulo se evalúa la influencia de los factores ambientales, el tiempo de muestreo y el genotipo sobre la calidad nutricional y nutracéutica de la fresa, en base a los cambios en la concentración de metabolitos primarios (azúcares y ácidos orgánicos) y secundarios (compuestos fenólicos) y su bioactividad (actividad antioxidante). El estudio de los metabolitos se realizó en tres variedades de fresa cultivadas en sistema de cultivo sin suelo bajo diferentes condiciones agronómicas (detalladas en la parte experimental del capítulo II). Las tres variedades fueron elegidas en base a su sensibilidad a las condiciones ambientales: Palomar (muy sensible), Festival (sensible) y Camarosa (resistente). A lo largo de la campaña se realizaron tres muestreos: diciembre (producción extra temprana), enero y marzo (producción temprana). Los análisis se llevaron a cabo mediante cromatografía líquida (HPLC-DAD) y espectrofotometría uv-vis. Los resultados obtenidos mostraron que la variedad Festival fue el genotipo con mayor contenido de azúcares y mayor valor para la proporción azúcares totales / ácidos totales, mientras que la variedad Camarosa fue más rica en antocianos. Con respecto al tiempo de muestreo, se observaron que la fresa cosechada en enero y marzo tenía mayor calidad. La fresa cosechada en enero fue más rica en compuestos fenólicos mientras que la fresa cosechada en marzo fue más rica en compuestos relacionados con el sabor (azúcares y ácidos orgánicos).

En el tercer capítulo se ha utilizado una estrategia metabolómica no dirigida (untargeted metabolomic) para caracterizar los metabolitos primarios en diferentes variedades de fresa y estudiar también sus alteraciones en respuesta

a diferentes condiciones de cultivo y al estrés ambiental. Se analizaron tres variedades de fresa con diferente sensibilidad a los factores ambientales (Camarosa, Festival y Palomar) cultivadas en sistemas sin suelo, usando varias condiciones agronómicas: tres niveles de conductividad eléctrica (EC = 1, 2, and 3 dS/m), dos tipo de cobertura (tapada ó destapada) y tres tipos de sustratos (fibra de coco, perlita y lana de roca). Los metabolitos primarios fueron perfilados por cromatografía de gases acoplada con espectrometría de masas (GC-MS). Los resultados obtenidos mostraron la existencia de una alteración significativa en los metabolitos primarios en las tres variedades de fresa estudiadas, incluyendo azúcares (fructosa, glucosa), ácidos orgánicos (ácido málico, ácido cítrico) y aminoácidos (alanina, treonina, ácido aspártico), entre otros.

ABSTRACT

Abstract

The direct relationship between the quality of strawberry and its metabolites content determines the need to study its chemical composition using analytical methodology that allows accurate identification and quantification and reproducible of the largest number of compounds related to organoleptic, nutritional and nutraceutical properties.

The economic importance of strawberries in Huelva and the competitiveness in the sector makes necessary the development of new production methods based on manipulating natural resources (light, water and nutrients) for improving plant resistance, productivity, quality and nutraceuticals properties. In this sense, soilless culture seems a promising alternative to increase sustainability and competitiveness of strawberry cultivation. Also, the introduction of early varieties that enable off-season production is another challenge posed by the sector.

Fruit quality, including nutritional and nutraceutical properties, is influenced by the variety, cultural practices, climate and the region of production as well as the sampling time, degree of maturity and biotic and abiotic stressors. Therefore, many variables can affect significantly the dietetic values of fruit. Numerous studies have explained the relationship between fruit quality and these factors.[1,2] For these reasons in this doctoral thesis it has been studied and analyzed several varieties of strawberries grown in soilless system in different agronomic conditions, and conventional system in order to evaluate the effect of these agricultural and environmental conditions on the quality of strawberry.

The results of this memory have been structured in three chapters. The first one provides a more thorough knowledge of organic and inorganic composition of strawberries for assessing the effect of production methods on fruit quality. For this proposal, two groups of samples were used: The samples representing group I consisted of six varieties, i.e.Camarosa, Candonga, Chiflón, BG 269, Tamar and Diamante cultivated in a soilless system using coconut fiber as substrate. Fruit of five varieties cultivated using conventional practices, i.e. Camarosa, Candonga, Medina, Marina and Ventana, represented group II. In order to evaluate the quality of the strawberry some chemical parameters (soluble solids, sugars, organic acids and mineral elements) were determined in strawberries of several cultivars. In addition, differences between strawberries cultivated in soilless systems versus the conventional crop system were also investigated applying multivariate statistical techniques. The analyses

were carried out using liquid chromatography (HPLC-DAD-RID) for determination of sugars and organic acids, and mineral contents were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Differences among cultivars were observed based on the mineral composition and glucose and fructose concentrations in both cultivation systems. However, no significant differences between cultivars were detected in sourness. Tamar and Camarosa were the sweetest cultivars among those grown in soilless and soil system, respectively. Both varieties also had the highest total sugars/total acids ratio. The comparison between strawberries cultivated in soil and soilless systems also revealed significant differences in the mineral composition, content of sugars and related parameters.

In the second chapter the influence of environmental factors, seasonal and genetic factors on nutritional and nutraceutical quality of strawberry fruits are evaluated. Also it will allow to correlate them with changes in concentration of primary (sugars and organic acids) and secondary (phenolic compounds) metabolites and their bioactivity (antioxidant activity). The study of metabolites was performed in three strawberry varieties grown in soilless system under different agronomic conditions. The study of metabolites was performed in three strawberry varieties grown in soilless system under different agronomic conditions (detailed in experimental part of chapter II). The three varieties were chosen basing on their sensitivity to environmental conditions: Palomar (very sensitive), Festival (sensitive), and Camarosa (resistant). Throughout the campaign, three samplings were performed: December (extra-early production), January (early production), and March (early production). The analyses were carried out using liquid chromatography (HPLC-DAD) and UV-vis spectrophotometry. The results showed that variety Festival was the sweetest genotype and also had the highest total sugars/total acids ratio, whereas Camarosa was richest in anthocyanins. Considering the harvest time, it has been observed that the strawberry harvested in January and March (early production) had higher quality. Fruits harvested in January were richer in phenolic compounds whereas fruits harvested in March were richer in taste-related compounds (sugars and organic acids).

In the third chapter, an untargeted metabolomic strategy was used to characterize the primary metabolome of different strawberry cultivars and to study metabolite alterations in response to different crop conditions and environmental stress. For this purpose, it were analyzed three varieties of strawberries with different sensitivity to environmental factors (Camarosa, Festival and Palomar) grown in soilless systems, and using various agronomic

conditions: three levels of electrical conductivity (EC = 1, 2, and 3 dS/m), two different types of coverage (covered or uncovered) and Three types of substrates (coconut fiber, perlite and rock wool). Primary metabolites were profiled by gas chromatography mass spectrometry (GC-MS). The results showed significant alterations in primary metabolites between the three strawberry cultivars studied in this work, including sugars (fructose, glucose), organic acids (malic acid, citric acid) and amino acids (alanine, threonine, aspartic acid), among others.

INTRODUCCIÓN

Introducción

I. Historia del cultivo de fresa

La primera vez que se mencionó la fresa en Europa, fue en la época romana, cuando se relacionó con propiedades medicinales (Apulius, Virgilio, Ovidio). Hasta la Edad Media, la especie de fresa que se cosechaba era *Fragaria vesca*, la fresa salvaje. En esa época, la especie *F. moschata*, tuvo mucho éxito debido a que producía una fruta más grande. El cultivo de la fresa en Europa sólo apareció claramente en la mitad del siglo XIV. En 1368, el rey Carlos V mandó a su jardinero, Jean Dudoy, plantar 1200 plantas de fresa en los jardines reales del Louvre en París. Otro testimonio de que la fresa era una fruta que se cultivaba, era su disponibilidad en los puestos de Londres en 1430. En los años 1500, las referencias de prácticas del cultivo de esta fruta aumentaron. Existen registros que datan del siglo XIV en los que se describe el manejo del cultivo de las fresas en "Agricultura y Casa rústica", la popularidad del consumo y cultivo de esta fruta se generalizó a finales del siglo XVI.

En España, existen referencias a su cultivo desde 1539. Gabriel Alonso de Herrera dijo que las fresas eran las plantas favoritas en pequeños huertos y jardines, donde crecían exuberantemente. Es muy probable que las especies cultivadas en España fuesen *F.vesca y F.moschata*.

Botánicamente, la fresa fue descrita en detalle por primera vez en 1484 en "Herbarius Latino Monguntiae." Los médicos y los "apotecarios" la utilizaron con propósitos medicinales. Fue en este momento, que los botánicos describen por vez primera las tres especies presentes en Europa: *F. vesca, F. moschata y F. viridis*. La primera descripción de *F. vesca* se remonta al siglo XVI y fue realizada por Jerome Bock en 1532.[3] Posteriormente, Duchesne hace una descripción muy detallada en su libro "Historia de las fresas".[3]

En el siglo XVIII, la presencia simultánea de dos especies de origen americano, *F. virginiana y F. chiloensis* permitió obtener plantas con mejores características combinando las ventajas de ambas especies, el aroma, el tamaño del fruto de *F. chiloensis* y la capacidad de producción de estolones de *F. virginiana*.

En el año 1766, Duchesne fue el primero en plantear la hipótesis de que estas nuevas plantas eran híbridos interespecíficos de *F. chiloensis* polinizadas por *F. virginiana* y las denominó Fragaria x ananassa porque la fruta tenía el olor de la piña. Desde 1800, los agricultores no han parado de mejorar la fresa hasta obtener los cientos de variedades existentes en la actualidad.

La obtención de Fragaria x ananassa Duch generó un interés económico más importante que *F. vesca, F. moschata o F. virginiana*. El descubrimiento de nuevas especies permitieron realizar otros cruzamientos pero la Fragaria x ananassa Duch actualmente es la especie más ampliamente cultivada.

II. Descripción morfológica

El género Fragaria (Fragaria epp., del latín fragans., oloroso) pertenece a la familia Rosáceas. Botánicamente, es una falsa fruta ya que en realidad se trata de un receptáculo carnoso sobre el que están dispuestos los aquenios en los bolsillos más o menos profundos. Tiene un color rojo o amarillo blanquecino dependiendo de la variedad y una forma ovoide oblonga más o menos redondeada. La fresa es de tipo baya con gran importancia económica en todo el mundo. Su adaptabilidad se debe principalmente a que sus requerimientos de frío durante la latencia suelen ser muy corto en climas cálidos, y al pequeño tamaño de la planta, lo que le da una buena protección invernal en condiciones más frías. Además, la corta duración de la maduración de la fresa (30-40 días) permite que se cultive en un clima mucho más frío que otras especies frutales. En climas subtropicales como California o España, las fresas pueden ser cultivadas anualmente y se producen desde principios de primavera hasta finales de otoño.

La fresa es una planta herbácea perenne en la que los diversos órganos están muy cerca uno del otro, incluyendo la parte aérea y la parte subterránea, a diferencia de los árboles. Sin embargo, las yemas no son nunca escamosas y su delimitación en relación con otras partes de la planta no es fácil. Una yema terminal garantiza el desarrollo de nuevas hojas durante el crecimiento, y también asegura la reproducción. En la base de los pecíolos de las hojas, las yemas axilares inhibidas aseguran, si es apropiado, el desarrollo de estolones o corazones secundarios cuyos meristemos pueden convertirse ellos mismos en criadores (Figura 1).

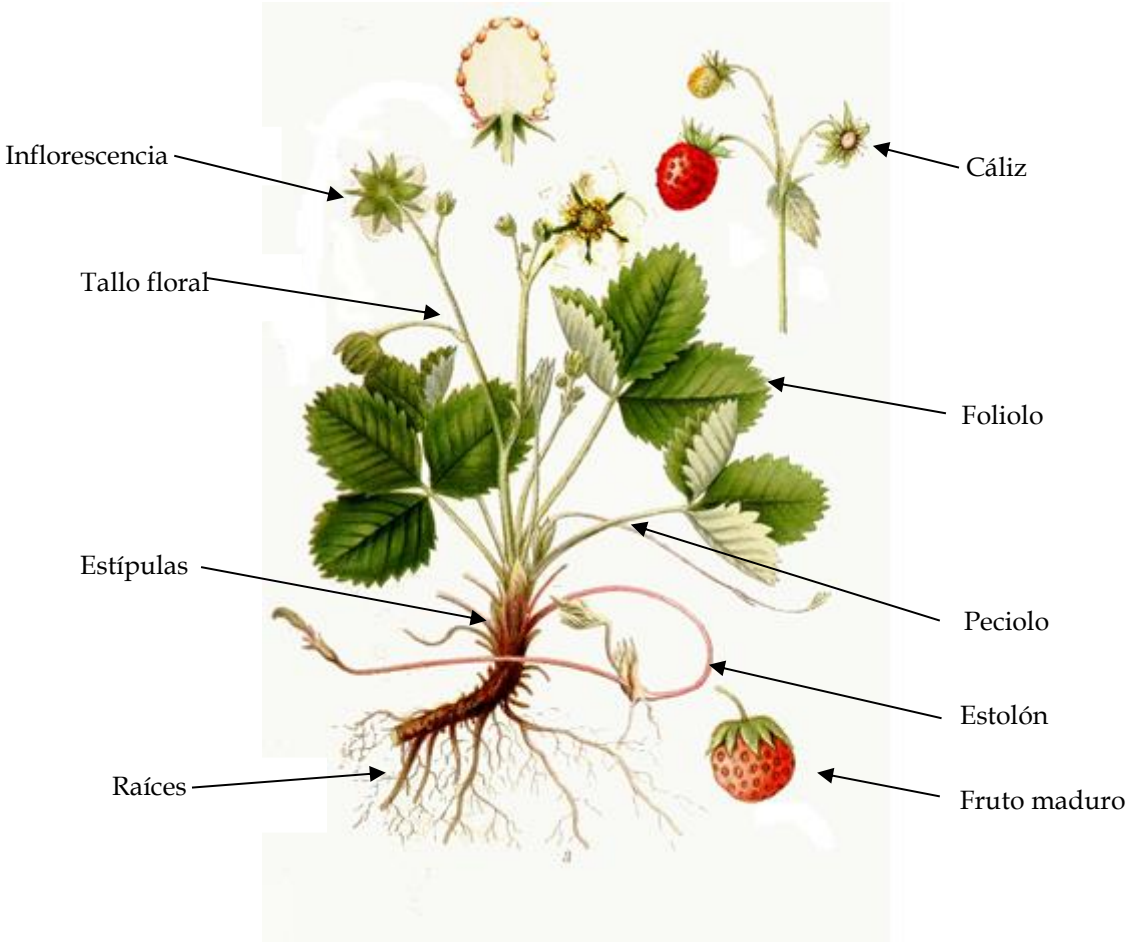


Figura 1- Aspecto general de una planta de fresa

III. La importancia económica de la fresa

La fresa es una fruta demandada y apreciada para consumo fresco y la elaboración de postres o incorporada en otras preparaciones. La producción de fresas en el mundo alcanzó 4.516.810 de toneladas en 2012, más de cinco veces la producción de fresa en 1960.[4] España es el tercer país productor del mundo. España, Estados Unidos y México son los primeros países exportadores. Los mejores compradores de las fresas españolas son Francia, Alemania, Reino Unido e Italia.

El sector de la fresa en España está concentrado, en un 96%, en la provincia de Huelva. Este dinámico sector se ha convertido en las últimas décadas en uno de los principales motores de desarrollo económico para la provincia de Huelva. La producción total de fresa durante la campaña 2014-2015 fue de 288.660 toneladas, ligeramente superior a la del pasado año (un 3% más). La facturación, por su parte, ha ascendido a los 355 millones de euros con un incremento del 20,70 % respecto a lo facturado en la campaña anterior,[5]

además el sector de las berries genera casi 90.000 puestos de trabajo, lo que da idea de la importancia económica del cultivo para la provincia onubense.

IV. Sistemas de cultivo utilizados en Huelva

IV.1. Sistema de cultivo sin suelo

El cultivo sin suelo es un sistema de cultivo en el que las plantas realizan su ciclo de producción completo sin que su sistema de raíces haya estado en contacto con su medio ambiente natural, el suelo.[6] Los cultivos sin suelo se pueden clasificar en hidropónicos (cultivos en agua) y cultivos en sustratos. Esta técnica inicialmente fue utilizada en el laboratorio para estudiar en detalle el funcionamiento de las plantas. Actualmente, el incremento en la utilización de esta técnica en una gran cantidad de especies cultivadas dentro de invernaderos, ha motivado los avances en la productividad y mejora de la calidad de la cosecha.[7] Estos avances son el resultado de una mayor disponibilidad de agua y de elementos minerales, suministrados en forma de una solución nutritiva que asegura las necesidades completas de la planta en sustratos inertes.

El bromuro de metilo (BM) ha sido muy utilizado como fumigante en agricultura en estructuras, mercancías almacenadas y tratamientos de cuarentena. Tiene un gran espectro de actividad y su aplicación es muy fácil, se considera que es un producto económico debido a que elimina todos los organismos vivos con los que entra en contacto. Debido a esto gran cantidad de sistemas de producción agraria se han vuelto dependientes de la fumigación con bromuro de metilo. Pero en 1992 se reconoció oficialmente que el BM es uno de los responsables del deterioro de la capa de ozono, por este motivo, se llegaron a acuerdos para disminuir la aplicación de BM. En 1998, la Unión Europea adoptó una normativa que obligaba a una reducción del 25% en la producción y suministro de BM. Dada la prohibición del uso de BM como desinfectante en 2007, el sistema de cultivo sin suelo se convierte en la única alternativa para algunas producciones. Algunos investigadores mostraron que el cultivo de fresa sin BM era posible utilizando el cultivo sin suelo con turba como sustrato,[8] y otros consideran el cultivo sin suelo y la utilización de fumigantes químicos para la producción de fresa como una alternativa al BM.[9,10,11]

En Europa, casi todos los cultivos en sistema de cultivo sin suelo bajo invernaderos se concentran en cuatro países; Holanda, con las mayores áreas, seguida de Francia, Bélgica y Gran Bretaña. En el sur de Europa, el desarrollo de este sistema se ha extendido, tras la adaptación de esta tecnología a las condiciones climáticas locales. En España el crecimiento de la superficie destinada a los cultivos sin suelo ha sido espectacular, pasando de 2000

hectáreas cultivadas en 1994 a las aproximadamente 5700 hectáreas de cultivos en 2010.[12]

El inicio del cultivo de fresa sin suelo en Huelva es muy reciente, fue en 1996 en las instalaciones de la empresa Famidan SL donde se realizó la primera plantación comercial de fresa sin suelo, aproximadamente unos 200 m^2.[13,14] Desde entonces la evolución del cultivo sin suelo no ha parado. La superficie de cultivo sin suelo de fresa se aproxima a las 300 ha, algo más del 3% de la superficie total de cultivo.[15]

El sistema de cultivo sin suelo ofrece numerosas posibilidades, que pueden ser sistemas suspendidos o apoyados, sistemas cerrados o abiertos (con o sin recirculación de la solución nutritiva) y sistemas con o sin sustratos. En Huelva el sistema más extendido para el cultivo de fresa es el sistema con sustrato, apoyado y sin recirculación de la disolución nutritiva.[16] También existe un sistema de cultivo sin suelo en el que no se utiliza sustrato denominado New Growing System® (NGS), basado en un cultivo hidropónico puro, actualmente utilizado en menor medida. Los sustratos empleados por los productores onubenses han sido de lo más variado, así, podemos destacar, fibra de coco, perlita, turba, lana de roca, compost de corcho, y distintas mezclas, siendo los más utilizados fibra de coco y perlita.

Por lo general, en el sistema sin suelo de fresón en Huelva se utiliza el sistema de plantación otoñal con planta fresca a raíz desnuda, obtenida en viveros de altura, de forma similar a como se viene realizando en el cultivo convencional. Con este tipo de planta se puede empezar a recoger desde finales de diciembre a finales de enero, dependiendo de la fecha de plantación (principios o finales de octubre).[16] La producción de fresas en sistema de cultivo sin suelo permite una producción uniforme de fresas de alta calidad durante todo el año. Con este sistema de producción, la recogida de la fruta es muy fácil, la incidencia de la enfermedad se reduce, y los rendimientos y la calidad de la fruta aumentan ya que representa una alternativa al uso de fumigantes.

Ventajas del cultivo sin suelo

- No hay riesgo de sequía o por el contrario de ahogar las plantas. El sustrato absorbe el agua necesaria y permite drenar el exceso.
- El sustrato es dosificado en la disolución nutritiva adaptado para optimizar el crecimiento de las plantas.
- Los microorganismos y enfermedades no son propensos a crecer porque es un ambiente casi estéril. También los insectos y parásitos no se instalan ni en el suelo ni en las plantas

- El sustrato es más ligero que el suelo, requiere menos trabajo y mantenimiento y es más fácil de manejar que los suelos.

Desventajas del cultivo sin suelo

- La principal desventaja de sistema de cultivo sin suelo es el coste considerable de instalación necesaria.
- Las plantas deben ser controladas continuamente a nivel de la temperatura, la luz, el pH y el contenido de sodio.
- Se requiere un trabajo importante en material plástico : Lonas, túneles, bolsillos de sustrato, etc

IV.2. Sistema del cultivo convencional

El sistema de cultivo convencional de la fresa en Huelva es de ciclo anual con planta fresca procedente de viveros de altura y variedades de día corto. Se plantan en octubre en lomos acolchados con plástico negro opaco (2 líneas por planta), el riego localizado (una cinta o un tubo de bajo coste) se instala durante el acolchado y se han generalizado los macrotúneles de plástico para el forzado de fruta extra temprana.[17]

En el año 2000, el cultivo de la fresa alcanzó la mayor superficie cultivada, con 8.750 hectáreas, mientras en 2015 el cultivo de la fresa viene experimentando un retroceso de la superficie cultivada con 6.340 ha (6.980 ha en 2014) según los datos aportados por las empresas asociadas a Freshuelva (Asociación Onubense de Productores y Exportadores de Fresa).

Tras el levantado del cultivo, la primera fase de la nueva campaña es la preparación del suelo. El objeto es homogeneizar el suelo tras el cultivo anterior y facilitar las labores de desinfección con el fin crear condiciones favorables para el buen desarrollo de los cultivos, es decir, el crecimiento de las raíces y de la planta, y en la mayoría de casos, para la formación del fruto.

La influencia del suelo, su estructura física y contenido químico es una de las bases para el desarrollo del fresón. Éste, prefiere los terrenos ligeros, equilibrados, ricos en materia orgánica, un nivel adecuado de nitrógeno, fósforo y potasio, aireados, bien drenados, pero con cierta capacidad de retención de agua y con un pH adecuado que oscila entre 6 y 6,5. Para ello la preparación del terreno es muy importante. Así, antes de la plantación se realiza la incorporación y enterrado del abonado/enmienda orgánica, con el fin de mejorar la estructura del suelo y para alcanzar el nivel óptimo de materia orgánica del mismo. Se utiliza normalmente estiércol de gallina (25.000 kg ha^{-1}) y cubierta posterior con una lámina de polietileno transparente de 40 µm de espesor, manteniendo dicho plástico en el suelo 4 semanas. Actualmente se

realizan ensayos con el objetivo de optimizar la técnica de biosolarización, utilizando diferentes dosis de gallinaza fresca; utilizando otros materiales orgánicos como biofumigantes: orujillo de olivo, glicerina, vinaza de remolacha o restos vegetales de algunas brasicáceas.[18]

Después de la fase de preparación del terreno, se procede a la desinfección de suelo, al alomado, a la colocación del sistema de riego localizado y al acolchado. Todas estas operaciones se realizan a la vez y en el mismo pase utilizando un apero alomador plurifuncional que se acopla a un tractor. Las dimensiones medias de los lomos suelen oscilar entorno a 50 cm en la meseta superior, 60 cm en la base y 35 cm de altura, siendo la separación entre ejes de lomos de 1,1 m.

En el sistema convencional de cultivo de fresa en Huelva, se utiliza un film de polietileno negro (opaco) para el acolchado. Este último tiene muchos beneficios: ahorro y conservación de agua, control de malezas y de insectos, mayor eficiencia en los métodos de desinfección química del suelo y ahorro de fertilizantes, incremento de la temperatura del lomo, incremento de la mineralización y la disponibilidad de nitratos, mayores rendimientos y frutas de mayor tamaño, limpieza y sanidad.

Antes de la instalación del cultivo, la desinfección de suelo es una operación muy importante realizada de forma simultánea al alomado y acolchado. Durante la década de los 90, el bromuro de metilo fue el fumigante más importante en la fresa de Huelva y casi el 100% del suelo dedicado al cultivo de la fresa en Huelva era fumigado con BM.[19] Dada la prohibición definitiva de BM en 2007, el Proyecto INIA (Instituto Nacional de Investigación y Tecnología Agraria) ensayó entre los años 1997 y 2009 más de 30 nuevas alternativas químicas y no químicas,[20,21] algunas de ellas por primera vez en el mundo y en Europa para el caso de la fresa y viveros de fresa (1,3-dicloropropeno (1,3D) + cloropicrina (PIC) (61%+35%), BM + PIC (67%-33%) ò (50%-50%), solarización + inyección con BM a baja dosis, solarización + inyección con metam sodio a baja dosis e inyección de metam sodio). A día de hoy, el 1,3-dicloropropeno, dazomet y cloropicrina se utilizan como desinfectantes químicos del suelo por los productores freseros. En Huelva la técnica de desinfección mayoritariamente usada en el cultivo de fresa, es la mezcla de 1,3-dicloropropeno-cloropicrina, que se inyecta bajo lomos acolchados.[22] Se ha demostrado que entre las alternativas químicas, la combinación de 1,3-dicloropropeno (1,3-D) más cloropicrina, dazomet y dosis reducidas de metam sodio cuando se aplican con solarización resultan tan eficaces como el BM.[23]

La plantación en el cultivo de la fresa se puede realizar desde el principio de la primavera hasta el final del otoño. La elección de la fecha de plantación va en función de diversos factores como el tipo de planta que se utilice, la técnica de cultivo, el clima de la zona y el tipo y características de las variedades utilizadas.

En cuanto al número de plantas cultivadas, esta encabezada por `Splendor´ con un 26%, le siguen `Sabrina´ con un 24 % y `Florida Fortuna´ con el 16 %. El resto del abanico varietal esta compuesto por: 7% de `Primoris´, 6% de `Sabrosa´ (Candonga), 6% de `San Andreas´, 5% `Benicia´ y el 10% restante de otras entre las que están `Antilla´, `Camarosa´o `Ventana´.[15] La obtención de nuevas variedades de fresa extra temprana permite a los empresarios agrícolas onubenses irrumpir en los mercados internacionales mucho antes de lo que hasta ahora ha venido siendo habitual. La plantación de la variedad temprana de fresa "Splendor" ha hecho que los freseros de la provincia de Huelva hayan podido comenzar la recolecta antes de final de año, concretamente en el mes de diciembre, con una producto "de excelente calidad".

V. Compuestos bioactivos del fruto de fresa

Los compuestos bioactivos son aquellos constituyentes extranutricionales que se encuentran en pequeñas cantidades en los alimentos, que influyen en la actividad celular y en los mecanismos fisiológicos y con efectos beneficiosos para la salud. Mejoran ciertas funciones y reducen el riesgo de algunas enfermedades. Existe una gran variedad de compuestos bioactivos con distintas estructuras químicas y actividades biológicas (Tabla 1).

Tabla 1- Compuestos bioactivos de los alimentos

Compuestos bioactivos de los alimentos				
Compuestos fenólicos	Terpenoides	Minerales	Lípidos	Y otros
Flavonoides Ác. Fenólicos Lignanos Cumarinas Taninos	Carotenoides Saponinas Terpenos simple Tocoferoles	Ca Zn Cu k	Ác. Grasos Insaturados Fitoesteroles Esfingolípidos	Ác. Ascórbico Oligosacáridos Polisacáridos Aminoácidos

V.1. Valor nutricional y valor nutracéutico

Los fresones son frutas muy poco energéticas, cuyo principal componente después del agua lo constituyen los hidratos de carbono. Son muy ricas en vitamina C, con un porcentaje incluso superior al que posee la naranja. También

son una buena fuente de fibra. Entre los minerales, los más elevados son el potasio y el fósforo seguidos del calcio, magnesio.

Tabla 2- Datos de composición de fresa [24]

	Por 100 g de porción comestible
Energía (Kcal)	40
Proteínas (g)	0,7
Lípidos totales (g)	0,5
Hidratos de carbono (g)	7
Fibra (g)	2,2
Agua (g)	89,6
Calcio (mg)	25
Hierro (mg)	0,8
Yodo (µg)	8
Magnesio (mg)	12
Zinc (mg)	0,1
Sodio (mg)	2
Potasio (mg)	190
Fósforo (mg)	26
Vitamina C (mg)	60
Vitamina A (µg)	1
Vitamina E (mg)	0,2

Además de sus características nutricionales, otro determinante crucial de la calidad de la fruta es su valor nutracéutico. Un alimento nutracéutico se define como cualquier alimento que ejerce una acción benéfica en la salud y en la prevención de las enfermedades en el ser humano. En la década pasada, se puso de manifiesto la importancia de los antioxidantes para la salud humana, demostrándose su capacidad potencial para reducir el riesgo de enfermedades cardiacas y de cáncer.[25] La fresa es un fruto rico en componentes fenólicos, pigmentos y vitaminas, estos tres se han identificado como componentes nutraceúticos, por lo que la fresa se ha identificado como un alimento funcional y nutraceútico.

El concepto de alimentos funcionales fue adoptado por primera vez en Japón. En la década de los 80, las autoridades japonesas reconocieron que la mejora de la calidad de vida era necesaria en la creciente población de las personas mayores, con el objetivo de desarrollar alimentos que posibilitan el ahorro en la salud pública.[26,27] Uno de los principales retos a los que se enfrenta la ciencia de los alimentos funcionales es la ausencia de una definición de los mismos. Este vacío ha generado una gran confusión de términos, que ha sido aprovechado por algunas empresas de alimentación en sus labores de

marketing. Según el ILSI (International Life Sciences Institute), los alimentos funcionales se consideran como alimentos destinados para ser consumidos dentro de una dieta equilibrada y que contienen compuestos biológicamente activos (minerales, vitaminas, ácidos grasos específicos, fibras dietéticas, fitoquímicos y antioxidantes) que pueden mejorar la salud y reducir el riesgo de enfermedades, es decir, todos los alimentos que demuestren satisfactoriamente que ejercen un efecto beneficioso sobre una o más funciones selectivas del organismo, más allá de los efectos nutricionales habituales. Estos alimentos pueden ser alimentos naturales, es decir alimentos tradicionales (aceite de oliva, fruta, legumbres, etc.), o alimentos a los que se le han agregado o eliminado un componente por alguna tecnología o biotecnología.

Los flavonoides y los compuestos fenólicos en general son sustancias importantes en los alimentos de origen vegetal, al ser responsables de algunas de sus características organolépticas y tecnológicas, y estar relacionados con efectos beneficiosos para la salud.

V.2. Los compuestos fenólicos en la fresa

Los compuestos fenólicos son una clase importante de metabolitos secundarios en plantas. Son por lo tanto, compuestos no esenciales para la supervivencia de la planta o partes de la misma. Los compuestos fenólicos de plantas se describen como las sustancias derivadas de la vía metabólica del ácido Shikímico. En la actualidad, más de 8.000 moléculas se han aislado e identificado.[28] Según sus características estructurales se dividen en una docena de clases químicas, todas las cuales tienen un punto común: la presencia en su estructura, al menos, de un anillo aromático de 6 carbonos, con un número variable de grupos hidroxilos (OH).[29] Los compuestos fenólicos comprenden desde moléculas simples, como los ácidos fenólicos, hasta sustancias altamente polimerizadas, como los taninos.

Las investigaciones realizadas durante los últimos diez años han demostrado que los compuestos fenólicos no son, de ninguna manera, productos inertes del metabolismo. Son responsables en los tejidos vegetales de cambios cuantitativos y cualitativos significativos y se involucran en los procesos vitales de lo más diverso. Su modo de acción y su significado fisiológico aún no está suficientemente claro.

Los polifenoles se dividen en varias categorías: flavonoides, cumarinas, lignanos, taninos, quinonas, ácidos fenólicos, xantonas y otros floroglucinoles donde los flavonoides son el grupo más común y ampliamente distribuido (Tabla 3).

Tabla 3- Principales clases de compuestos fenólicos en plantas [30]

Nº de carbonos en la estructura base	Esqueleto	Clases
6	C6	Fenoles simples
		Benzoquinonas
7	C6-C1	Ácidos fenólicos
8	C6-C2	Acetofenonas
		Ácidos fenilacéticos
9	C6-C3	Ácidos hidroxicinámicos
		Cumarinas
		Cromonas
10	C6-C4	Naftoquinonas
13	C6-C1-C6	Xantonas
14	C6-C2-C6	Estilbenos
		Antraquinonas
15	C6-C3-C6	Flavonoides
18	(C6-C3)2	Lignanos
		Neolignanos
30	(C6-C3-C6)2	Biflavonoides
n	(C6-C3)n	Ligninas
	(C6-C3-C6)n	Taninos condensados

Según Horst y Lajolo *et al*.[31] los compuestos fenólicos se pueden clasificar en dos grandes grupos: flavonoides y no flavonoides.

V. 2. 1. Los compuestos flavonoides

El término flavonoide derivado del latín "flavus" significa "amarillo". Estas moléculas tienen diversas estructuras químicas y características propias. Los flavonoides son muy abundantes en las frutas, verduras, cereales, bebidas como el té y otras partes de plantas.[32] Se consideran como los pigmentos cuasi universales de los vegetales que pueden estar implicados en el proceso de fotosíntesis,[33] en la regulación de los genes y en el metabolismo de crecimiento.[34] Actualmente, se conocen cerca de 4.000 compuestos flavonoides[35] y todos tienen el mismo esqueleto básico con quince átomos de carbono que están dispuestos en una configuración de C6-C3-C6 (Figura 2) de tipo 2-fenil-benzopirano que es un sinónimo de la estructura 2-fenil-cromano.[36]

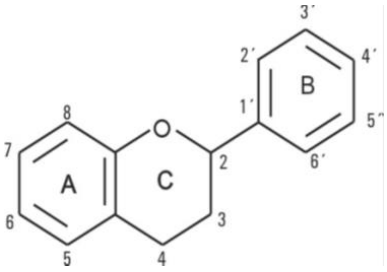
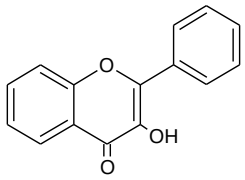


Figura 2- Estructura general de los flavonoides

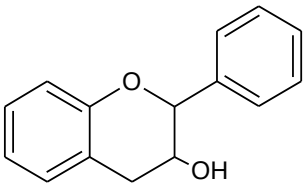
La naturaleza química de los flavonoides depende de su clase estructural, del grado de hidroxilación y metoxilación, del grado de polimerización, de las sustituciones y de las conjugaciones en el anillo C, es decir, de la presencia del doble enlace C2-C3, del grupo 3-O y la función 4-oxo.[32,36]

Los flavonoides se pueden encontrar en forma libre o unidos a azúcares formando heterósidos resultantes de la combinación del grupo reductor de un monosacárido y una sustancia no glúcida, la aglicona o genina, con la eliminación de agua. Los azúcares que aparecen unidos con más frecuencia a las geninas son D-glucosa, D-galactosa, L-ramnosa, L-arabinosa, D-xilosa y ácido D-glucurónico. Pueden aparecer como O-heterósidos o como C-heterósidos. A veces se encuentran en forma de dímeros (biflavonilos) que se han unido mediante un enlace entre los carbonos 5' y 8.

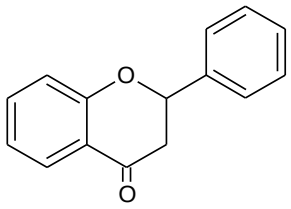
En base a su esqueleto, los flavonoides se pueden dividir en diferentes clases: flavonoles, flavanoles, flavanonas, chalconas, antocianidinas, flavonas, isoflavoindes, dihidroflavoneles, flavandioles, biflavonoides, auronas,[34,35] cuyas estructuras se muestran en la Figura 3.



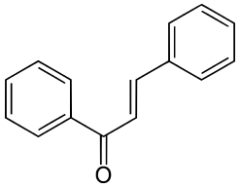
Flavonoles



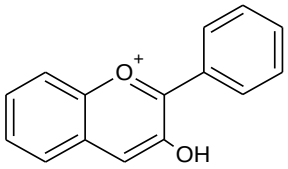
Flavanoles



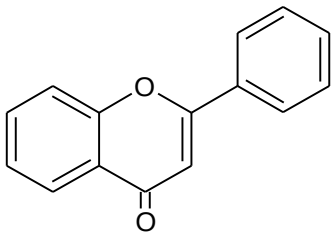
Flavanonas



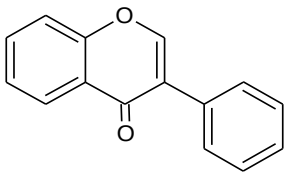
Chalconas



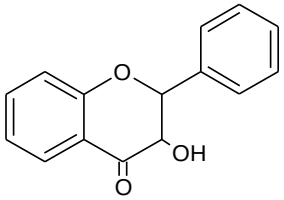
Antocianidina



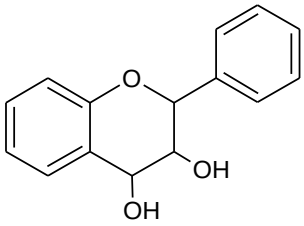
Flavonas



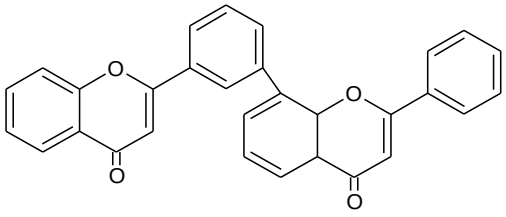
Isoflavoindes



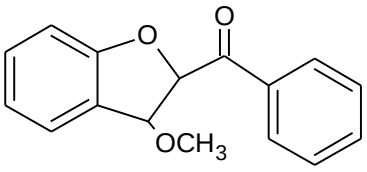
Dihidroflavoneles



Flavandioles



Biflavonoides

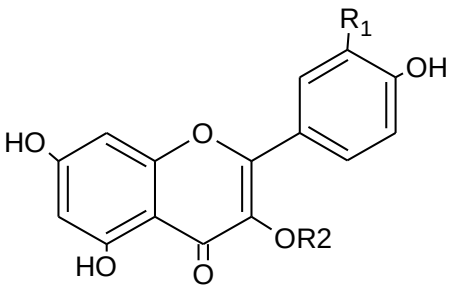


Auronas

Figura 3- Estructuras de los flavonoides

Flavonoles

Los flavonoles son los flavonoides más abundantes en las fresas. Los compuestos más representativos de esta familia son kaempferol y quercetina. Esta última es conocida por poseer un fuerte poder antioxidante debido a su estructura química que favorece la captación de radicales libres. La glicosilación con glucosa o ramnosa es muy común. Los derivados de la quercetina son predominantes en las fresas.



Flavonoles	R1	R2
Kaempferol	H	H
Kaempferol-3-O-glucósido	H	Glc
Kaempferol-3-O-glucurónido	H	Gluc
Quercetina	OH	H
Quercetina-3-O-glucósido	OH	Glc
Quercetina-3-O-glucurónido	OH	Gluc
Isorhamnetina	OCH_3	H
Isorhamnetina-3-O-glucósido	OCH_3	Glc

Figura 4- Estructuras químicas de algunos flavonoles

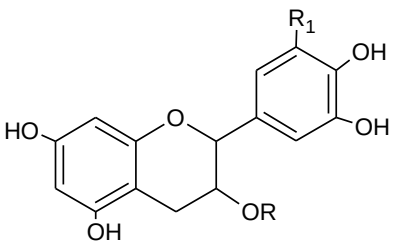
Flavan-3-oles y proantocianidinas

Los taninos condensados, catequinas o proantocianidinas están muy presentes en nuestros alimentos, jugando un papel importante en la calidad organoléptica y nutricional de los mismos. Son responsables en gran medida de la textura, color y sabor astringente y amargo de algunos alimentos como el té, el café y el chocolate. Los taninos condensados son totalmente diferentes a los taninos hidrolizables debido a que no tienen azúcar en su molécula y su estructura es similar a la de los flavonoides. Se trata de polímeros flavánicos obtenidos de flavan-3-oles unidos entre sí por enlaces carbono-carbono. Las proantocianidinas se han aislado e identificado en todos los grupos de plantas.[37] En la naturaleza se pueden encontrar como monómeros o condensados entre sí, formando compuestos con grado de polimerización diverso.

Las unidades de monómero

Las formas naturales monómeros de flavan-3-oles se diferencian por la estereoquímica de los carbonos asimétricos C-2 y C-3 y por el nivel de la hidroxilación del anillo B (Figura 5). Los principales monómeros son (+)-

catequina, (-)-epicatequina y (-)-epicatequina galato. El (-)-epicatequina galato resulta de la esterificación de epicatequina con ácido gálico.



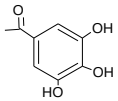
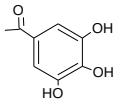
Flavan-3-ols	R1	R
(+)-Catequina	H	H
(-)-Epicatequina	H	H
(-)-Epicatequina galato	H	
(-)-Epigalocatequina galato	OH	

Figura 5- Estructura química de los principales flavan-3-ol monómeros

Los taninos condensados (tipo A y B)

Los taninos condensados son oligómeros o polímeros de flavan-3-oles que tienen la capacidad de liberar las antocianinas, por calentamiento en medio ácido, al romperse el enlace interflavánico.[38] Hay dos tipos de proantocianidinas de acuerdo con la naturaleza de la antocianina liberada, las procianidinas (polímeros de catequina y epicatequina), liberan la cianidina, y las prodelfinidinas (polímeros galocatequina y epigalocatequina) que liberan la delfinidina.

Las proantocianidinas se distinguen por el número de unidades de monómeros y el tipo de conexión entre ellos. Estos dímeros de procianidinas se pueden clasificar en dos categorías: proantocianidina de tipo B y proantocianidina de tipo A. El tipo B se caracteriza por un enlace interflavánico que puede ser C4-C8 o C4-C6, de conformación *trans* con respecto al hidroxilo en posición C-3. Las proantocianidinas de tipo A contienen un enlace éster adicional entre el carbono C-2 y el hidroxilo 5 o 7 del anillo A (Figura 6).

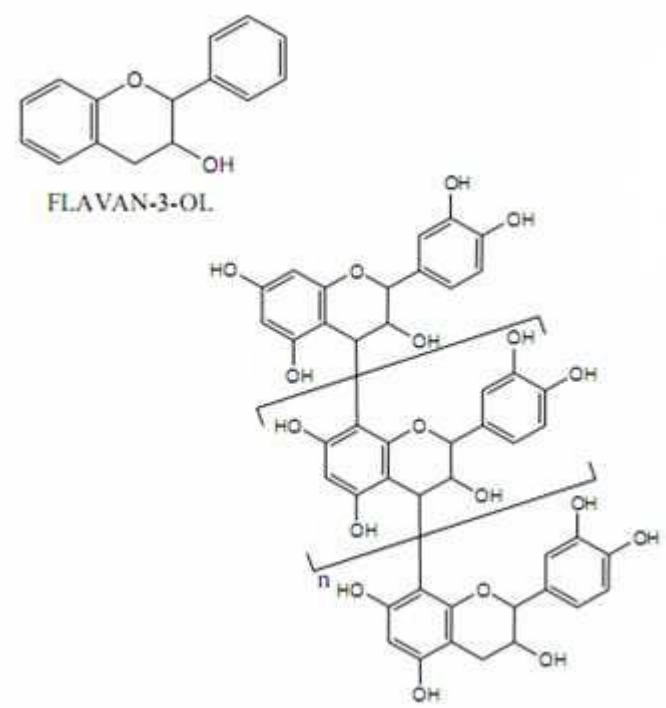


Figura 6- Estructura de taninos condensados

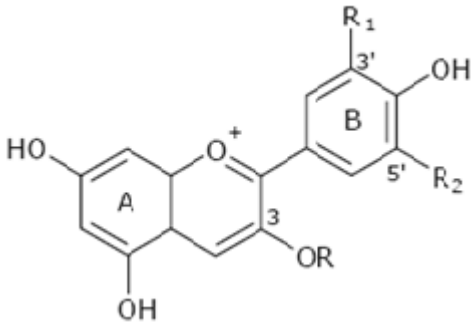
Antocianos

Las antocianinas (del griego anthos = flor + kyáneos = azul) son los colorantes más importantes y generalizados en las plantas Habitualmente se encuentran en disolución dentro de las vacuolas celulares, aunque a veces pueden estar localizadas en vesículas esféricas existentes en el interior de la célula, llamadas antocianos plastos.[39] Estos pigmentos son conocidos por ser responsable de los colores que van del azul al rosa pasando por rojo, púrpura y violeta en los pétalos y las hojas de las plantas superiores. Están presentes en todas las partes de las plantas superiores: tallos, hojas, flores, polen, frutos, semillas. Los antocianos se encuentran localizados en las partes exteriores de la fruta y juegan un papel fundamental en la coloración de las fresas rojas. Su estructura básica está caracterizada por un núcleo "flavona" generalmente glucosidada en la posición C-3. Las antocianinas se diferencian por su grado de hidroxilación y metilación, también por la naturaleza, el número y la posición de los azúcares unidos a la molécula. La aglicona o antocianidina es el grupo cromóforo del pigmento. Existen seis antocianidinas más comunes en las plantas: cianidina, peonidina, delfinidina, malvidina, petunidina y pelargonidina, esta última es mayoritaria en la fresa.

Químicamente las antocianinas son glucósidos de las antocianidinas, es decir, están constituidas por una molécula de antocianidina, que es la aglicona, a la que se le une un azúcar por medio de un enlace β-glucosídico. La estructura

química básica de estas agliconas es el iónflavilio,[40] también llamado 2-fenil-benzopirilio, que consta de dos grupos aromáticos: un benzopirilio (A) y un anillo fenólico (B); el flavilio normalmente funciona como un catión.[40]

El principal pigmento antociánico de la fresa es la pelargonidina-3-glucósido (Pg 3-Glc), identificada por primera vez por Robinson *et al*.[41]Posteriormente, Lukton *et al*.[42] identificaron la cianidina-3-glucósido (Cy-3-Glc), Hong *et al*.[43] identificaron la pelargonidina-3-rutinósido (Pg-3-rut), como pigmentos mayoritarios (Figura 7).



Antocianos	R_1	R_2
Cianidina-3-O-glucósido	OH	H
Pelargonidina-3-O-glucósido	H	H
Peonidina-3-O-glucósido	OCH_3	H
Malvidina-3-O-glucósido	OCH_3	OCH_3
Delfinidina-3-O-glucósido	OH	OH

Figura 7- Las estructuras químicas de antocianos (R= glucósido)

A pesar de las ventajas que las antocianinas ofrecen como posibles sustitutos de los colorantes artificiales, su incorporación a matrices alimenticias o productos farmacéuticos y cosméticos son limitadas debido a su baja estabilidad durante el procesamiento y el almacenamiento.[44,45] Factores como su misma estructura química, pH, temperatura, presencia de oxígeno y ácido ascórbico, concentración y actividad de agua de la matriz, determinan la estabilidad del pigmento.

El pH tiene efecto en la estructura y la estabilidad de las antocianinas además es un factor importante en el cambio del color. El ión oxonio o catión flavilio de color rojo, se encuentra en su forma más estable en el medio ácido a valores de pH inferiores a dos y se convierte, en el medio básico, en base carbinol (incoloro), en chalcona (amarillo) o base quinona (azul) (Figura 8).

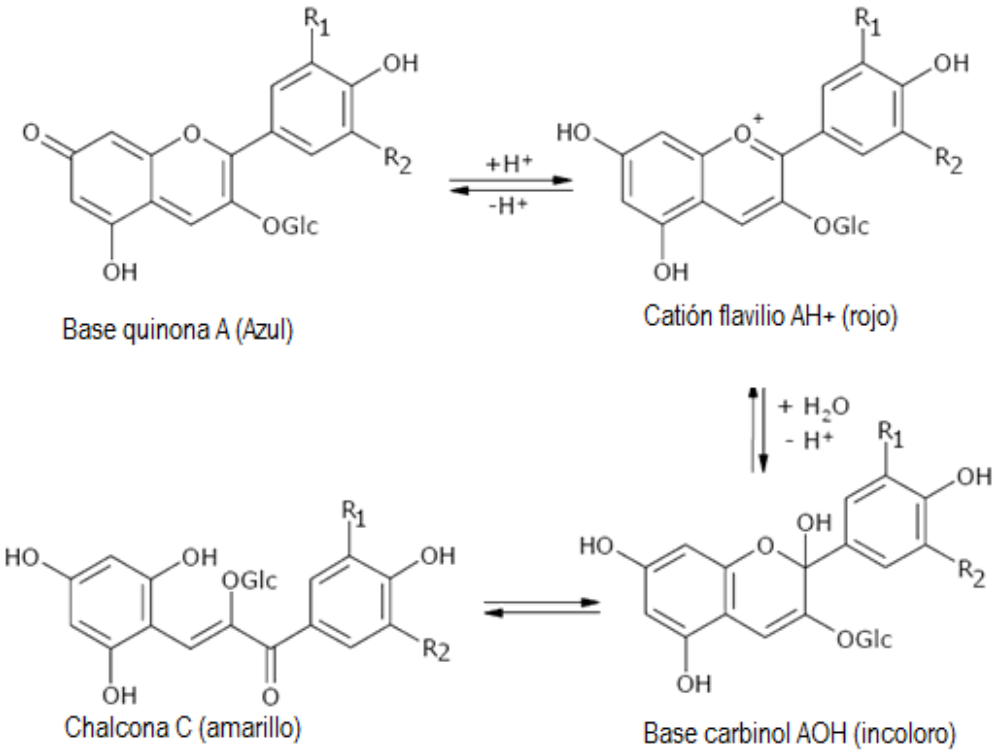


Figura 8- Transformación de la estructura de malvidina-3-O-glucósido en un medio acuoso.[46]

Síntesis de antocianina

La ruta de biosíntesis de las antocianinas es muy compleja y con muchas ramas que conducen a la síntesis de muchos metabolitos diferentes,[47] que todavía no son bien conocidas. La ruta biosintética de antocianinas según los supuestos actuales se resume en la Figura 9. La fenilalanina es el precursor de la mayoría de compuestos fenólicos, incluyendo antocianinas, a través del metabolismo de fenilpropanoides.[46] Un estudio realizado por Aoki et al[48] demostró que la actividad PAL (fenilalanina amonio liasa) en frutos de fresa maduros es mucho mayor que en los frutos verdes. Así, se encontró una correlación muy estrecha entre la actividad de la PAL y la concentración de antocianinas, que se incrementa durante la maduración. La fenilalanina amonio liasa (PAL) es la primera enzima implicada en la biosíntesis. Cataliza la producción de ácido cinámico a partir de fenilalanina. El ácido cinámico se convierte entonces en ácido p-cumárico por cinamato-4-hidroxilasa (C4H). La enzima 4-cumarato CoA ligasa (4CL) conduce a la síntesis del éster p-cumaril-

CoA por la unión de CoA al ácido p-cumárico. La chalcona es el primer flavonoide sintetizado por condensación de p-cumaril-CoA con tres moléculas de malonil por la chalcona sintasa (CHS). La chalcona isomerasa (CHI) transforma luego la chalcona en flavanonas y la flavonona 3-hidroxilasa (F3H) conduce a la formación de dihidroflavonoles por hidroxilación de flavononas. Las flavanonas o dihidroflavonones pueden sufrir las hidroxilaciones en su anillo B bajo la acción de flavonoide 3'-hidroxilasa (F3'H) y flavonoide 3'5'hidroxilasa (F3'5'H). Estas enzimas determinan también el tipo de antocianinas producidas. La actividad de la F3'H conducen a la acumulación de antocianinas de tipo cianidina mientras que la de F3'5'H conducen a las antocianinas de tipo delfinidina en las plantas.[49]

La dihidroflavonol reductasa (DFR) cataliza la primera etapa de conversión de dihidroflavonoles en antocianinas. Esta enzima es la causa de una reducción en la posición 4 del ciclo C (Figura 2) de los dihidroflavonoles que conduce a la formación de leucoantocianidinas. Los pasos que conducen a la formación de antocianinas aún no están claros. Strack et al[46] sugiere un mecanismo en tres etapas hipotéticas implicando una hidroxilasa y dos deshidratasas. La estructura de flavilio lábil producido es posteriormente estabilizado mediante la glicosilación en la posición 3 del anillo C (Figura 2) catalizada por la UDP-glucosa: flavonoide-3-O-glucosiltransferasa (UFGT). La delfinidina-3-glucósido y cianidina-3-glucósido son las primeras antocianinas sintetizadas y se consideran como precursores de otras formas.[49] El orden exacto de los cambios posteriores de antocianinas que involucran los glicosilaciones, acilaciones y metilaciones, es aún desconocido.

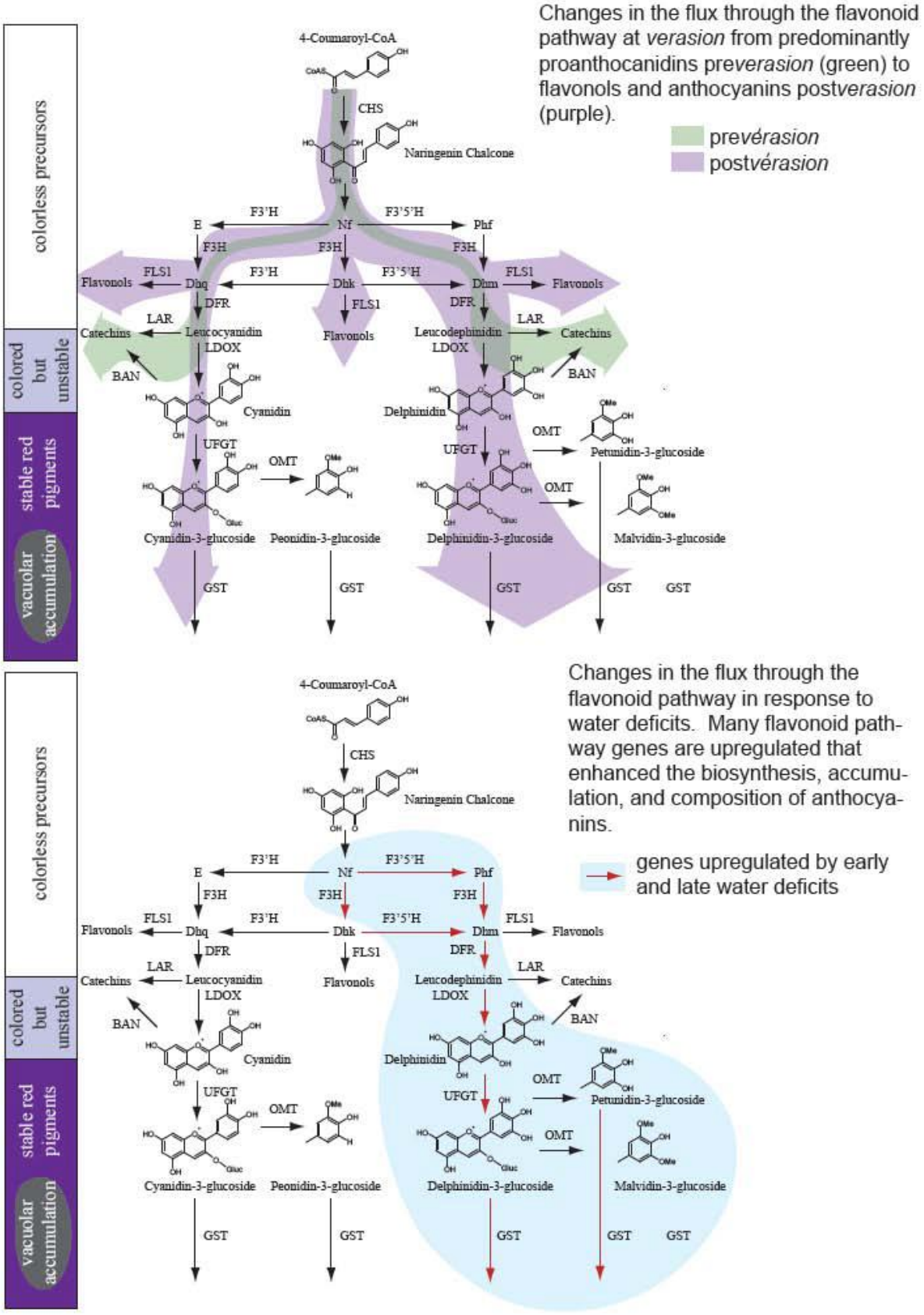


Figura 9- Ruta de biosíntesis de las antocianinas a partir de fenilalanina.[50]

V. 2. 2. Los compuestos no flavonoides

Los ácidos fenólicos

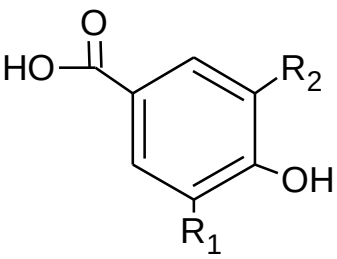
Los ácidos fenólicos incluyen ácidos hidroxibenzoicos con 7 átomos de carbono, y ácidos hidroxicinámicos con 9 átomos de carbono. Están formados básicamente por un anillo aromático, un grupo alifático y un ácido carboxílico en el extremo. Son denominados hidroxi –cinámicos o hidroxi –benzoico por la sustitución del grupo -OH (hidroxilo) en el anillo aromático.

Los ácidos hidroxibenzoicos

Los ácidos hidroxibenzoicos tienen una estructura general C1-C6 y derivan directamente del ácido benzoico. Las variaciones en las estructuras de los diferentes ácidos hidroxibenzoico se encuentran en las hidroxilaciones y las metilaciones del anillo aromático.[51] Encontramos especialmente cuatro ácidos fenólicos: p-hidroxibenzoico, vanílico, siríngico y protocatéquico. Pueden estar presentes en forma soluble combinados con azúcares o ácidos orgánicos, así como en las fracciones de las paredes celulares, por ejemplo la lignina.[46,52] Un ácido hidroxibenzoico también muy común es el ácido salicílico (2-hidroxibenzoato). El ácido gálico es un derivado trihidroxílico involucrado en la formación de galotaninos hidrolizados.[46] Su producto de condensación dimérica (ácido hexahidroxidifenico) y dilactona (ácido elágico) (Figura 10b) son metabolitos comunes en la planta. El ácido elágico esta normalmente presente en los elagitaninos en forma de éster.[53,54]

Ácidos hidroxibenzoico	R_2	R_1
Acido p-hidroxibenzoico	H	H
Protocatéquico	OH	H
Vanílico	OCH_3	H
Siríngico	H	OCH_3
Ácido gálico	OH	OH

a)



b)

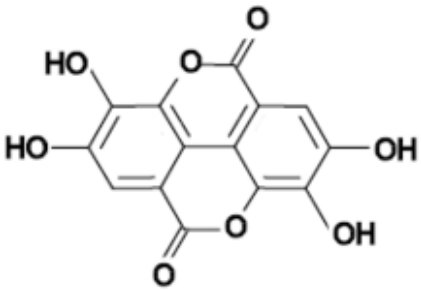
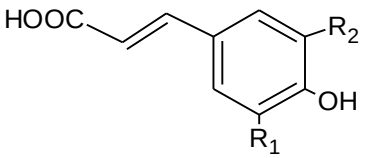


Figura 10- Estructura química básica de los ácidos hidroxibenzoicos (a), Ácido elágico (b).

Los ácidos hidroxicinámicos

Los cuatro ácidos hidroxicinámicos más conocidos y de mayor abundancia en la naturaleza son los ácidos p-cumárico, cafeico, ferúlico y sinápico,[51] por lo general se encuentran en diversas formas conjugadas, siendo las más habituales los esteres de hidroxiácidos tales como el ácido quínico y ácido tartárico, así como sus derivados de azúcar. Otro derivado es el ácido clorogénico, que se presenta en altas concentraciones en las fresas.



Ácidos hidroxicinámicos	R_1	R_2
P-cumárico	H	H
Cafeico	OH	H
Ferúlico	COH_3	H
Sinápico	COH_3	COH_3

Figura 11- Estructuras de ácidos hidroxicinámicos.

Los taninos hidrolizables

Los taninos hidrolizables incluyen los galotaninos y elagitaninos. Estos son oligo o poliéster de un azúcar y de un número variable de unidades de ácido fenólico. El azúcar siempre es la glucosa. El ácido fenólico es el ácido gálico en el caso de los galotaninos o el ácido hexahidroxidifénico (HHDP) y sus derivados en el caso de elagitaninos (Figura 12).

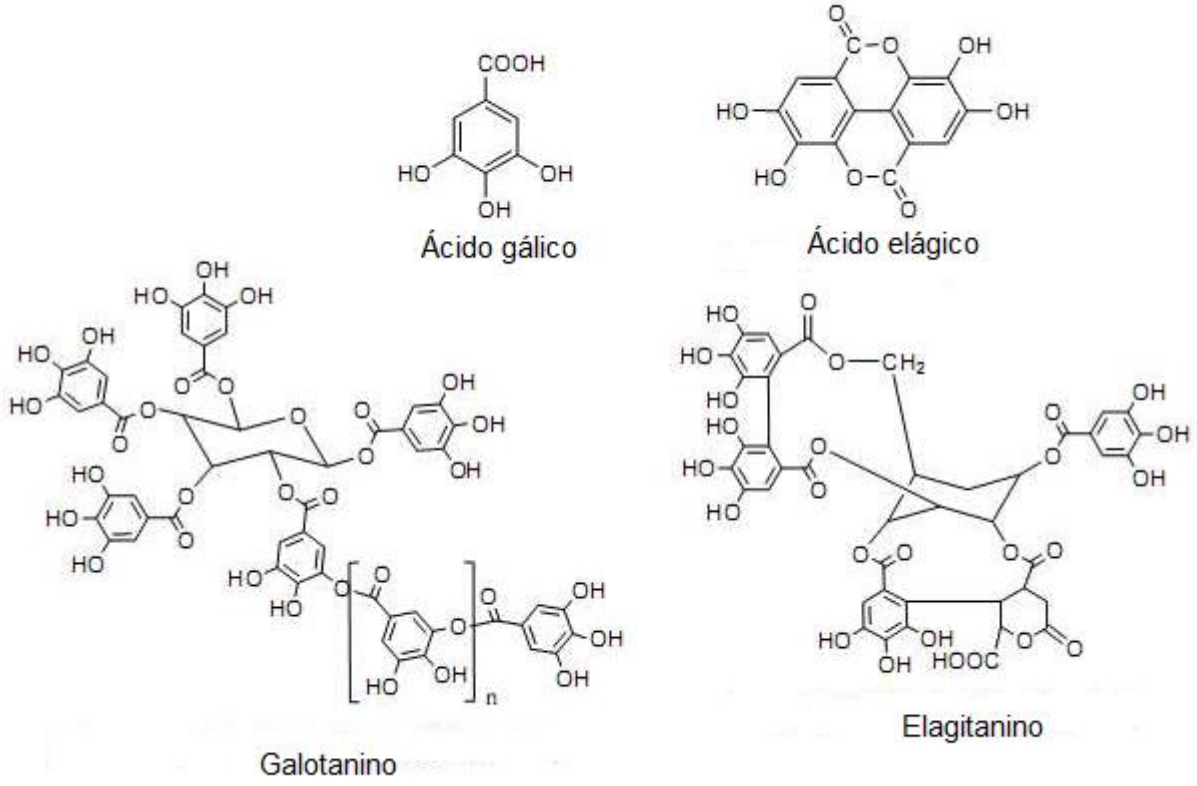


Figura 12- Estructura de los taninos hidrolizables

Los estilbenos

Estos compuestos son muy escasos en nuestra alimentación. El más conocido de ellos es el resveratrol, que ha sido ampliamente estudiado por sus propiedades anticancerígenas demostradas en el estudio de las actividades biológicas de las plantas medicinales.[55]

V. 3. Potencial antioxidante y los radicales libres

Existe hoy en día un creciente interés en el estudio de los radicales libres. Esto no es sólo debido a su condición en los fenómenos agudos tales como traumatismo, isquemia, sino también por su implicación en muchas enfermedades crónicas asociadas con el envejecimiento tales como el cáncer, las enfermedades cardiovasculares, inflamatorias y la degeneración del sistema inmune.[56]

El estrés oxidativo se define como un profundo desequilibrio entre los sistemas oxidantes y antioxidantes del organismo a favor de los primeros, lo que conduce a un daño celular irreversible.[57] El estrés oxidativo es una función del organismo que es normal siempre y cuando no exceda de ciertos límites. De hecho, todos los organismos vivos que consumen oxígeno producen radicales

libres (RL), que son pequeñas especies químicas muy oxidadas por contacto con el oxígeno. Normalmente nuestras células pueden deshacerse fácilmente de ellas.

Un radical libre es una molécula (orgánica o inorgánica), en general extremadamente inestable y, por tanto, con gran poder reactivo. Se distingue por la presencia de un electrón desapareado en la capa electrónica más externa. Esta propiedad hace que estas sustancias sean muy reactivas debido a la tendencia de este electrón a volverse a emparejar, lo que desestabiliza otras moléculas. Las moléculas transformadas a su vez se vuelven radicales libres y por lo tanto se forma una reacción en cadena que en definitiva se convierte en una reacción oxidativa.

Los RL nocivos se producen en el cuerpo durante el metabolismo normal, y su producción aumenta cuando aumenta el consumo del oxígeno. Varios son los mecanismos y sistemas responsables de la producción de radicales libres que se han identificado hasta ahora, entre ellos se pueden mencionar: las fugas de electrones de la cadena respiratoria de las mitocondrias, los procesos inflamatorios producidos por las células fagocíticas activadas del sistema de xantina deshidrogenasa / oxidasa durante la isquemia-reperfusión,[58,59] la exposición a las agresiones ambientales, tales como agentes infecciosos, contaminación, rayos UV, humo del cigarrillo y radiación.[60]

La reducción de diversos radicales por los polifenoles se ha estudiado en profundidad con el fin de determinar los compuestos responsables de la actividad antioxidante. Gracias a su bajo potencial redox, los polifenoles, sobre todo los flavonoides (Fl-OH) son termodinámicamente capaces de reducir rápidamente los radicales. Las propiedades antioxidantes de los polifenoles varían en función de su estructura química. La posición y el grado de hidroxilación juegan un papel significativo en la actividad antioxidante de los polifenoles. Así, los polifenoles con un grupo catecol (un anillo aromático con dos grupos hidroxilo adyacentes) tienen un potencial antioxidante elevado. Por lo que todos los flavonoides que se encuentran hidroxilados en posición 3 y 4 poseen una actividad antioxidante significativa.

El alto contenido en polifenoles y vitamina C de la fresa le confiere una alta capacidad antioxidante, es decir, una alta capacidad de neutralizar el efecto del estrés oxidativo de los radicales libres, lo cual es muy beneficioso para la salud humana.

V. 4. Azúcares y ácidos orgánicos

El contenido de azúcar es muy importante desde el punto de vista nutricional y en combinación con los ácidos orgánicos, contribuyen al buen sabor de la fresa.[61] Los azúcares y ácidos orgánicos son los principales constituyentes solubles de las fresas y tienen un efecto importante en el sabor, la madurez de la fruta, y en el grado de aceptabilidad por el consumidor. La valoración organoléptica está influenciada por las cantidades totales y relativas de azúcares y ácidos en las fresas maduras. Wozniak et al[62] demostraron una correlación entre la calidad sensorial global de algunos cultivares de California y la relación azúcares / ácido en la maduración de la fruta.

La sacarosa producida por fotosíntesis en las hojas, es el carbohidrato principal que será transportado en la planta. Sin embargo, la glucosa y fructosa son los dos azúcares principales que se acumulan en las fresas y el contenido total de azúcar puede cambiar durante el período del crecimiento. Zhang *et al*.[63] demostraron que el contenido de azucares se incrementa dramáticamente durante el crecimiento y desarrollo de la fresa. Por ejemplo, el contenido de azúcar soluble puede llegar a ser de aproximadamente de 500 mg g^{-1} DW.[64] La acumulación de azucares está directamente relacionada con la intensidad de la luz, el agua y el estrés biótico.

El ácido cítrico y el ácido málico son ácidos orgánicos mayoritarios en las fresas, mientras el ácido succínico, oxálico y tartárico están presentes en una cantidad mucho menor. La concentración de los ácidos varía durante el desarrollo y maduración de las fresas y alcanza una concentración máxima en la maduración.[63]

V. 5. Aminoácidos

Los aminoácidos son sustancias orgánicas nitrogenadas de peso molecular muy elevado. Son de vital importancia en el metabolismo de los seres vivos, ya que constituyen las unidades estructurales de las proteínas. Por otro lado, los aminoácidos se encuentran en las células y fluidos intercelulares de las plantas y juegan un papel esencial en muchos procesos fisiológicos: fase de crecimiento, el desarrollo y la formación de la fruta. El número de aminoácidos existentes en la naturaleza es de unos 20, y los que más comúnmente se encuentran en las fresas son ácido aspártico, serina, alanina, prolina, valina, treonina, tirosina y lisina.[63] Los aminoácidos en la fresa son donadores de nitrógeno (N) orgánico para la formación de diversas moléculas biológicas; como es el caso de los compuestos volátiles. La diversidad en el tipo de ésteres encontrados en el perfil aromático de la fresa se debe a la composición de aminoácidos lo que explica la diferencia tan marcada en el sabor y aroma, entre diferentes variedades.[65]

V. 6. Minerales

Los minerales son una gran familia de elementos inorgánicos que se encuentran en nuestros alimentos. Estos elementos de la tierra son esenciales para el funcionamiento del organismo participando en numerosas reacciones químicas en el cuerpo. Se clasifican en dos categorías: mayoritarios y oligoelementos. Los macro-elementos son el calcio (Ca), magnesio (Mg), fósforo (P), potasio (K) y sodio (Na). Son denominados "macro" porque representan una parte significativa del contenido del organismo, un hombre adulto sano contendrá, por ejemplo, en la estructura de su esqueleto más de 1 kg de calcio. Los oligoelementos, llamados "traza", debido a que están presentes en bajas concentraciones en los organismos, son esenciales en los procesos biológicos, pero a niveles altos pueden ser tóxicos (zinc (Zn), cobre (Cu), selenio (Se), cromo (Cr), molibdeno (Mo), níquel (Ni), arsénico(As)). Otros oligoelementos como el plomo (Pb), cadmio (Cd) y mercurio (Hg), no tienen función biológica conocida, son considerados como contaminantes tóxicos para los seres vivos, aún en concentraciones muy bajas. Algunos de estos minerales tienen efectos beneficios sobre la salud,[66] tal como por ejemplo, el selenio y el zinc que poseen propiedades antioxidantes que podrían ayudar a reducir el riesgo de desarrollar enfermedades crónicas (cáncer, enfermedades cardiovasculares), o el magnesio que previne ciertas complicaciones del infarto de miocardio.

Las fresas son una buena opción entre los alimentos ya que contienen cantidades abundantes de varios componentes esenciales, como el potasio, calcio y magnesio. La necesidad del organismo humano por algunos minerales varía dependiendo del sexo y de la edad (Tabla 4).

Tabla 4- El contenido de los minerales en la fresa en comparación con la ingesta recomendada.[24]

Componente	Contenido en 100 g de fresas	Recomendaciones día-hombres (20-39 años)	Recomendaciones día-mujeres (20-39 años)
Energía (Kcal)	40	3000	2300
Calcio (mg)	25	1000	1000
Hierro (mg)	0.8	10	18
Magnesio (mg)	12	350	330
Zinc (mg)	0.1	15	15
Potasio (mg)	190	350	350
Sodio (mg)	2	<2000	<2000

El cultivo, la variedad, la fertilización, la región y las condiciones climáticas, así como el tiempo de muestreo y el grado de madurez afectan considerablemente a la concentración de los minerales en las fresas.[67]

VI. Factores que influyen sobre la calidad de fresa

En la etapa de precosecha se determina la calidad del producto en el momento de la recolección, dando lugar al comportamiento en la vida útil poscosecha. Los factores precosecha que influyen sobre la calidad pueden variar en su intensidad y hacerlo en forma individual o por interacción con otros factores. Estos factores pueden ser de diferente origen: genéticos como las características de cultivo y manipulación genética; biológicos como agentes patológicos; ambientales y de cultivo como las condiciones climáticas y meteorológicas (intensidad de luz, lluvia…) la disponibilidad de agua y de nutrientes, características del suelo, prácticas de cultivo utilizadas y condición de fertilización; y fisiológicos como la influencia del estado de madurez. Por lo tanto estos factores afectan considerablemente a los valores dietéticos de la fruta y numerosos estudios se han centrado en explicar las relaciones entre la calidad de la fresa y estos factores. Tulipani *et al*.[68] afirman que el contenido en fitoquímicos, la capacidad antioxidante y la composición nutricional de la fresa están fuertemente influenciados por factores pre y postcosecha. Algunos autores han observado que el contenido de compuestos fenólicos varía significativamente cuando comparan diferentes variedades de fresa (Skupién and.[69-72] En comparación con las prácticas culturales, Jin *et al*.[73] estudiaron dos variedades de fresa, en sistema de cultivo ecológico y convencional, encontraron diferencias significativas en el contenido en flavonoides a favor del cultivo ecológico. Así también en comparación con las fresas cultivadas mediante prácticas tradicionales, las cultivadas en sistemas sin suelo presentan generalmente un contenido más bajo de azúcares solubles y un contenido más alto de ácido málico, aunque la acidez total es prácticamente igual para ambos sistemas.[74] La acumulación de compuestos fenólicos varía durante el proceso de maduración del fruto de fresa, las antocianinas y los flavonoloes alcanzan la máxima concentración en el estadio maduro.[63,71,75]

VII. La metabolómica aplicada a las plantas

VII. 1. Historia y definiciones

Desde principios de la década de los noventa, las innovaciones tecnológicas han supuesto una revolución en la manera en la que los sistemas biológicos son visualizados y estudiados.[76] Los avances en genética han llevado a la secuenciación completa de genomas de organismos (genómica), las técnicas de biología molecular han permitido el análisis exhaustivo de la expresión

génica (transcriptoma), y la espectrometría de masas ha contribuido al estudio completo de las proteínas presentes en el organismo y la expresión de los mismos (proteómica). Para completar el proceso de integrar estos diferentes niveles de información biológica y las interacciones, se ha llevado acabo un enfoque conocido bajo el término "metabolómica". El término 'metaboloma' se utilizó por primera vez en 1998 para describir el conjunto de metabolitos de bajo peso molecular presente en el organismo.[77] Más tarde, en 2001, Oliver Fiehn introdujo el término 'metabolómica' definido como el análisis integral y cuantitativo de todos los metabolitos de un sistema biológico.[78]

La Metabolómica es el estudio de las pequeñas moléculas orgánicas, denominadas metabolitos, presentes en un sistema biológico (células, fluidos biológicos, tejidos, órganos) como productos del metabolismo. Los metabolitos son moléculas de bajo y medio peso molecular (<1500 Dalton) que intervienen en los procesos celulares y revelan cómo está funcionando el metabolismo en un órgano determinado o en un ser vivo.

En los estudios metabolómicos se utilizan principalmente dos enfoques: uno llamado el enfoque dirigido (targeted analysis) que se interesa en una vía metabólica particular, y el otro se llama "El enfoque no dirigido" (untargeted analysis) definiendo una huella característica de todos los metabolitos.

El enfoque dirigido (targeted analysis) en el cual se obtiene información cualitativa y/o cuantitativa de un conjunto limitado de metabolitos previamente conocidos, a menudo correspondiente a una familia de compuestos químicos. La identificación de los metabolitos inicialmente se establece mediante el empleo de las bases de datos disponibles y usando compuestos puros. A menudo se basan en realizar una extracción selectiva de un grupo determinado en función de su significancia biológica con el problema investigado. Los análisis realizados en este contexto son cuantitativos, precisos y sensibles.

El enfoque no dirigido (untargeted analysis) se lleva a cabo en tres etapas correspondiente al tratamiento de la muestra, tratamiento de datos e identificación de metabolitos alterados tal como se observa en el esquema de la Figura 13.

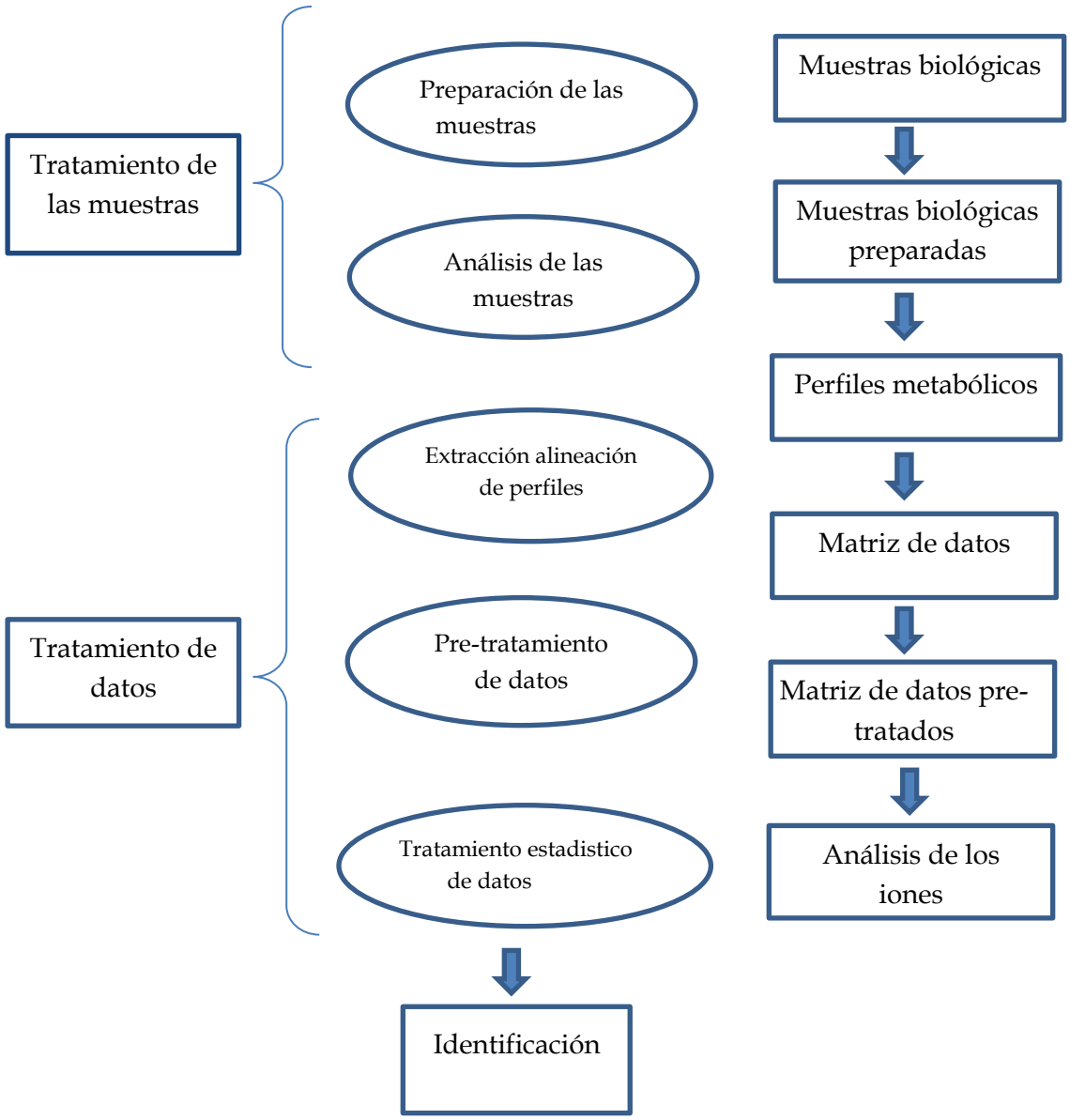


Figura 13- Mapeo de los pasos del enfoque no dirigido

El enfoque no dirigido es el análisis de la modificación del conjunto de pequeñas moléculas contenidas en la muestra biológica inducidas por un estímulo, tal como una intervención nutricional, medicamentos o una perturbación genética. Este enfoque recientemente ha demostrado ser particularmente prometedor para la comprensión de metabolismo de los nutrientes, los efectos biológicos y el papel que pueden desempeñar en la prevención nutricional de enfermedades. El objetivo de este enfoque es acceder a todos los metabolitos en muestras biológicas. Las técnicas empleadas generan

una gran cantidad de datos en tiempos de análisis relativamente cortos. Las herramientas estadísticas y la bioinformática permiten comparar los perfiles metabólicos completos y extraer información biológica. Sin embargo, hay dos factores limitantes: en primer lugar la diversidad físico-química y la amplia gama de concentración de los metabolitos analizados, y en segundo lugar el volumen y la complejidad de los datos obtenidos. Por lo tanto, los desarrollos tanto para la etapa de procesamiento de la muestra como para la de procesamiento de datos, son necesarios.

VII. 2. Preparación de la muestra vegetal en la metabolómica

La preparación de la muestra es un paso fundamental en estudios metabolómicos, debido a que la calidad de los datos que deben ser tratados posteriormente depende, en gran medida de su preparación.[79] Esta estapa se inicia con el cultivo y la recolección de plantas. En general, las plantas se cultivan en un ambiente controlado en términos de temperatura, humedad y luz para reducir la variabilidad biológica en los individuos. En la cosecha, se debe tener cuidado para evitar reacciones de oxidación. Con ese fin, las plantas pueden ser congeladas inmediatamente después de la recolección en un baño de nitrógeno líquido, y se almacenan a baja temperatura (-80 ° C) hasta su uso. Es habitual secar las plantas, pero durante esta etapa, el riesgo es mayor debido a que es posible que se reanuden los procesos metabólicos.[80] Una solución puede ser la liofilización, que permite evitar la descongelación del material vegetal. La ausencia de agua reduce la actividad enzimática.[81] Sin embargo, la liofilización de los tejidos puede causar la adsorción irreversible de los metabolitos en las paredes y membranas celulares.[82] También es posible extraer la planta fresca congelada directamente, preferiblemente por calentamiento para inhibir la reactivación de las enzimas.[82] La extracción debe ser simple y rápida, ya que un gran número de muestras suelen ser tratados en los estudios metabolómicos. Se pueden emplear diversas técnicas con objeto de aumentar la velocidad de procesado de muestras como la extracción con microondas[83] y extracción con ultrasonido.[84] Los disolventes de extracción son seleccionados en base a sus propiedades físico-químicas, en particular, su polaridad y su selectividad, de forma que cubran un rango de polaridad lo más amplio posible. Sin embargo, es necesario tener en cuenta también, la compatibilidad de los metabolitos extraídos con el método de análisis propuesto. El uso de etapas adicionales de purificación permite eliminar los compuestos interferentes potenciales, por ejemplo, por medio de la extracción líquido-líquido (LLE) o extracciones en fase sólida (SPE) sobre diversos soportes (gel de silica C18 y resina de intercambio iónico).

VII. 3. Técnicas analíticas

El tamaño exacto del metaboloma sigue siendo hasta hoy desconocido, incluso para los organismos más conocidos. Sin embargo, algunos autores estiman que existen más de 3.000 metabolitos en los seres humanos[85] y más de 200.000 en el reino vegetal.[86] Los metabolomas son sistemas complejos y dinámicos. Por lo tanto, la distribución de muchos metabolitos está sometida a variaciones temporales y espaciales. Los componentes difieren tanto en su tamaño, naturaleza de sus grupos funcionales, volatilidad y polaridad como en su concentración fisiológica. Hasta la fecha, no hay un método analítico universal, capaz de centrarse en todas estas moléculas. Cada método analítico tiene sus ventajas y limitaciones. La elección del método analítico utilizado para el enfoque metabólico depende de los resultados esperados y las posibles vías metabólicas de interés, y también se requiere velocidad, selectividad y sensibilidad.

Muchos métodos han sido aplicados en los estudios metabolómicos: La espectroscopía de Infrarrojo por Transformada de Fourier (FT-IR),[77] la absorción UV-visible y fluorescencia,[87] la electroforesis capilar acoplada a espectrometría de masas (CE-MS),[88] pero los más ampliamente utilizados en la actualidad son la resonancia magnética nuclear (RMN) y espectrometría de masas (MS). Estos métodos pueden ser utilizados en combinación con técnicas de separación tales como cromatografía de gases (GC) o la cromatografía líquida (LC) en el caso de MS y sobre todo con la LC en el caso de RMN. La elección del acoplamiento utilizado dependerá de los compuestos a estudiar (Figura 14). Por ejemplo, la GC permite analizar principalmente los compuestos de bajos pesos moleculares y no polares (volátiles)) mientras que la LC/MS acoplada al electrospra y permite la ionización de moléculas polares y masas moleculares entre 10^1 y 10^6.

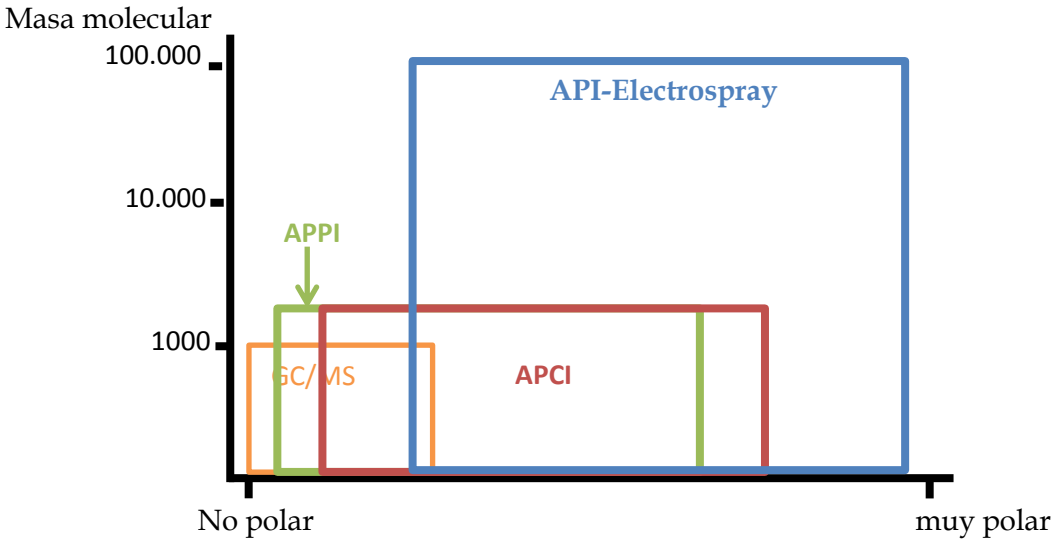


Figura 14- La elección de la técnica de acoplamiento de acuerdo con la naturaleza de los compuestos de ensayo (de polaridad y peso molecular).

VII. 3. 1 Resonancia magnética nuclear (RMN)

La RMN de protón (1H-RMN) es la técnica más antigua utilizada para el estudio del perfil de metabolitos en el campo de las plantas, con las primeras aplicaciones publicadas en los años 1970.[89,90] RMN tiene una serie de ventajas: rápida, no destructiva, fácil de implementar, robusta y genera espectros ricos en informaciones estructurales directas. Se considera más reproducible que la espectrometría de masas, en particular para los estudios que se prolongan durante largos periodos de tiempo. Se requiere sólo una mínima preparación de la muestra y permite una cuantificación absoluta de los metabolitos presentes en la muestra analizada. Además, no requiere preselección de las condiciones empleadas, lo que le da una ventaja sobre las técnicas cromatográficas en los que los metabolitos observados dependen de las columnas y condiciones de elución. Se publicaron muchos estudios metabolómicos aplicados a las plantas y basados en RMN.[91-96] Krishnan *et al*.[97] han propuesto un enfoque de dos etapas para la identificación de metabolitos, basado en una huella dactilar metabólica para la discriminación de los grupos y la identificación de regiones importantes del espectro, permitiendo la identificación de 20-40 metabolitos en el extracto no fraccionado. Sin embargo, la presencia de un gran número de picos en un espectro de protón puede comprometer la identificación de los metabolitos específicos. Para superar este inconveniente, es posible utilizar RMN en dos dimensiones, pero se alarga el tiempo de análisis.[98] La baja sensibilidad es la principal limitación de esta técnica.[99] Se utiliza principalmente para controlar los cambios metabólicos experimentados por un sistema biológico, indicadores de la enfermedad o un modo de acción.

VII. 3. 2 Espectrometría de masas MS

La espectrometría de masas está basada en la obtención de iones a partir de las moléculas orgánicas en fase gaseosa. Una vez obtenidos estos iones se separan de acuerdo con su masa y su carga y finalmente se detecta por medio un dispositivo adecuado.

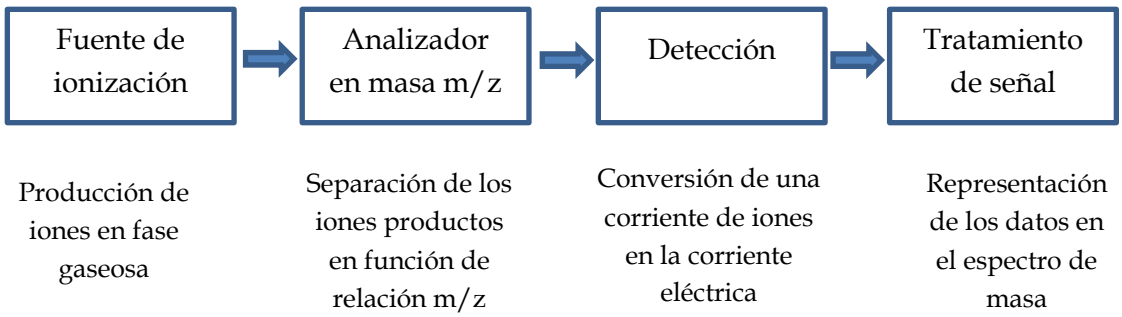


Figura 15- Diagrama de los diferentes elementos de un espectrómetro de masas.

Básicamente, un espectrómetro de masas se compone de los siguientes elementos (Figura 15): un sistema de introducción del analito, una fuente para ionizar los analitos, uno o más analizadores para separar los diferentes iones generados en la fuente, un detector para contar los iones, Finalmente, un sistema de procesamiento de datos que permite la visualización y el registro de espectros de masas y el control de los parámetros de adquisición.

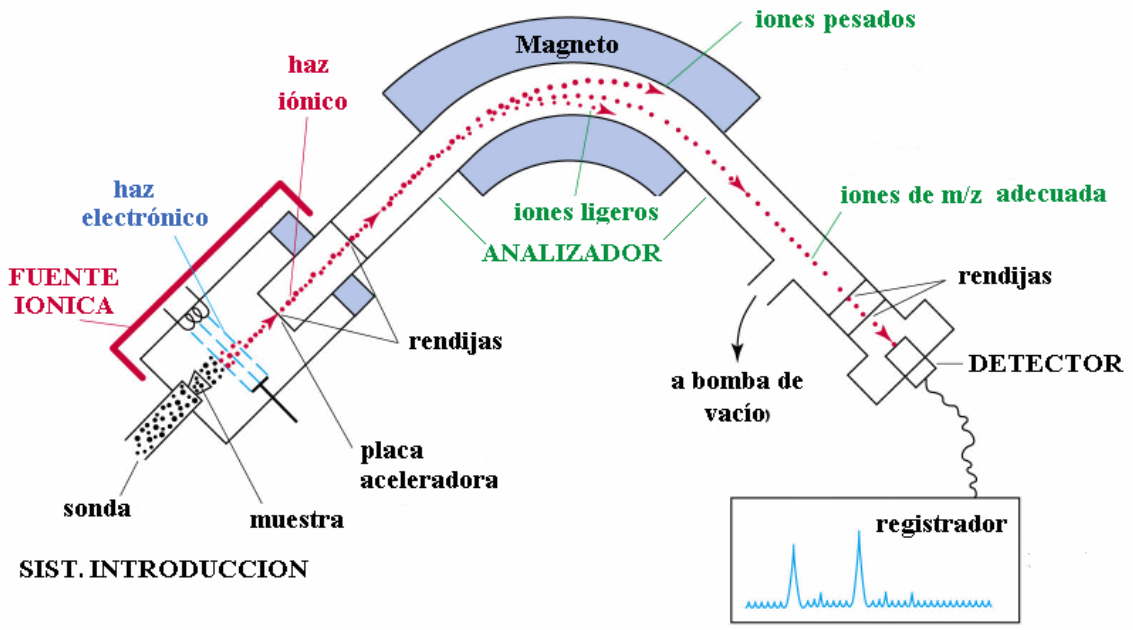


Figura 16- Esquema de un espectrómetro de masas

La espectrometría de masas ofrece varias ventajas para los estudios de metabolómica. Es sensible y permite la detección de especies a nivel de µM, proporcionando acceso a muchas clases de metabolitos. Además, permite la identificación de los metabolitos a través de la obtención de su peso molecular con gran precisión dependiendo del tipo de analizador, y mediante el estudio de su espectro de fragmentación, que se puede comparar con la de un compuesto de referencia que proporcionada información estructural.

VII. 3. 2. 1. Acoplamiento GC-MS

Desarrollada en los años 1960, la cromatografía de gases acoplada a espectrometría de masas ofrece numerosas ventajas como una buena resolución cromatográfica, la detección específica de analitos, y la capacidad de cuantificar los analitos con una buena sensibilidad. A principios de los años 1980, esta técnica se aplicó por primera vez al análisis de las planta.[100] Aunque el uso de columnas capilares permite una buena capacidad de pico, en el caso de muestras biológicas complejas, el poder de separación de la columna es a menudo insuficiente. Por lo tanto, se ha desarrollado el método de GC-2D (GC x GC), donde cada pico de una primera columna, generalmente apolar, se transfiere a continuación a una segunda columna polar para someterse a la segunda dimensión de separación.[101]

La energía transmitida a los analitos con la fuente de impacto de electrones es muy importante, que conduce a una fragmentación altamente reproducible, que ha llevado a la creación de bibliotecas de espectros universal. La existencia de estas bases de datos de espectros de más de 100.000

compuestos (National Institute of Standards and Technology (NIST), por ejemplo), y la obtención directa de información estructural facilitan la identificación de los metabolitos. La oportunidad de consultar a estas bases de datos presente una cierta ventaja en comparación con la LC-MS y esto explica en parte por qué muchos de estudios metabolómicos continúan siendo llevados a cabo por GC-MS.

Sin embargo, el análisis por GC-MS requiere que los analitos sean térmicamente estables y volátiles. Los compuestos no volátiles de bajo peso molecular (<500 Da) pueden ser analizados después de la derivatización química apropiada, pero es un proceso largo que puede introducir fuentes de error, lo cual puede conducir a reacciones de degradación, al mismo tiempo que puede afectar al aspecto cuantitativo, el equilibrio de la reacción puede modificarse en algunas muestras. El GC está acoplado con mayor frecuencia con analizadores de tipo cuádruplo o de tiempo de vuelo utilizando una fuente de impacto electrónica (IE), una alternativa a IE es la ionización química (CI), la cual produce iones por la colisión de las moléculas con los iones primarios en la fuente.

Las primeras aplicaciones metabolómicas reales fueron publicadas en el año 2000: en un estudio realizado por Roessner *et al*.[102] se detectaron más de 300 metabolitos en un extracto de patata. En Arabidopsis, 326 compuestos fueron cuantificados por Fiehn.[103]

VII. 3. 2. 2 Acoplamiento LC-MS

Los acoplamientos entre la cromatografía líquida y espectrometría de masas aparecieron posteriormente a los de las cromatografías de gases. A diferencia de la GC-MS donde el acoplamiento es relativamente fácil, la combinación de la LC a la MS técnicamente es más compleja, ya que la detección por MS requiere la producción de iones en la fase gaseosa. Sin embargo, la LC se aplica a menudo para el análisis de compuestos de bajo volatilidad. El problema se complica además por la necesidad de deshacerse del disolvente de elución incompatible con el alto vacío requerido por las fuentes de ionización históricamente disponibles en GC-MS. Esto explica por qué la LC-MS ha requerido más tiempo antes de establecerse como una técnica esencial en el análisis de muestras en general y también en metabolómica. Actualmente el éxito de la LC-MS es debido en gran parte al desarrollo de fuentes de ionización a presión atmosférica (API), tales como electrospray (ESI), la ionización química a presión atmosférica (APCI) o fotoionización a presión atmosférica (APPI). En resumen, se puede decir que el APCI, en el que la ionización tiene lugar en la fase gaseosa, se utiliza principalmente para el análisis de moléculas no polares y pequeño tamaño, mientras que el ESI, que es una técnica de ionización en fase condensada, es más adecuado para los compuestos polares en una gama más

amplia de pesos moleculares. El APPI a menudo supera la APCI y ESI para la ionización de las moléculas no polares y generalmente es más sensible.[104] Así, la LC-MS permite el análisis de moléculas con diferentes propiedades fisicoquímicas y detecta fácilmente metabolitos de 1.000 Da o más.

Por lo tanto, muchos grupos de investigación comenzaron a desarrollar enfoques metabolómicos basados en métodos de LC-MS.[105] La principal desventaja de la ionización en LC-MS es su propensión a los efectos de matriz, especialmente la supresión de ionización cuando dos o más compuestos co-eluyen. Esto último es especialmente frecuente en ESI, e introduce además de perdida de sensibilidad, una dificultad adicional para la cuantificación de los metabolitos.

VII. 4. Tratamiento de datos

Los estudios metabolómicos producen una gran cantidad de datos a menudo complejos para interpretar. Además, el tamaño de los ficheros informáticos generados por los instrumentos actuales es considerable. Por tanto, es necesario el desarrollo de herramientas de procesamiento de los datos y de estadística compatibles con la evolución de las tecnologías y problemas biológicos dirigidos. Estas nuevas maneras de tratar los datos se recogen bajo el término de quimiometría.[106] La idea central que se desprende de esta disciplina se encuentra en la transformación de la cantidad de datos disponibles en información relevante que puede generar conocimiento. Las herramientas de análisis de datos se pueden dividir en dos categorías que distinguen los llamados enfoques supervisados, de los enfoques no supervisados. Dentro de estos dos enfoques, los métodos pueden a su vez dividirse en modelos lineales y no lineales. En el campo de la metabolómica, el primer paso a menudo implica el uso de métodos lineales no supervisados. El más utilizado es el análisis de componentes principales (PCA),[107] generalmente usado para identificar diferencias entre muestras, que variables contribuyen a estas diferencias, y si estas variables contribuyen de la misma manera, es decir, están correlacionadas o son independientes. Este método descriptivo permite visualizar los datos originales en un espacio de 2 o 3 dimensiones reduciendo la dimensionalidad de conjunto de datos complejo. Así, el método genera nuevas variables denominadas componentes principales (CPs), que son las combinaciones lineales de las variables originales. CPs son ortogonales entre sí y forman un nuevo sistema de coordenadas para la proyección de la muestra. Dentro de los métodos supervisados PLS-DA (Partial Least Squares Discriminant Analysis) y más recientemente O-PLS-DA (Orthogonal-PLS-DA) son los más utilizados.[108] PLS-DA es un método de regresión lineal usando la información de pertenencia de un individuo a su clase, consiguiendo de esta manera una mejor separación entre los diferentes grupos de observaciones

mediante rotación de los componentes principales de forma que se obtenga una separación máxima entre clases, las cuales, a diferencia del PCA, están identificadas. Antes de la aplicación de estas herramientas estadísticas, los filtros deben ser utilizados para reducir la cantidad de información y limpiar el ruido de fondo de los datos. Estos filtros permiten evaluar el valor predictivo de cada variable a través de un análisis de varianza (ANOVA).

VII. 5. Aplicación

Los campos de aplicación de la metabolómica son muy diversos, entre ellos podemos citar la toxicología, la patología, la nutrición y un nivel más fundamental de la comprensión global de los sistemas vivos. La metabolómica rápidamente ha encontrado múltiples aplicaciones en las ciencias de los alimentos y en agricultura. Así, proporciona una información muy valiosa relativa a la biodiversidad genotípica y fenotípica,[109] los cambios bioquímicos asociados con los ciclos vegetativos y reproductor durante el crecimiento de la planta y maduración del fruto [63,110] y la comparación, describiendo diferencias y similitudes, de composición de diversas especies y variedades. Las técnicas de CG se han utilizado para obtener perfiles metabolómicos que permitan comparar sistemas de mejora genética incluyendo mutaciones inducidas[111] y diferentes prácticas culturales como cultivo convencional frente al orgánico.[112,113,114] Los perfiles metabolómicos son también valiosos en ingeniería metabólica para conseguir cultivos con alto contenido de metabolitos nutricionalmente importantes o como herramientas para mejorar sus características agronómicas o su calidad nutricional y organoléptica,[115] consiguiendo alimentos que combinen una apariencia atractiva y un buen sabor con altos niveles de fitonutrientes como flavonoides y carotenoides. Otra aplicación interesante es la caracterización de la respuesta de las plantas a un amplio rango de estresantes bióticos y abióticos y su efecto sobre la productividad, aspecto con una gran trascendencia económica. Así se ha estudiado las alteraciones en el metaboloma de un amplio número de especies en respuesta a diferentes condiciones de estrés, siendo las relativas a la temperatura y nutrición (fundamentalmente nutrientes como nitrato, fosfato y sulfuro combinados con niveles altos y bajos de oxígeno y carbono) las mejores caracterizadas,[116-120] junto a los estresantes bióticos.[121]

En el campo de la ciencia de los alimentos y de la nutrición, la metabolómica permite estudiar muchos aspectos de la "nutrición molecular" incluyendo el análisis de los constituyentes de los alimentos, calidad y detección fraudes alimentarios, monitorización del consumo de alimentos y monitorización fisiológica en los estudios de intervenciones alimentarias o dietéticas.[122-124] Con el desarrollo de la metabolómica, los alimentos y bebidas son ahora analizados con un mayor detalle de forma que cientos o incluso miles

de compuestos químicos son detectados y/o identificados en ciertos alimentos. De esta forma tenemos la oportunidad de comprender los detalles moleculares de porque ciertos alimentos o bebidas tienen un sabor, textura, aroma o color únicos. Igualmente, se pueden identificar de manera precisa los ingredientes bioactivos y comprender mejor sus efectos potenciales beneficiosos (o dañinos). Así, los perfiles metabolómicos se han utilizado en fresas para estudiar el desarrollo del fruto mediante una combinación de CG-MS y UPLC-QTOF-MS,[125] cubriendo no sólo los metabolitos primarios sino cerca de 100 metabolitos secundarios incluyendo derivados fenólicos. Esto representa un paso significativo más allá del estado actual de conocimientos basados en determinar diferencias en clases químicas específicas tales como flavonoides, y aminoácidos debidas a la variación genética o el estrés.

OBJETIVOS

Objetivos

El objetivo general de esta Tesis Doctoral es caracterizar las fresas cultivadas en sistemas sin suelo desde el punto de vista organolétptico, nutricional y nutraceútico mediante metodología analíticas clásicas y metabolómicas estudiando la influencia de las distintas condiciones de cultivos sobre la calidad de la fresa.

Para ello en este estudio se han planteado los siguientes objetivos específicos:

1. Estudiar la composición orgánica e inorgánica de variedades de fresas cultivadas en sistema convencional (suelo) y sistema sin suelo.

2. Evaluar la influencia de factores genéticos, estacionales y ambientales sobre metabolitos primarios, segundarios y bioactividad (actividad antioxidante) de fresas cultivadas en sistema sin suelo.

3. Determinar el perfil matabolómico de fresas cultivadas sin suelo con distintas sensibilidad al estrés ambiental, utilizando CG-MS combinado con técnicas quimiométricas.

4. Estudiar la ateración de metabolitos primarios en respuesta a diferentes condiciones de cultivo y estrés medioambiental.

5. Investiragar la influencia de la variedad y sistema de cultivo mediante la aplicación de técnicas estadísticas multivariante.

CAPÍTULO I

Influence of cultivar and culture system on nutritional and organoleptic quality of strawberry

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Abstract

BACKGROUND: Strawberries are widely appreciated and consumed as a pleasant tasting fruit either in fresh form or as processed product. Of the many factors which can affect the quality of fruit (sensorial and nutritional), the cultivar is especially important, but it is also affected by crop conditions. The main characteristics related to the fruit quality and nutritional attributes were assessed in several varieties of strawberry grown in soilless and soil culture. The effect of different cultivars and cropping systems on selected quality parameters, were assessed by applying multivariate statistical methods, such as principal component analysis and partial least square discriminant analysis (PLS-DA).

RESULTS: Differences among cultivars were observed based on the mineral composition and glucose and fructose concentrations in both cultivation systems. However, no significant differences between cultivars were detected in sourness. Tamar and Camarosa were the sweetest cultivars among those grown in soilless and soil system, respectively. Both varieties also had the highest total sugars/total acids ratio. The comparison between strawberries cultivated in soil and soilless systems also revealed significant differences in the mineral composition, content of sugars and related parameters.

CONCLUSION: Good discrimination was observed between cultivars of conventional crops, whereas no clear separation between cultivars grown in the soilless system was achieved by PLS-DA. PLS-DA also allowed differentiation of samples by type of crop (soilless vs. conventional crop). This information could be useful for selection of growing conditions and high-quality cultivars.

Keywords: strawberry; quality; cultivars; soilless system

1. Introduction

Strawberry (Fragaria x ananassa Duch.) is a very important crop in Europe and the USA owing to its nutritional and sensorial characteristics and its profitability. Modern production methods allow strawberry cultivation under a wide range of environmental conditions, making the fruit available on the market almost independent of the season. However, this could affect its final price. For this reason strawberry and other small fruits could represent an important and valuable portion of daily fresh food consumption.

Strawberry is widely cultivated in several areas under different cultivation conditions, and every year new varieties come into trade and cultivation. In Spain, strawberry is one of the most important crops, especially in the south of Huelva (southwest of Spain), because of soil and climatic conditions and water quality. Strawberry production in this region accounts for 35% of strawberry production in the European Union (EU) and 90% of national production, Camarosa being the most used variety, followed by Candonga, Festival and Ventana.[1] Nowadays, the largest production of strawberries is in tunnels on soil beds. Nevertheless, they can also be produced in soilless growing systems[2], which allow cultivation to be developed independently from environmental conditions.

Soilless culture has been strongly developed in the southern regions of Spain, France and Italy as a potential alternative to methyl bromide (MB). This soil fumigant was definitively phased out for strawberry in the EU in 2007. Since then, several chemical and non-chemical alternatives to MB have been developed.[3] The replacement of soil crops with soilless crops may have important advantages: reduction in water requirements, prevention from loss of nutrients and pesticides if recirculation is done, and product quality improvement.[4] The differences in strawberry quality as a function of the soilless cultivation system or substrate type have been studied in depth in previous papers, in which it was concluded that coconut fiber was the best substrate for the production of strawberry in soilless culture.[2,5,6]

The final goal of modern agriculture is production of fruit with high organoleptic, nutritional and nutraceutical qualities, free from biotic and xenobiotic contaminants. On the other hand, consumers highly value quality fresh foods, so the influence of factors such as cultivation method and genotype on organoleptic and nutritional characteristics should be investigated.

Quality, safety and sensorial attributes such as color, flavor and taste are the most important characteristics that determine the value of food. These characteristics are influenced by their chemical composition. Strawberries consist of 95% water and 5% dry matter, containing different percentages of proteins, lipids, sugars, vitamins and minerals. This composition is affected by a

number of pre-harvest and post-harvest factors. Among the preharvest factors, the variety,[7,8] handling during production,[9] climate and production region,[10,11] as well as sampling time and the degree of ripeness,[12,13] influence nutritional composition. As an example, it has been recently concluded that strawberries of the Camarosa variety cultivated in soil, following conventional practices under plastic tunnels, have higher pH values, soluble sugar (SS) content, and total soluble solids (TSS) and SS/titratable acidity (TA) values than strawberries cultivated in a soilless system.[6] With regard to mineral content, no significant differences in relation to culture system were found, except for N and P.

The aim of the present study was to provide a more thorough knowledge of organic and inorganic composition of strawberries and to assess the effect of production methods on fruit quality. For this, some chemical parameters (soluble solids, sugars, organic acids and mineral elements) were determined in strawberries of several Spain cultivars. In addition, differences between strawberries cultivated in soilless systems versus the conventional crop system were also investigated applying multivariate statistical techniques.

2. Material and methods

2. 1. Samples

Strawberry fruits (Fragaria x ananassa Duch.) were harvested in the same experimental and professional plantations located in Huelva (southwest Spain) at commercial ripeness (>75% of the surface showing red color). The strawberry samples used for the analysis of the fruit quality were divided into two groups. The samples representing class I consisted of six varieties, i.e. Camarosa, Candonga, Chiflón, BG 269, Tamar and Diamante cultivated in a soilless system. Fruit of five varieties cultivated using conventional practices, i.e. Camarosa, Candonga, Medina, Marina and Ventana, represented class II.

In soilless growing systems, plants were grown under an automated polycarbonate-covered greenhouse in Palos de la Frontera (Huelva,) using elevated horizontal troughs filled with coconut fiber as substrate. Radiation source in the greenhouse consisted of natural daylight. Temperature ranged from 25 °C during the day to 8 °C overnight. These values were similar to those applied in conventional culture. Relative humidity was held at 75%. Nutrient solution was made with four commercial fertilizers: KNO_3, KH_2PO_4, $MgSO_4$, $Ca(NO_3)_2$ and other microelements. The greenhouse was managed by the University of Huelva, where testing of different soilless cultivars and types of substrates has been carried out for several years.

In the conventional crop, tray plants were grown in double rows under a high tunnel in Moguer (Huelva), with drip irrigation and fertilization practices

according to standard recommendations for strawberry production in the zone.[14] The samples were collected from only one commercial farm, allowing all strawberry plants to be grown under the same conditions in terms of soil, fertilization, irrigation and illumination. This farm was chosen for its proximity to the experimental greenhouse. The varieties were chosen for their commercial interest and economic importance in the area.

For each cultivar, several fruits were collected to generate a representative pooled fruit sample. Strawberries were packed into polystyrene punnets (capacity 500 g) and shipped to the laboratory. Fruits were washed and sepals dissected. Samples were then homogenized in a blender and stored at −4 °C for further analysis. Analyses were carried out within a 6 month time period after harvest.

2. 2. Physicochemical parameters

Four analytical parameters were determined following recommendations from official methods of food analysis:[15] water content, pH, TA and TSS. All determinations were performed in triplicate from the pool of fruit for each cultivar.

2. 3. High-performance liquid chromatographic analysis for sugars and organic acids

Approximately 1 g of homogenate was accurately weighed, diluted to 10.0 mL with ultrapure water (Millipore, Bedford, MA, USA) and centrifuged at 10 000 rpm for 10 min (BHG-Hermle Z 365, Wehingen, Germany). The liquid supernatant was filtered through a 0.45 µm filter (Hydrophilic PVDF, Millipore) prior to injection into the high-performance liquid chromatographic system. Analyses were carried out in an Agilent series 1100 chromatograph (Agilent Technologies, Inc., Palo Alto, CA, USA) equipped with a manual injector (Rheodyne), and a 20 µL loop. A Metacarb 87 H hydrogen-form cation-exchange resin-based columns (300 × 7.8 mmi.d.) packed with sulfonated polystyrene was used for separation. Two detectors, photodiode array detector and refractive index detector were used for the simultaneous detection of the sugars and organic acids.

In a single chromatographic run, three sugars (glucose, fructose and sucrose) and six acids (oxalic, citric, tartaric, malic, succinic and lactic) were separated in 15 min using sulfuric acid as mobile phase. The flow rate was 0.5 mL min^{-1}, and UV detection was at 210 nm for organic acids. Sugars were detected by refractive index.

Acids were identified by comparison of both retention time and UV–visible spectrum with those of reference compounds. Sugars were identified by comparison of retention time with that of standard compounds.

2. 4. Determination of mineral elements

Major (Ca, Mg, K, P and S) and trace (Al, As, Cd, Cr, Cu, Fe, Hg, Ni, Pb, Ba, Mn, Na, Sr, V and Zn) minerals were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) using a Jobin-Yvon Ultima 2 ICP optical emission spectrometer and an ultrasonic nebulizer (U6000 AT+, CETAC, Omaha, NE, USA). The instrument was operated under the following conditions: radio frequency, 27 MHz; operating power, 1200 W; plasma argon flow rate, 2 L min^{-1}; auxiliary gas flow rate, 2 L min^{-1}; nebulizer gas flow rate, 0.02 L min^{-1}; nebulizer pressure, 1 bar; rinsing time, 35 s; rinsing pump speed, high; transfer time, 60 s; stabilization time, 20 s; and transfer pump speed, high. A 10% v/v solution of nitric acid (Panreac, Spain) was used for sample digestion. ICP Multi Element Standard IV and VI CertiPUR (Merck) were used to prepare reference solutions. The ashes were obtained by calcination (600 °C) of 20 g samples of strawberry. After the addition of 1 mL nitric acid and 2 mL distilled water, the ashes were dissolved and filtered. The final solution was transferred to a 10 mL volumetric flask.

2. 5. Statistical analysis

Experimental data were subjected to analysis of variance (ANOVA), along with the LSD test using Statistica software (StatSoft, Tulsa, OK, USA) to determine the presence of significant differences among cultivars and cultivation techniques. Two pattern recognition methods – principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA), were also carried out using Statistica software and SIMCA-P v11.5 software (UMETRICS, Umeå, Sweden), respectively. Both techniques were utilized to check the possibility of identifying variables able to discriminate strawberries on the basis of cultivar or growing system. PCA is one of the most powerful and common techniques used for reducing the dimensionality of large sets of data without loss of information. New variables obtained from linear combination of the original ones were calculated in such a way as to keep most of the information present in the original dataset in the least possible number of new variables or principal components (PCs). PLS-DA is a supervised pattern recognition method based on searching an optimal set of latent variables (or components) to discriminate between the previously defined categories. The PLS-DA method consists of a classical PLS regression where the dependent variable Y is categorical and represents sample class membership. It can be conveniently used when the number of objects is fewer than the number of

variables. The principle of PLS is to find the components in the matrix X (matrix of predictors) which describe, as much as possible, the relevant variations in the input variables and, at the same time, have maximal correlation with the target value in Y (matrix of responses), giving less weight to the variations that are irrelevant or noisy.[16] That is, PLS searches for a set of components that performs simultaneous decomposition of X and Y with the constraint that these components explain, as muchas possible, the covariance between X and Y. The optimal number of components is, in general, achieved by cross-validation techniques. The overall quality of PLS-DA models is described by R_X^2 and Q^2 values. R_X^2 is defined as the proportion of variance in the data explained by the models and indicates goodness of fit. Q^2 is a measurement of the predictive ability of the model.

The PCA and PLS-DA output consisted of scores plots to visualize the contrast between different samples and loading plots to explain the class separation. Prior to both PCA and PLS-DA, variables with non-normal distribution, Na, K, Ca, Cr and N, were subjected to log-transformation. All data were mean-centered and scaled to 'unit variance'.

3. Results and discussion

In this study, the chemical composition of six strawberry varieties cultivated in a soilless system and five strawberry varieties grown as a conventional crop was assessed. Tables (I.1–I.5) show the means and standard deviations based on three replicate samples of the data obtained from analysis of various physicochemical parameters (sugars, organic acids and minerals) determined in strawberries expressed on a fresh basis. The several groups of samples were compared by ANOVA to determine whether mean values for chemical composition differ significantly with the variety and culture system.

3. 1. Effect of the variety on chemical composition and organoleptic quality

Total soluble solids (TSS), titratable acidity (TA) and the ratio TSS/TA are important factors for evaluating fruit quality. In this work, TSS, TA, the ratio TSS/TA and pH values were examined and the data obtained are shown in Table I.1. Statistical analysis showed that there are significant differences between cultivars. The highest TSS value was obtained for cv. Tamar cultivated in soilless system (class I), followed by Candonga variety. In soil culture (class II), cv. Camarosa had the highest TSS and TA values, followed by cv. Medina. Galletta *et al*.[17] reported that TSS value in strawberry fruit is generally in the range 7–12% depending on variety. In the present study, the values for TSS ranged from 3% to 6% in class I and from 4% to 5% in class II, and were slightly lower than those cited in the literature.[7,12,18–21]

Table I.1- Physicochemical parameters determined in strawberry fruits of different.

Strawberry variety	Moisture (%)	pH	TA (%)	TSS (°Brix)	TSS/TA
Soilles culture					
Camarosa	86.87±2.41	3.53± 0.03^{a}	0.53±0.06^{a}	3.54±0.27^{a}	7.16±0.74^{ab}
Chiflón	92.13±4.17	3.59± 0.05^{a}	0.50±0.10^{a}	3.75±0.47^{a}	7.52±0.95^{ab}
Candonga	90.33±3.41	3.62± 0.04^{a}	0.84±0.08^{b}	4.49±0.39^{ab}	5.41±0.50^{a}
Tamar	89.96±4.22	3.82± 0.05^{b}	0.62±0.10^{a}	5.66±0.49^{b}	9.15±0.98^{b}
Diamante	92.71±4.16	3.56± 0.05^{a}	0.40±0.11^{a}	3.45±0.45^{a}	8.60±0.80^{ab}
BG 269	91.43±4.20	3.51± 0.05^{a}	0.56±0.10^{a}	3.69±0.47^{a}	6.69±0.64^{ab}
Soil culture					
Camarosa	84.67±2.92	3.59±0.02^{a}	0.74±0.06	4.98±0.10	7.04±0.62
Ventana	91.30±3.02	3.77±0.03^{b}	0.55±0.03	4.10±0.08	8.29±0.97
Medina	91.89±3.01	3.66±0.01^{c}	0.63±0.04	4.57±0.07	7.41±0.50
Candonga	89.58±2.98	3.73±0.02^{bd}	0.50±0.03	4.47±0.09	9.02±0.93
Marina	91.85±3.01	3.71±0.01^{cd}	0.58±0.05	4.50±0.10	7.80±0.94
Analysis of variance (p-value)					
Class I	0.777	0.004	0.048	0.031	0.000
Class II	0.462	0.003	0.670	0.330	0.877

Varieties and grown system.

Letters are comparisons of cultivars within a cropping system. Means with the same letter are not significantly different (P=0.05) by LSD test.

Paranjpe *et al*.[22] measured much higher levels of TSS in seven cultivars of strawberry grown under protected culture in Florida, ranging from 10% to 11% for fruit of cv. Camarosa. The values of TA (1.3%) were also higher than the mean value (0.53% and 0.74% in class I and II, respectively) reported in this work for the same variety. Voća *et al*.[23] also found for Elsanta variety grown in soilless system values for TSS (6.23%) and TA (7.35 g L^{-1}) very close to the mean values obtained in soilless culture in this work.

Differences between results found in the literature and those shown here may be due to the variation in genotype, production area, soil and climatic conditions and/or agricultural practices. Some of the differences may also stem from the variation in sample preparation and analysis procedures.

The pH value of the strawberries did not change significantly between varieties cultivated in soilless system and was quite similar in the two crop systems: 3.6 ± 0.1 and 3.7 ± 0.1 in class I and II, respectively. These values were similar to those reported by Kallio *et al*.[24] in other strawberry varieties grown under different conditions, and they were close to the average value for ripe strawberry: pH 3.3.[25]

The ratio TSS/TA ranged from 5.41 to 9.15 for strawberries cultivated in soilless system (class I), and therefore the value of 8.5 reported by Voća *et al*.[23] (in a study of Elsanta variety grown in soilless system) is included. In soil systems, the highest TSS/TA ratio was detected in cv. Candonga (9.02 ± 0.93), whereas the lowest was detected in cv. Camarosa (7.04 ± 0.62). These values are within the range reported in the literature (8.5–17).[21] This relation can be considered as a maturation index of fruit with high relevance on fruit quality for fresh consumption.

The soluble sugars identified and quantified in strawberry fruits were fructose, glucose and sucrose. The amounts of these sugars are displayed in Table I.2 and total sugars contents are shown in Table I.3. The concentration of glucose and fructose were found to be significantly different ($P < 0.05$) between varieties in both classes, in agreement with other authors.[11,13,21,26] The total amount of sugars was between 29 and 49 g kg^{-1} (class I) and between 35 and 43 g kg^{-1} (class II), which are at a lower level than that reported by other authors on different cultivars from different parts of the USA.[20,26] and Europe.[9,11,18,19,24,27] Fructose was the major sugar (51–57% of total sugars) in all cultivars, glucose being the second one (33–46% and 33–42% in class I and II, respectively). In general, fruit contained a lower sucrose concentration compared to fructose and glucose, and its participation in total sugar content ranged from<LD (below the detection limit) in Tamar (class I) and Candonga and Marina (class II) to11% in Chiflón. Results are in good agreement with those reported by Sturm *et al*.[18] and Wang *et al*.[26]

Table I.2- Mean content of soluble sugars and organic acid in strawberry fruits of different varieties and grown system expressed in g Kg^{-1} of fresh fruit weight.

Strawberry variety	Sucrose	Fructose	Glucose	Citric acid	Succinic acid	Malic acid
Soilless culture						
Camarosa	1.56±0.60	17.09±1.27^{a}	11.86±1.39^{a}	6.32±0.35^{a}	0.95±0.06^{a}	1.43±0.14^{ab}
Chiflón	3.79±1.04	17.90±2.19^{ab}	10.67±2.41^{a}	6.12±0.61^{a}	1.41±0.10^{b}	1.77±0.25^{a}
Candonga	1.23±0.85	22.16±1.79^{bc}	15.42±1.97^{a}	6.15±0.50^{a}	0.89±0.08^{ac}	1.33±0.20^{ab}
Tamar	ND	26.65±2.20^{c}	22.39±2.40^{b}	6.17±0.60^{a}	0.69±0.11^{cd}	0.98±0.22^{b}
Diamante	2.78±1.03	15.59±1.95^{a}	11.30±1.78^{a}	4.14±0.47^{b}	0.50±0.07^{d}	1.67±0.21^{ab}
BG 269	2.91±1.04	16.70±1.97^{ab}	12.20±1.83^{a}	5.52±0.52^{ab}	1.23±0.12^{b}	1.89±0.25^{a}
Soil culture						
Camarosa	3.20±0.12^{a}	22.07±0.45^{a}	17.83±0.31^{a}	5.90±0.56	0.79±0.09^{a}	1.36±0.25
Ventana	3.76±0.13^{b}	19.65±0.33^{b}	11.45±0.25^{b}	6.44±0.57	1.01±0.10^{ab}	1.21±0.20
Medina	3.72±0.10^{b}	19.00±0.28^{b}	12.99±0.27^{b}	6.15±0.50	1.31±0.12^{b}	1.18±0.22
Candonga	ND	22.13±0.42^{a}	16.44±0.30^{a}	5.61±0.47	1.00±0.10^{ab}	1.43±0.27
Marina	ND	22.30±0.40^{a}	16.35±0.31^{a}	6.31±0.49	1.10±0.11^{ab}	1.01±0.19
Analysis of variance (p-value)						
Class I	0.183	0.021	0.033	0.134	0.000	0.177
Class II	0.000	0.040	0.002	0.854	0.137	0.841

Letters are comparisons of cultivars within a cropping system. Means with the same letter are not significantly different (P=0.05) by LSD test.
ND: not detected

In strawberries grown in soilless crop, the levels of sugars varied between 10.67 (cv. Chiflón) and 22.39 g kg^{-1} (cv. Tamar) for glucose and between 15.59 (cv. Diamante) and 26.65 g kg^{-1} (cv. Tamar) for fructose. Montero *et al*.[28] found that glucose and fructose were present in equal concentrations (close to 30 mg g^{-1} FW) in ripe strawberry fruit of cv. Chandler grown in a greenhouse

hydroponic culture. The quantity of sucrose determined by these authors (~5 mg g^{-1} FW) was higher than our results (1.23–3.79 g kg^{-1}).

For strawberries cultivated in the soil system (class II), Camarosa berries were found to be the richest in terms of total sugar (17.83 g kg^{-1} glucose and 22.07 g kg^{-1} fructose); the contents varied between 11.45 and 17.83 g kg^{-1} for glucose, between 19.00 and 22.30 g kg^{-1} for fructose and between <LD and 3.76 g kg^{-1} for sucrose. These results were similar to the values reported by other authors,[9,11,12,21,26,29] except for sucrose, for which they obtained higher values.

Organic acids are minor components of strawberry fruit, but they are important attributes of flavor that, in combination with sugars, have an impact on the sensory quality of strawberry fruit. Citric acid was the major organic acid found in the analyzed strawberries (Table I.2), in agreement with results found in the literature.[9,11,18,28,30] The highest citric acid concentration was seen in cv. Camarosa (6.32 g kg^{-1}) and Ventana (6.44 g kg^{-1}) in class I and II, respectively. Sturm *et al*.[18] found much higher levels of citric acid in 13 cultivars of strawberry, ranging from 4.4 to 10.5 g kg^{-1}. Skupien and Oszmianski[30] also report similar contents of citric acid (0.85–1.50 g 100 g^{-1} fresh weight) for six cultivars of strawberry grown in Poland. The values measured in the sample fruit in our research were lower than the mean values measured by these authors. However, our results show values which are included in the range reported by them, and they are in agreement with values found by Crespo *et al*.[11]

Other organic acids, such as malic and succinic acids were found in lower concentrations. The content of succinic acid was similar to that measured by Skupien and Oszmianski[30] but they reported a higher value of malic acid (0.49–1.28 g 100 g^{-1} fresh weight). Nevertheless, Sturm *et al*.[18] could not determine malic acid owing to co-elution problems with fructose.

Amounts of citric and malic acids were reported as 3.21 and 1.11 g kg^{-1} in Oso Grande variety grown in Huelva by Perez *et al*.[29] The content of malic acid was similar to our results (0.98–1.89 and 1.01–1.43 g kg^{-1} for class I and II, respectively) but the content of citric acid was lower than our values. Pelayo *et al*.[20] found values of citric acid (6.6 g kg^{-1}) and malic acid (2.3 g kg^{-1}) in cv. Diamante slightly higher than those measured by us in the same variety (Table I.2). The amount of tartaric acid was lower, to the limit of detection, in all analyzed samples. However, this acid was detected by Sturm *et al*.,[18] although only in some varieties.

No significant differences in sourness between varieties were detected when ANOVA was applied, as can be seen in Table I.2 only the content of succinic acid was different, depending on the variety grown in the soilless system. Wang *et al*.[26] reported differences in organic acid contents among the

genotypes examined. Kallio *et al*.[24] found no statistical differences between varieties in the content of citric acid, whereas malic acid varied statistically between the samples.

Sweetness is one of the major attributes appreciated by consumers. The good taste of strawberry is a balance among sweetness due to sugars and the sourness of organic acid. Sugars differ in sweetness perception by the human tongue. For instance, fructose is perceived to be sweeter than sucrose and glucose less sweet than sucrose. To equalize contributions of sugars to human taste perception, a relative sweetness index was calculated based on the relative amount and sweetness properties of each individual sugar.[31] The total sweetness perception was calculated based on the fact that fructose is 2.3 and sucrose 1.35 times sweeter than glucose. Results are shown in Table I.3. As can be observed, there were significant differences between cultivars independent of the crop system. Tamar and Camarosa were the sweetest cultivars among those grown in soilless culture and soil system, respectively. Both varieties also had the highest total sugars/total acids ratio; therefore these cultivars were seemingly more palatable and balanced. However, this should be confirmed by consumer panels.

The inorganic composition of strawberry fruits was also studied, due to the fact that minerals are essential dietary nutrients and they play an important role in maintaining fruit quality and determining nutritive value. The data on major and minor minerals are shown in Tables I.4 and I.5. As can be seen, N, K, P, S, Ca, Na and Mg were the most abundant elements in all strawberry samples (Table I.4). Nitrogen was, quantitatively, the most important element (9.8 and 11.8 g kg^{-1} in class I and II, respectively), and accounted for 88–90% of the total mineral content; potassium, with an average content of 945.15 (soilless) and 816.45 (soil) mg kg^{-1}, represented 8.5% and 6.2% of the total in class I and II, respectively; phosphorous, calcium and magnesium were present in moderate amounts in strawberries, with average concentrations of 18.00, 134.28 and 104.59 mg kg^{-1}, respectively, in soilless culture and 19.79, 190.40 and 108.71 mg kg^{-1}, respectively, in soil culture. Sodium ranged between 6.75 and 20.10 mg kg^{-1} (class I), and in soil culture the highest value was found for cv. Marina (14.98 mg kg^{-1}) and the lowest in cv. Candonga (<LD). Other elements such as Fe, Cu, Zn and Mn showed a low average value (Table I.5) and represented less than 1% of the total mineral content. Hakala *et al*.[32] compared the results of different studies on the mineral content of different varieties of strawberries, finding that these results are very similar to each other, and they are in agreement with those reported in the present study, except for K and Mg, whose mean contents found by us were lower. Concentration of cadmium, cobalt, nickel, lead and mercury were below detection limits in all analyzed

samples. These elements can become highly toxic if they accumulate in biological systems; therefore it is important to determine their concentration in food.

Table I.3- Mean content of several parameters related to organoleptic characteristics in strawberry fruits of different varieties and grown system.

Strawberry variety	Sweetness index	Total sugars	Total acids	Total sugars/total acids
Soilless culture				
Camarosa	53.26±3.25^{a}	30.50±2.38^{a}	8.70±0.34^{a}	3.49±0.31^{a}
Chiflón	56.94±4.44^{a}	32.35±4.12^{a}	9.31±0.59^{a}	3.47±0.54^{ab}
Candonga	68.04±5.46^{ab}	38.80±3.37^{ab}	8.37±0.48^{a}	4.79±0.44^{bc}
Tamar	83.67±6.57^{b}	49.03±4.13^{b}	7.84±0.37^{ab}	6.25±0.50^{c}
Diamante	50.91±3.19^{a}	29.67±3.12^{a}	6.31±0.38^{b}	4.70±0.48^{abc}
BG 269	54.54±4.07^{a}	31.81±4.09^{a}	8.64±0.48^{a}	3.68±0.34^{ab}
Soil culture				
Camarosa	72.91±6.87^{a}	43.10±0.87^{a}	8.05±0.36	5.67±0.36
Ventana	61.32±4.89^{b}	35.30±0.83^{b}	8.68±0.38	3.99±0.30
Medina	61.24±4.87^{b}	35.31±0.80^{b}	8.65±0.42	4.09±0.34
Candonga	67.34±4.98^{ab}	38.57±0.67^{ab}	8.04±0.30	4.79±0.41
Marina	67.64±4.77^{ab}	40.81±0.74^{ab}	8.42±0.45	4.59±0.38
Analysis of variance (p-value)				
Class I	0.028	0.031	0.046	0.013
Class II	0.056	0.034	0.970	0.333

Letters are comparisons of cultivars within a cropping system. Means with the same letter are not significantly different (P=0.05) by LSD test.

Differences among strawberry cultivars were observed based on the mineral composition. In the soilless system only the contents of N and Sr were significantly different (Tables I.4 and I.5). Nevertheless, significant differences

in the levels of N, S, Ca, K, Mg, Na, Al, Fe and Ba were found between the five varieties in class II, as can be observed in Tables I.4 and I.5.

3. 2. Multivariate analysis

PCA was applied to visualizing data trends. In order to obtain some knowledge about the properties of the studied system and to try to interpret the chemical significance of principal components, the Varimax rotation technique was applied.

Table I.4- Mean content of minerals in strawberry fruits of different varieties and grown system expressed in mg Kg^{-1} of fresh fruit weight, except nitrogen (g Kg^{-1}).

Strawberry variety	N	P	S	Ca	K	Mg	Na
Soilles culture							
Camarosa	10.2±0.5^{a}	18.04±0.61	39.21±4.53	146.00±15.35	846.96±100.11	95.87±5.28	10.46±2.84
Chiflón	14.1±0.8^{b}	17.63±1.06	39.33±7.84	122.19±26.59	997.28±173.39	118.94±9.14	13.29±4.90
Candonga	13.2±0.6^{b}	16.89±0.86	41.57±6.40	137.49±21.71	1055.32±141.57	92.47±7.46	20.10±4.02
Tamar	8.4±0.9^{c}	18.74±0.95	46.83±6.58	130.90±22.21	993.47±172.22	116.37±8.52	12.47±3.51
Diamante	1.4±0.4^{d}	16.83±0.82	44.21±6.77	128.22±21.36	789.22±99.21	92.13±4.61	6.75±1.31
BG 269	12.1±0.10^{a}	19.85±0.85	47.20±5.69	140.87±20.43	988.69±112.36	111.81±8.29	16.58±3.12
Soil culture							
Camarosa	11.5±0.4^{a}	18.51±0.59	51.34±0.22^{a}	147.13±8.21^{a}	944.72±66.24^{ab}	109.63±1.25^{a}	8.11±0.21^{a}
Ventana	13.8±0.6^{b}	21.30±0.62	60.39±0.25^{b}	131.03±7.47^{a}	1176.94±70.28^{a}	122.93±1.79^{b}	9.92±0.31^{ab}
Medina	12.9±0.5^{ab}	18.10±0.41	42.64±0.19^{c}	136.58±6.28^{a}	704.88±51.06^{b}	88.61±1.01^{c}	11.05±0.32^{b}
Candonga	6.8±0.1^{c}	20.50±0.65	28.70±0.26^{d}	344.30±9.26^{b}	205.91±29.63^{c}	124.37±1.59^{b}	ND
Marina	13.0±0.4^{ab}	20.53±0.58	59.47±0.29^{b}	192.91±8.69^{c}	1049.80±62.42^{a}	98.04±1.13^{ac}	14.98±0.45^{c}
Analysis of variance (p-value)							
Class I	0.000	0.328	0.914	0.969	0.766	0.123	0.364
Class II	0.002	0.309	0.000	0.000	0.002	0.002	0.000

Letters are comparisons of cultivars within a cropping system. Means with the same letter are not significantly different (P=0.05) by LSD test
ND: not detected

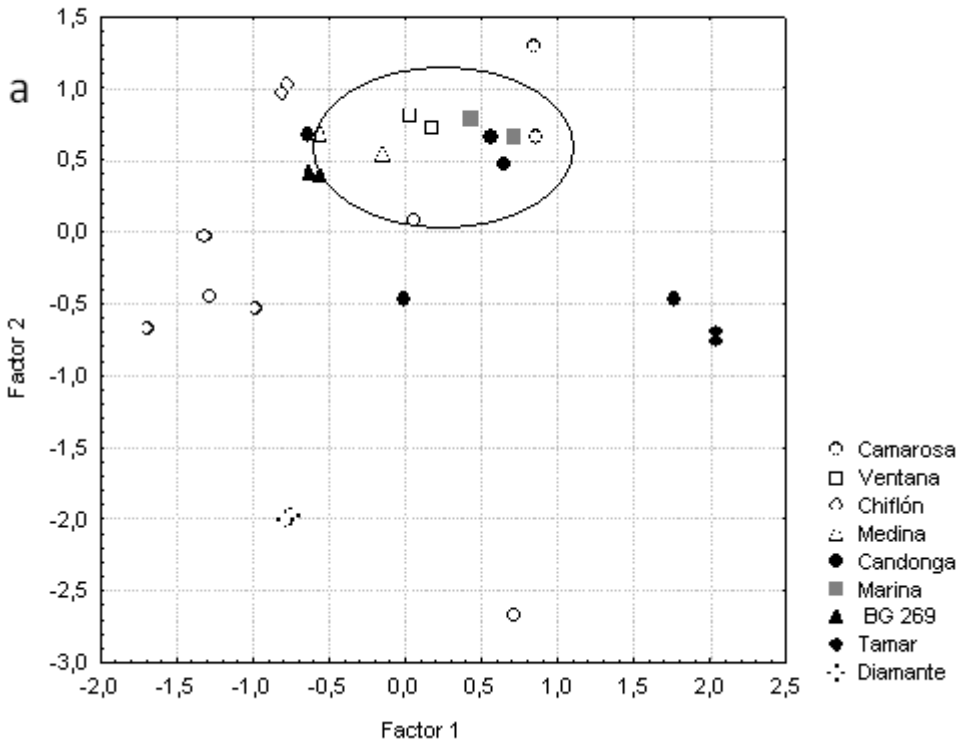
Table I.5- Mean content of minor minerals in strawberry fruits of different varieties and grown system expressed in mg Kg^{-1} of fresh fruit weight.

Strawberry variety	Al	Cr	Cu	Fe	Zn	Ba	Mn	Sr
Soilles culture								
Camarosa	0.47±0.07	0.07±0.02	0.59±0.05	4.81±0.14	3.66±0.10	0.15±0.03	4.55±0.10	1.12±0.14^{a}
Chiflón	0,26±0.04	0.06±0.03	0.38±0.03	2.63±0.08	1.66±0.08	ND	1.77±0.08	0.36±0.25^{b}
Candonga	0.61±0.10	0.05±0.02	0.66±0.06	6.14±0.16	3.97±0.10	0.15±0.03	2.71±0.06	0.56±0.20^{b}
Tamar	0.55±0.06	0.10±0.02	0.44±0.05	5.60±0.10	2.00±0.08	0.03±0.01	3.07±0.08	0.39±0.22^{b}
Diamante	0.34±0.04	0.05±0.02	0.56±0.07	3.32±0.10	2.01±0.08	0.06±0.01	4.24±0.11	1.66±0.28^{a}
BG 269	0.52±0.08	0.14±0.04	0.47±0.04	5.11±0.14	3.22±0.11	ND	3.67±0.10	0.44±0.19^{b}
Soil culture								
Camarosa	0.47±0.03^{a}	0.10±0.04	0.61±0.05	4.63±0.10^{a}	4.32±0.11	0.12±0.01^{a}	5.06±0.10	1.01±0.37
Ventana	0.44±0.02^{a}	0.03±0.01	0.45±0.03	3.75±0.09^{a}	2.31±0.08	0.22±0.02^{b}	2.76±0.08	0.25±0.10
Medina	0.51±0.04^{ab}	0.07±0.02	0.79±0.06	3.99±0.10^{a}	2.96±0.08	0.20±0.01^{b}	2.58±0.06	0.28±0.09
Candonga	0.60±0.07^{b}	0.07±0.02	0.79±0.07	6.02±0.15^{b}	5.15±0.10	0.47±0.08^{c}	1.92±0.06	0.41±0.06
Marina	0.61±0.07^{b}	0.07±0.02	0.70±0.07	6.02±0.14^{b}	3.84±0.10	0.47±0.08^{c}	2.70±0.08	0.35±0.05
Analysis of variance (p-value)								
Class I	0.365	0.406	0.234	0.340	0.756	0.119	0.065	0.010
Class II	0.061	0.572	0.172	0.003	0.410	0.000	0.684	0.610

Letters are comparisons of cultivars within a cropping system. Means with the same letter are not significantly different (P=0.05) by LSD test.
ND: not detected

Eight PCs with eigenvalues higher than 1.0 were extracted (Kaiser Criterion), which account for 90% of the total variance. PC1 is the most important, with 25% of the total variance. The variables which contribute most to the first component are pH value, sweetness index, glucose and fructose contents and TSS/TA ratio (Fig. I.1b). Therefore, factor 1 can be used as an indicator of sweetness, while factor 2 is an indicator of sourness, since it is positively correlated with the content of citric and succinic acids. PC2 is also

correlated with manganese and strontium concentrations, but negatively. Figure I.1(a) shows the sample scores plot for the first two principal components for discrimination of strawberry samples on the basis of cultivar. From examining sample distribution, a rough division can be seen in the plot. Strawberries cultivated in soil are located along PC1 in a way that reflects their sweetness and in the positive part of PC2 and can be described as higher sourness. Thus cv. Medina is less sweet and has lower scores on PC1. Strawberries cultivated in soil also have higher nitrogen, phosphorus and barium contents than the other strawberries cultivated in the soilless system, which appear as a less homogeneous group. As can be observed in Fig. I.1(a), Tamar, Camarosa, Candonga and Diamante cultivars, cultivated in the soilless system, are located in the negative part of PC2, and so have a lower sourness level than Chiflón, BG269 and all cultivars cultivated in soil. According to loading values, Tamar and Candonga are sweeter cultivars than Diamante and Camarosa, which have higher contents of manganese and strontium.



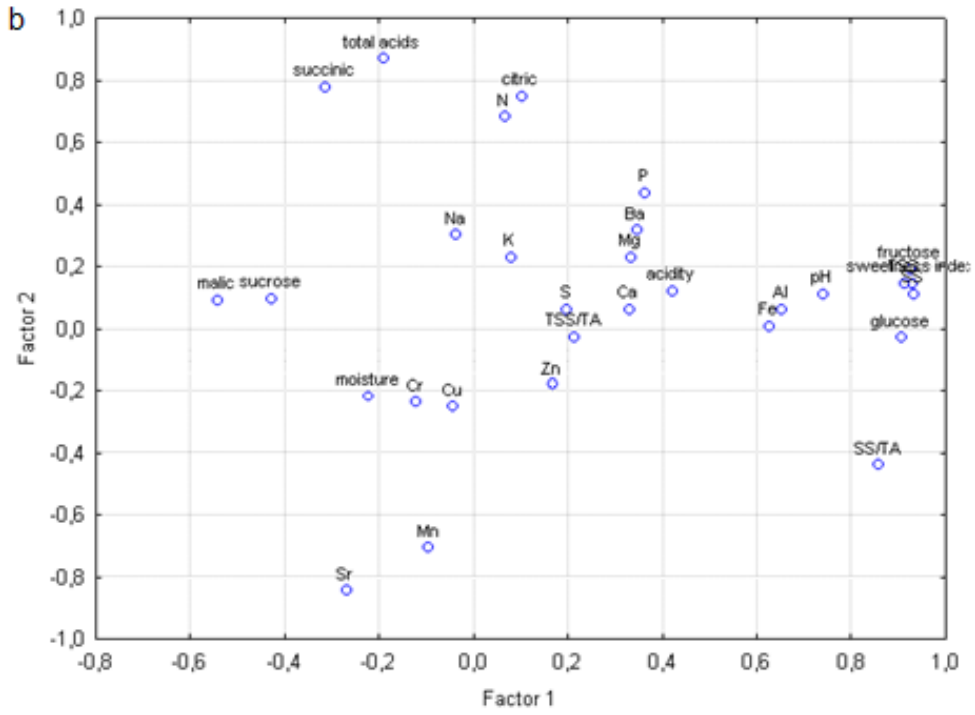


Figure I.1- Scores plot (a) and loadings plot (b) of first two principal components obtained by PCA of quality attributes of 27 strawberry samples. Ellipse highlights samples of strawberries grown in soil.

The data were also subjected to supervised chemometrics using PLS-DA. PLS-DA is a supervised method that explains the maximum variation in the dataset (X) using a dummy matrix (Y), which can be used to sharpen the separation between groups or observations. First, PLS-DA was carried out using the same data matrix as that used for PCA for all strawberry samples in order to discriminate between cultivars, but the model was not satisfactory. This two-component model had a low predictive power, Q^2 <0.05, indicating that the model was poor, and did not predict better than chance. Hence two models were constructed for strawberries cultivated in soil and soilless systems, respectively. In the first case, a three-component model was obtained with good quality of fit and validity (R^2=0,618) and prediction (Q^2= 0,0713). Figure I.2(a) shows the scores plot obtained for strawberries grown in soil, grouped according to the first two components. As can be seen, the separation between the five cultivars was satisfactory and they were clearly discriminated. The evaluation of PLS-DA loadings plot (Fig. I.2b) allowed a good understanding of the variables that contribute most to the discrimination of each class. Fructose and glucose were responsible for the separation of Camarosa, Candonga and Marina from Ventana and Medina cultivars. Succinic acid content and pH value contributed to the differentiation of Ventana and Medina cultivars from Camarosa. On the other hand, Ca, Mg, Ba, Fe, Al and Cu mostly contributed to

separation of strawberries of Candonga cultivar. The PLS models also provided the possibility of obtaining a quantitative measure of the discriminating power of each variable by the means of variable importance for the projection (VIP). This is a weighted sum of squares of PLS loadings taking into account the amount of explained Y-variance of each PLS component. X-variables, characterized by VIP values, which are larger than 1, had major importance for modeling the responses, whereas variables with VIP values smaller than 0.5 had little or no influence on the model. In this model, all the considered variables had good discriminatory power, being characterized by VIP values higher than 1.0 (data not shown).

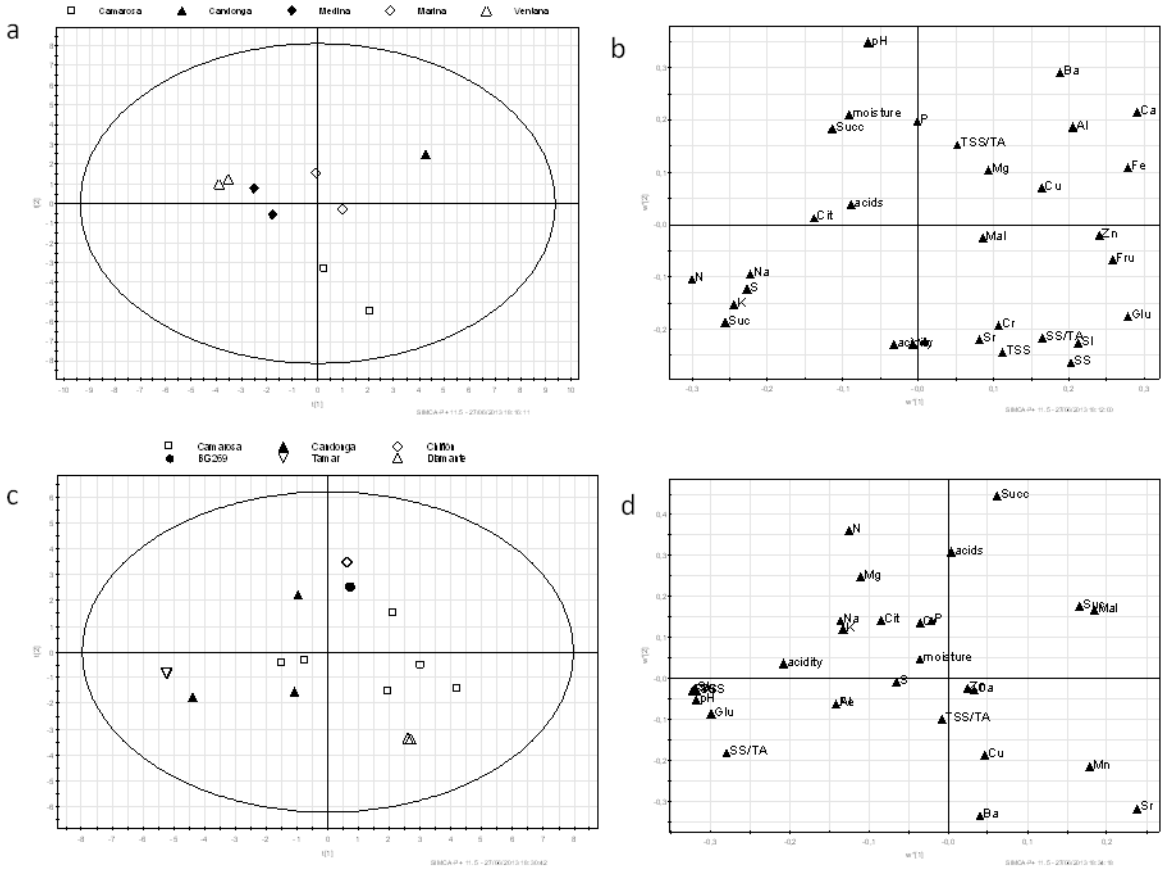


Figure I.2- PLS-DA scores (a, c) and loadings (b, d) plots based on chemical composition of strawberry cultivars grown in soil (a, b) and soilless crops (c, d). Fru, fructose; Glu, glucose; Suc, sucrose; Succ, succinic acid; Cit, citric acid; Mal, malic acid; SI, sweetness index.

A second model was obtained with data from strawberries grown in soilless cultures. This two-component model explained 46.5% of the variance

(R^2 = 0.465), but the PLS-DA scores plot showed a poor separation between cultivars, resulting in low predictability, as indicated by Q^2 of −0.104. Although the model cannot distinguish between Camarosa and Candonga cultivars, the t1 and t2 components allowed discrimination between ''Tamar'' (positioned on the left side of the plot), Diamante (positioned on the right side of the plot) and Chiflón and BG2692 cultivars, situated at the top of the plot. By comparing the scores plot (Fig. I.2c) with the loadings plot (Fig. I.2d) the variables contributing the most to the discrimination between cultivars were glucose content, pH value, sweetness index, and total sugars and TSS contents, which had a high contribution in discrimination of strawberries from cv. Tamar. Total acids and succinic acid were the most important variables for the Chiflón and BG269 cultivars, and Mn and Sr for cv. Diamante. All these variables had VIP values higher than 1.0.

3. 3. Effect of cultivation system on the nutritional parameters

The effect of culture system (soil and soilless) was investigated in Camarosa and Candonga cultivars by ANOVA factorial, which was performed using the General Linear Model program. The multivariate test showed that both factors – variety and crop system – were significant ($P < 0.05$). Considering the univariate results for each dependent variable, it was concluded that there were differences between the two cropping systems in the content of acidity and N (cv.Camarosa) and acidity, N and Ba (cv.Candonga). The effect of cultivation system on the nutritional characteristics of strawberry is more significant in the Candonga variety than in Camarosa. Thus the results obtained by ANOVA showed that strawberry of cv. Candonga cultivated in the soilless system had a higher acidity value, nitrogen, potassium and sodium contents and less calcium and barium amounts than those cultivated in soil. Strawberries of cv. Camarosa, cultivated traditionally in soil, present slightly higher acidity and N concentration, but the content of Ca, Ba, Na and K are practically the same for both systems. On the other hand, magnesium concentration and pH value were higher in the soil system independent of cultivar. The influence of the strawberry growing system was also investigated by the multivariate chemometric method. From a data matrix of 27 samples and 30 variables, PCA and PLS-DA were carried out, but good discrimination between strawberries cultivated in soil and soilless crops was only achieved by PLS-DA (Fig. I.3a, b).

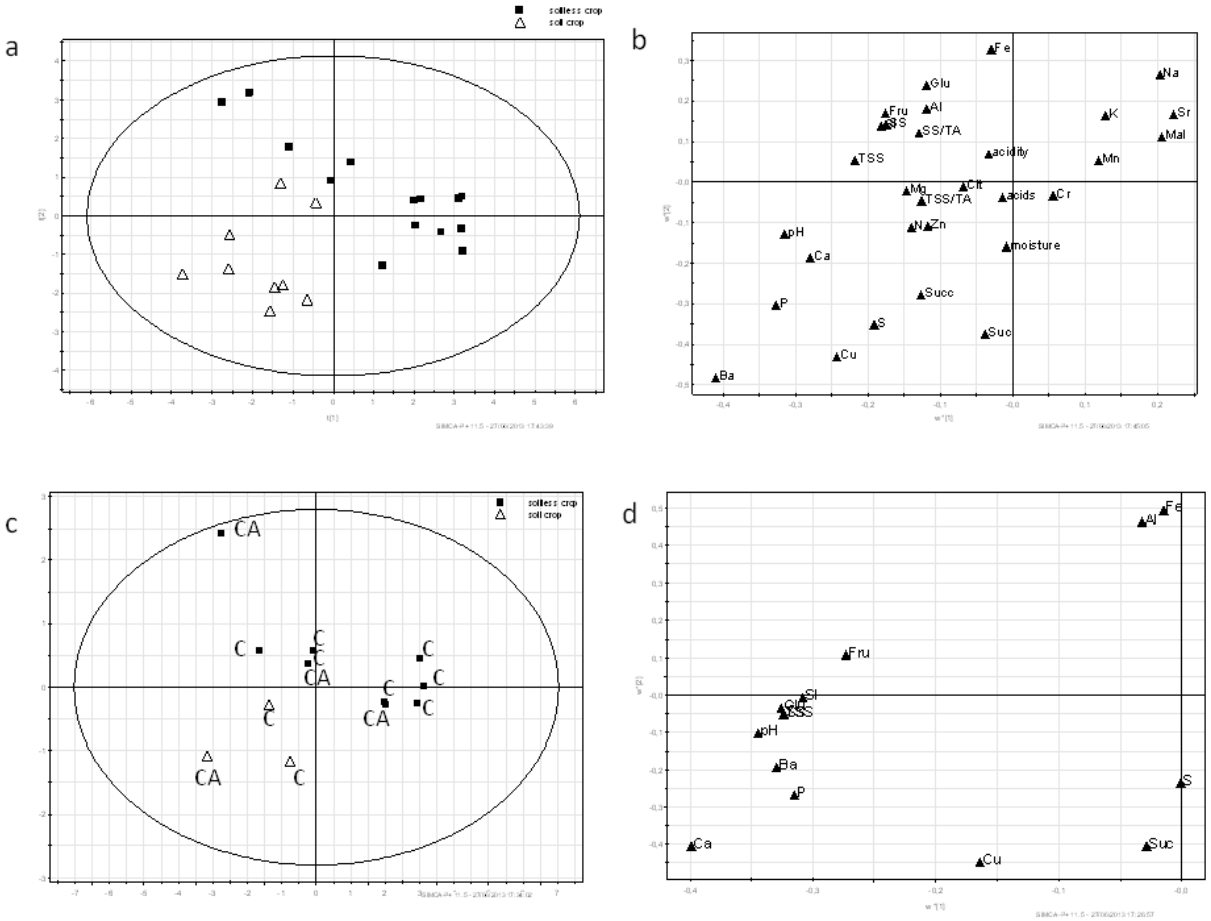


Figure I.3- PLS-DA scores (a, c) and loadings (b, d) plots based on chemical composition of strawberries grown in soil (open triangle) and soilless crops (box) with (a, b) all samples, and (c, d) only samples of 'Camarosa' (C) and 'Candonga' (CA) cultivars and PLS-VIP selection of variables. Fru, fructose; Glu, glucose; Suc, sucrose; Succ, succinic acid; Cit, citric acid; Mal, malic acid; SI, sweetness index.

The plot of X-scores showed the samples of strawberries grown in the soilless system distributed from the upper left to lower right side of the plot, and the soil samples situated at the lower left corner. The VIP scores indicated pH, Ca, P, S, Cu, Ba and sucrose as the most important parameters for strawberries grown in soil, whereas Fe, Al, glucose, fructose, total sugars, TSS and sweetness index were more relevant for strawberries grown in the soilless system. These variables with VIP scores higher than one were considered as input variables for a new PLS-DA model constructed with samples of Camarosa and Candonga cultivars. PLS-DA resulted in a two-component model and explained 55.9% of the variance. The R^2 and Q^2 values obtained by sevenfold cross validation were 0.559 and 0.118, respectively. Good discrimination was observed between the two sets of strawberries, with a similar distribution to that discussed above, as can be seen in Fig. I.3c. The loading plot showed how the first component (t1) was related to pH, glucose, TSS, sweetness index, Ba and P on the negative side where the soil samples were situated (Fig. I.3d). The

differences in mineral composition and sugar content and related parameters between strawberries cultivated in soil and soilless crops might be attributable to differences in agronomical practices (fertilization and irrigation) and environmental conditions.

4. Conclusion

The comparison of different cultivars of strawberry cultivated in two crop systems revealed differences in nutritional and organoleptic qualities, mainly in sweetness. Tamar and Camarosa were the sweetest genotypes among those grown in soilless and soil systems, respectively, as determined by the sweetness index. Both varieties also had the highest total sugar/total acid ratio; thus these cultivars were seemingly more palatable and balanced, although this information should be confirmed by sensory analysis. PCA and PLS-DA were performed to investigate differences between strawberry cultivars and crop systems. The variables with higher discriminating power can be identified by VIP value. Good discrimination was observed between cultivars of class II (conventional crop), whereas no clear separation between cultivars grown in the soilless system was achieved. In addition, good discrimination between strawberries cultivated in soilless systems and conventional crop system was also achieved by PLS-DA.

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CAPÍTULO II

Nutritional and nutraceutical quality of strawberries in relation to harvest time and crop conditions

Este capítulo está basado en el artículo: Akhatou, I and Fernández Recamales, M. A. (2014). Nutritional and Nutraceutical Quality of Strawberries in Relation to Harvest Time and Crop Conditions. J. Agric. Food Chem. 62: 5749−5760.

Abstract

Three strawberry varieties cultivated in soilless systems were studied for their content of primary and secondary metabolites in relation to harvest time and crop conditions. The three varieties were chosen based on their sensitivity level to environmental stress: Palomar (very sensitive), Festival (sensitive), and Camarosa (resistant). Throughout the campaign, three samplings were performed: December (extra-early production), January, and March (early production). Differences among cultivars and harvest times were observed based on the contents of sugars, organic acids, phenolic compounds, and antioxidant capacity. The higher levels for total anthocyanins and flavan-3-ols were found in Camarosa and Festival strawberries, both in the January harvest. The Palomar variety showed higher total sugar/total organic acids ratio in the March harvest. The influence of cultivation practices and environmental conditions was assessed by nested ANOVA and PLS-DA. Differences in the sugar and phenolic content were observed depending upon variety and coverage type. TEAC was most influenced by the substrate type.

KEYWORDS: strawberry, quality, metabolites, cultivars, soilless system, crop conditions

1.Introduction

Strawberries (Fragaria x ananassa Duch.) are one of the most popular berry fruits. They are widely used for industrial food processing but mainly consumed as fresh fruit. Its consumption has been increasing over the years due to the fact that epidemiological studies have revealed the relationship between diets which are rich in fruits and vegetables, and a lower incidence of some major human chronic diseases. Besides its proven health benefits,[1–3] strawberries have an attractive red color and flavor, and these attributes influence the choice of consumers and strawberries marketability.

The sugar content is very important from a nutritional point of view and, in combination with organic acids, contributes to strawberry taste and flavor. So, good taste is the result of a balance between sweetness provided by sugars and the sourness of organic acid. It depends not only on the total sugar and organic acid contents but also on the type and quantity of individual compounds. Different sugars differ from sweetness level percepted by human tongue. For this reason, the relative proportion of each sugar is important for sweetness perception and consumer acceptability. The strawberry color is related to the presence of pigments, among which, anthocyanins play an essential role. Moreover, anthocyanins and other phenolic compounds have attracted a great deal of attention due to their properties as antioxidants, which can protect the human body against cellular oxidation reactions through scavenging of free radicals. These antioxidants have been shown to reduce the risk of cardio- and cerebrovascular diseases, cancer and other agerelated diseases[4,5] and also contribute to the high nutritional quality of the fruit. However, it should also be considered that antioxidants protective effect can be dose-dependent and some of them may be harmful at high doses.[6]

Recent studies postulated that the antioxidant potential of anthocyanins and other phenolic compounds, as a basic mechanism of biological activity, is compromised by their low level of bioavailability especially when compared to the concentrations of endogenous antioxidants. Consequently, further research in this area is required in order to target other mechanisms of action that could be involved in the health-promoting effects beyond antioxidant activity.[2,3]

Strawberry is widely cultivated in several areas under different cultivation conditions and every year new varieties come into trade and cultivation. In Spain, strawberry is one of the most important crops, especially in the south of Huelva (southwest of Spain) due to soil and climatic conditions and water quality. Strawberry production in this region is accounting for 35% of strawberry production in the EU and 90% of national production. The economic importance of this crop and its great competitivity in the strawberry market drive strawberry producers to develop new growing methods such as soilless

culture,[7] and to use early varieties. These varieties come into market in winter with enhanced fruit quality, higher resistance to stresses (biotic and abiotic) as well as higher productivity.

The chemical composition of berry fruits is affected by a number of preharvest and postharvest factors. Among the preharvest factors, the variety is considered the main source of variation in composition. Their effects on the nutritional and sensorial quality are well-known[8–10] and several breeding and biotechnological programs are focused on them.[3] In addition, nutritional and nutraceutical quality is also influenced by crop conditions (environmental and cultivation techniques), sampling time and the degree of ripeness, among other factors.[11–17] Ripeness and maturity are the key factors that influence the taste of the fruit. In wild strawberry, climatic conditions, such as light, temperature, and rainfall are the main factors to be considered since fruit maturation is depending on these conditions. However, strawberry is currently cultured under controlled conditions, where light intensity is reduced by plastic covers; temperature is higher under plastic than in the outside mainly in coldest months and moisture is controlled by regular irrigation. Other factors such as soil composition and texture can affect phytochemical content and composition of fruits. The comparison between strawberries cultivated in soil and soilless systems revealed significantly differences in the mineral composition, content of sugars and related parameters.[18] Wang and Millner[13] studied the effect of matted row, black plastic mulch, and compost socks cultural systems on the phenolic and anthocyanin content of strawberry. They observed a higher phytochemical content in the compost sock system compared to the other two production systems.

Basic knowledge related to these aspects will allow the optimization of culture systems (environmental and agronomic conditions) and also may help to prevent some physiological disorders, such as the known as dry calyx.[19] This physiological disorder differs from salinity damage and its origin is unknown. For these reasons, the aim of the present work was to assess the influence of environmental, seasonal and genetic factors on nutritional and nutraceutical quality of strawberry fruits. Also, it will allow to correlate them with changes in concentration of primary (sugars and organic acids) and secondary (phenolic compounds) metabolites and their bioactivity (antioxidant activity).

2. Material and methods

2. 1. Experimental design and sampling

The study was conducted in experimental plantations managed by the University of Huelva, in southwestern Spain (latitude 37°14′N, longitude 6°53′W, and altitude 23 m) where strawberry plants (Fragaria x ananassa Duch.)

were grown in soilless systems. The assay consisted of two macrotunnels whose dimensions were 60 × 6 m. One of them was covered with plastic Tricoat 800 gauges on the arched structure of galvanized iron, and the other one was uncovered. Each macrotunnel contained three breeding lines of plants grown under different conductivities. Each line of breeding plants was composed of three strawberry cultivars. Finally, each cultivar was grown in three different commercial substrates.

The experimental design (Table II.1) was a strip-split-split-plot, where the main plot corresponds to crop covered (T) and culture exposed (D, without plastic cover). The subplot corresponds to the electrical conductivity of irrigation (EC = 1, 2, and 3 dS/m), the sub-sub-plot corresponds to the variety (Palomar, Festival and Camarosa) and the basic plot represents the substrate type (coconut fiber, perlite and rockwool). The three varieties were chosen basing on their sensitivity to environmental conditions: Palomar (very sensitive), Festival (sensitive), and Camarosa (resistant). Throughout the campaign, three samplings were performed: December (extra-early production), January (early production), and March (early production).

Table II.1- Description of the experimental design.

V1	Covered or uncovered macrotunnel																										
V2	EC1									EC2									EC3								
V3	Pal			Cam			Fes			Pal			Cam			Fes			Pal			Cam			Fes		
V4	C	P	R	C	P	R	C	P	R	C	P	R	C	P	R	C	P	R	C	P	R	C	P	R	C	P	R

Abbreviations:
V1= tunnel specifications. V2= Electrical conductivities. V3: Cultivars. V4: Substrate types
EC: electrical conductivity
Pal: Palomar. Cam: Camarosa. Fes: Festival
C: coconut fibers. P: perlite. R: rockwool

The strawberries were harvested at commercial ripeness, specifically when 75% of the surface showed a red color, which corresponds to stage 5 in terms of commercial criterion. For each treatment, several fruits were collected in our experimental plantations to generate a representative pooled fruit sample. Immediately after harvesting, fruits were sorted. Those showing damage, defects, or small size were removed. Then, sample lots were frozen in situ in a deep freezer and shipped to our laboratory in polystyrene punnets. Fruits were washed, sepals were dissected, and finally, fruits were gently homogenized by means of a kitchen mixer. The pastes obtained were subsequently stored for 2 months at −21 °C for further analysis.

2. 2. Reagents, materials, and apparatus

Methanol, ethanol, and acetonitrile were of HPLC grade. Trolox (6-hydroxy-2, 5, 7, tetramethylchroman-2-carboxylic acid) and ABTS (2, 2′-azino-bis (3- ethylbenzothiazoline-6 sulfonic acid)) were purchased from Sigma-Aldrich (Steinheim, Germany), and potassium persulfate was purchased from Panreac (Barcelona, Spain).

Standards (purity ≥99%) of fructose, glucose, malic acid, citric acid, oxalic acid, tartaric acid, and ascorbic acid were purchased from Merck (Darmstadt, Germany); sucrose was purchased from Panreac (Barcelona, Spain).

Polyphenol standards were supplied as follows: cyanidin-3-glucoside, pelargonidin-3-glucoside, peonidin-3-glucoside, malvidin-3-glucoside, petunidin chloride, procyanidin B1, luteolin, apigenin, quercetin-3-O-glucoside, kaempferol, and isorhamnetin were purchased from Extrasynthese (Genay, France); tyrosol, (+)-catechin, procyanidin B2, vanillic acid, m-coumaric acid, caffeic acid, and cinnamic acid were purchased from Fluka (Buchas, Switzerland); and epicatechin gallate, gallic acid, ellagic acid, p-coumaric acid, and quercetin were purchased from Sigma-Aldrich (Steinheim, Germany).

HPLC was used for separation, identification, and quantification of primary and secondary metabolites in strawberry. Chromatographic analyses were performed by means of an Agilent 1100 series HPLC system (Palo Alto, CA) equipped with a photodiode-array detector (PDA), which was set to scan from 200 to 770 nm, and RI detector.

Spectrophotometric determinations were carried out using a Helios Gamma UV−vis spectrophotometer (Thermo Fisher Scientific, USA) with 1 cm path length cuvettes.

2. 3. Antioxidant capacity assay

To assess strawberries antioxidant power, 0.5 g of homogenized fruit were extracted with 10 mL of methanol−water (80% v/v) and the pH value was adjusted to 5.0 ± 0.2 with NaOH (0.5M) and then centrifuged (at 10.000 rpm for 10 min at 4 °C). The supernatant was filtered through 0.45 µm filter and diluted with ethanol in 1:15 ratio.

The free-radical scavenging activity was determined by ABTS radical cation decolorization assay according to the method of Pellegrini *et al*.[20] The assay was carried out using a Helios Gamma UV−vis Spectrophotometer. The ABTS was dissolved in water to a 7 mM concentration. The ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration), and the solutions were allowed to react at room temperature in the dark for 16 h. The ($ABTS^{\bullet+}$) solution was diluted with ethanol to obtain an absorbance of 0.70(±0.01) at 734 nm. An

aliquot of 100 µL of the diluted extract was added to 2900 µL of diluted $ABTS^{\bullet+}$ solution and the absorbance was read exactly 6 min after initial mixing. The linear range of the calibration standard Trolox was 0.00−100 µM. Results from the assay were expressed in terms of micromoles of Trolox per gram of strawberry fresh weight (TEAC).

2. 4. Extraction and determination of sugars and organic acids

Different extraction procedures were applied to strawberry samples for the determination of individual sugars (fructose, glucose and sucrose) and organic acids (citric, malic, oxalic, tartaric and ascorbic). Homogenized fruits (5.0 g) were dissolved with 25 mL of methanol, sonicated for 30 min and then centrifuged 10 min at 10 000 rpm 4 °C. Supernatants were concentrated by means of a rotary evaporator at 40 °C and the residues were redissolved in 3 mL of 50% methanol. The concentrated extracts were filtered through 0.45 µm filters (Hydrophilic PVDF, Millipore Millex-HV, Bedford, MA) prior to their injection in HPLC system for sugars analysis.

Separation of sugars was carried out using an Ultrabase NH2 column (5 µm, 250 mm × 4.6 mm i.d.) with temperature maintained at 35 °C. Elution was performed at isocratic conditions with acetonitrile: water (70:30 v/v) as mobile phase, and at a constant flow rate of 0.5 mL/min. Refractive index (RI) detector was used for monitoring eluated sugars.

For extraction of organic acids, 1 g of strawberries was homogenized in 10 mL of phosphoric acid pH 2.20 (mobile phase) and then centrifuged at 10 000 rpm during 10 min. The supernatant was filtered through a 0.45 µm filter. The extract was diluted with phosphoric acid (pH 2.20) in 1:4 ratio (v/v).

Organic acids were analyzed by HPLC using an Ultrabase C18 column (2.5 µm, 100 mm × 4.6 mm i.d.) as stationary phase operating at a temperature of 50 °C. Organic acids were monitored at 210 nm and ascorbic acid at 254 nm. The mobile phase was phosphoric acid pH 2.20 prepared with Milli-Q water and the separation was carried out by isocratic elution, at a constant flow rate of 0.4 mL/min. The injection volume was set at 20 µL and the duration of analysis was 10 min.

Sugars in strawberry extracts were identified by comparison of their retention times to those of standards. The organic acids were identified by their retention times and UV spectra. For quantification, calibration curves were constructed using standards at 0.5−8 mg/L (ascorbic acid), at 1−60 mg/L (oxalic acid), at 20−100 mg/L (tartaric and malic acids), at 60−200 mg/L (citric acid), and at 1−50 g/L (sugars). Concentrations were expressed as g per kg fresh weight.

2. 5. HPLC analysis of phenolic compounds and anthocyanins

Samples were prepared as described above for extraction of sugars. Extracts were injected (20 µL) into a reverse phase Ultrabase C18 column (2.5 µm, 100 mm × 4.6 mm i.d.) following the methodology previously described by our previous studies,[12] but adapted to the new column. The modified method was further validated and their performance characteristics were confirmed. As for anthocyanins, the HPLC analyses was carried out according to the methodology described by Kallithraka *et al*.[21]

For analysis of colorless flavonoids and phenolic acids, elution solvents were water: methanol: acetic acid (93:5:2) as eluent A, and methanol: acetic acid (98:2) as eluent B. Applied elution conditions were as follows: 0−29 min, 40% B isocratic; 29−34.8 min, linear gradient 40−60% B; 34.8−37.7 min, linear gradient 60−75% B; 37.7− 40.6 min, linear gradient 75−100% B; 40.6−46.4 min 0% B. The flow was 0.8 mL/min, and the column temperature was set at 20 °C.

As for anthocyanins, the mobile phase consisted of 10% aqueous formic acid (A) and HPLC grade methanol (B). The gradient profile was as follows: 0−0.70 min, 5% B; 0.70−16.60 min, linear gradient 5- 50% B; 16.60−18.60 min, linear gradient 50−95% B; 18.60−20.60 min 95% B isocratic. Flow rate was 0.8 mL/min and temperature of the column was set at 30 °C.

Identification of phenolic acids and flavonoids was achieved by comparison of their retention times and UV spectra at specific wavelenght with those of appropriate standards. For quantification, the external standard method was applied considering the following wavelengths: 260 nm for ellagic acid and derivatives, 280 nm for benzoic acids and flavan-3-ols, 320 nm for cinnamic acids, and 360 nm for flavonols. Each standard was dissolved in methanol at a concentration of 1 mg mL^{-1}, and five diluted solutions from these stock solutions were used to prepare calibration curves of each standard (for duplicate). Anthocyanins were monitored at 520 nm. Calibration curves were constructed using standards at 1−50 mg/L (cyanidin-3-glucoside) and at 10−200 mg/L (pelargonidin-3-glucoside). The obtained linear equation was used to calculate the concentration of anthocyanins in strawberries.

2. 6. Statistical analysis

ANOVA followed by the Newman-Keuls post hoc test was applied in order to make multiple comparisons of mean values to evaluate if there were significant differences between the studied strawberries cultivars at different times of harvesting. A significant difference was statistically considered at the level of $p < 0.05$. Nested ANOVA was carried out for assessing the influence of different cultural and environmental factors. Data processing was performed using Statistica software package for statistical analysis (Statsoft, USA).

Pattern recognition (PR) techniques, including principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA), were also carried out using Statistica software (StatSoft, USA) and SIMCA-P v11.5 software (UMETRICS, Umeå, Sweden), respectively. PCA is one of the most powerful and common techniques used for reducing dimensionality of large sets of data without losses of information. New variables are obtained as a linear combination of the original ones. They are calculated in such a way to keep most of the original data set information, and mantain the least possible number of new variables or principal components (PCs). PLS-DA is a supervised pattern recognition method based on searching an optimal set of latent variables (or components) in order to discriminate between the previously defined categories. PLS-DA method consists of a classical PLS regression where the dependent variable Y is categorical and represents samples class membership. It can be conveniently used when the number of objects is fewer than the number of variables. The principle of PLS is to find the components in the matrix X (matrix of predictors) which describe, as much as possible, the relevant variations in the input variables and, at the same time, have maximal correlation with the target value in Y (matrix of responses). This gives less weight to the variations which are irrelevant or noisy.[22] That is, PLS searches for a set of components that performs simultaneous decomposition of X and Y with the constraint that these components explain, as much as possible, the covariance between X and Y. The optimal number of components is, in general, achieved by cross-validation techniques. The overall quality of PLS-DA models was described by R_X^2 and Q^2 values. R_X^2 is defined as the proportion of variance in data explained by models and indicates goodness of fit. Q^2 measures the predictive ability of the model.

The PCA and PLS-DA output consisted of scores plots and loading plots. They allow to visualize the contrast between different samples and to explain class separation, respectively. Prior to both, PCA and PLS-DA, data were mean-centered and scaled to "Unit Variance".

3. Results and discussion

3. 1. Chemical composition and antioxidant capacity

In this study, the chemical composition of three strawberry varieties cultivated in a soilless system was assessed. Table II.2 shows the mean, minimum, and maximum values of data obtained from analysis of the different chemical parameters. Sugars, organic acids, and phenolic compounds were determined in strawberries and expressed on a fresh basis. Table II.2 also shows antioxidant activity values.

Table II.2- Mean, minimum and maximum values of antioxidant activity and primary and secondary metabolites in three strawberry varieties.

Compounds	"Camarosa" (n=78)			"Festival" (n=69)			"Palomar" (n=60)		
	Mean	Minim	Maxim	Mean	Minim	Maxim	Mean	Minim	Maxim
TEAC	11.55	9.77	12.85	11.71	10.39	12.97	11.74	9.77	13.10
Cyanidin-3-O-glucoside	4.82	1.52	11.37	3.35	0.89	9.98	2.56	0.68	6.75
Pelargonidin-3-O-glucoside	125.14	47.13	208.75	102.44	27.81	188.96	100.08	16.11	170.13
Pelargonidin-3-rutinoside	56.69	18.72	103.32	35.25	10.55	66.78	31.13	7.75	57.02
Pelargonidin derivative	7.10	1.35	18.85	3.41	0.50	9.56	3.83	0.57	7.41
Procyanidin B1	52.98	8.07	108.11	60.17	5.89	116.54	39.20	8.49	109.77
Tyrosol	8.08	0.03	65.02	7.96	1.51	34.86	9.07	1.72	52.58
(+)-Catechin	38.02	9.27	64.49	39.92	4.64	72.84	34.45	4.04	195.28
Procyanidin B2	31.02	2.06	106.15	33.19	1.98	142.70	35.11	2.39	101.54
Vanillic acid	3.07	0.34	12.61	3.12	0.19	9.09	3.57	0.62	17.13
Epicatechin gallate	2.75	0.13	5.60	1.77	0.13	4.11	2.91	0.13	28.63
m-Coumaric acid	1.55	0.02	3.37	2.20	0.09	6.27	1.55	0.17	4.85
Gallic acid	4.07	0.49	14.03	4.67	0.85	26.66	5.31	0.64	17.30
Ellagic acid	9.33	3.72	24.86	10.07	4.67	24.92	8.69	3.98	21.32
Ellagic acid derivative	9.67	0.23	63.75	8.43	0.76	16.88	7.44	0.23	15.36
Caffeic acid	0.71	0.11	1.91	0.56	0.11	2.44	1.08	0.11	3.60
Caffeic acid derivative	2.83	0.95	5.20	1.91	0.42	3.64	2.72	0.11	5.83
p-Coumaric acid	1.60	0.08	7.58	1.53	0.08	4.39	2.20	0.08	8.92
Luteolin	1.05	0.00	5.28	0.91	0.00	3.28	0.79	0.00	2.12
Apigenin	1.72	0.85	3.38	2.39	1.33	10.74	2.47	1.07	5.02
Quercetin-3-O-galactoside	2.80	0.14	33.68	3.98	0.15	41.11	2.59	0.07	30.65
Quercetin	2.42	0.74	11.47	2.27	0.66	5.06	2.11	0.45	6.47
Kaempferol	0.95	0.42	2.56	0.97	0.37	4.46	1.00	0.50	3.07
Isorhamnetin	1.27	0.60	1.87	1.24	0.40	1.83	1.23	0.35	1.92
Quercitrin	0.64	0.03	2.78	0.87	0.00	5.18	0.72	0.02	2.11
Oxalic acid	1.41	0.44	2.92	0.88	0.15	1.47	1.16	0.25	2.52
Tartaric acid	2.10	1.03	3.04	2.10	1.47	2.78	1.88	1.17	3.01
Citric acid	6.36	4.62	8.41	5.70	4.16	7.54	5.48	3.28	7.24
Malic acid	3.16	1.06	5.85	3.12	1.15	5.24	3.14	1.25	4.64
Ascorbic acid	0.08	0.01	0.25	0.10	0.00	0.29	0.14	0.00	0.34
Fructose	19.54	13.45	24.57	21.29	15.70	25.76	18.94	8.14	23.33
Glucose	17.63	11.57	23.40	19.52	13.61	26.98	17.37	9.33	24.04
Sucrose	3.22	0.90	6.60	4.09	1.15	9.80	2.71	0.61	7.33

The soluble sugars identified and quantified in strawberry fruits were fructose, glucose, and sucrose. Fructose was the major sugar (50% of total

sugars) in all cultivars, glucose being the second one (45%). The relationship between the content of both sugars was about unity, regardless of the cultivar. In general, the analyzed fruits contained lower sucrose concentration compared to fructose and glucose, and its participation in total sugar content ranged from <LD (below the detection limit) to 20% of total sugar content. As regards the organic acids, citric acid was the major organic acid found in analyzed strawberries followed by malic acid (Table II.2). Other organic acids such as oxalic, tartaric, and ascorbic acids were found in lower concentrations. These results are in good agreement with those found in the literature.[13,15–17] The Festival variety presented the highest mean contents in the three sugar types (41.05 mg/g, as sum of sugars). The lowest level of total sugar was found in cv. Camarosa (38.92 mg/g), which had the highest contents of all acids (13.08 mg/g). In view of these results, Festival was the sweetest genotype and also had the highest total sugars/total acids ratio; therefore, this cultivar was seemingly more palatable and balanced. However, this should be confirmed by consumer panels.

Twenty five different phenolic compounds were identified and quantified. They were classified into five different groups: (a) phenolic acids and derivatives (gallic, vanillic, tyrosol, caffeic, m-coumaric, p-coumaric, caffeic derivative, ellagic and ellagic derivative acid); (b) flavonols (quercetin, kaempferol, hiperoside, isorhamnetin and quercetrin); (c) flavones (luteolin and apigenin); (d) flavan-3-ols (catechin, epicatechin gallate and procyanidins B1 and B2); (e) anthocyanins (pelargonidin-3-glucoside, cyanidin-3-glucoside and pelargonidin-3-rutinoside).

Total phenolic content (considering the sum of all the individual phenolics) ranged from 307.43 µg/g of fresh fruit (cv. Palomar) to 373.82 µg/g of fresh fruit (cv. Camarosa). Anthocyanins were the predominant group, accounting for 48.0% of the total phenolic content. This is in agreement with results found in the literature.[3,23] As also previously reported,[10,24] the three major anthocyanins were pelargonidin-3-glucoside (mean value of 110.14 µg/g), pelargonidin-3- rutinoside (mean value of 41.95 µg/g) and cyanidin-3-glucoside (mean value of 3.66 µg/g). The levels of pelargonidin-3-glucoside and cyanidin-3-glucoside were in agreement with those reported by Crespo *et al*.[15] Other minor anthocyanins pelargonidin derivatives were observed but they could not be quantified. Camarosa cultivar was the richest in anthocyanins, which agreed well with our previous study findings[12] and with results reported by other authors.[25]

Flavan-3-ols was the second most important group in quantitative terms and accounted for 37.0% of the total phenolic content. In absolute terms, the highest levels were found in cv. Festival (143.01 µg/g of fresh fruit, as mean

value) and the lowest in cv. Palomar (120.74 µg/g of fresh fruit, as mean value). Procyanidin B1 was the predominant flavanol in the three surveyed strawberry cultivars, with a mean value of 51.2 µg/g, following by catechin (37.6 µg/g) and procyanidin B2 (32.9 µg/g). Catechin has been reported to be the major flavan-3-ol in Spanish strawberries.[26] These authors found a global flavanols content of 4.9 mg/100 g, and the catechin content (1.57 mg/100 g) was lower than our results. However, Padula *et al*.[10] and Aaby *et al*.[27] reported higher contents of catechin in cv. Camarosa (5.86 mg/100g) and in Totem (6.2 mg/100 g) and Puget Reliance (9.0 mg/100 g) cultivars, respectively. With regard to the proanthocyanidins content, de Pascual *et al*.[26] reported the presence of four proanthocyanidins dimers (B1, B2, B3, and B4) in strawberry, being B3 the most abundant. Aaby *et al*.[23] quantified three procyanidin dimers, a trimer, and a pentamer in 27 strawberry cultivars using HPLC-DAD-MS^2. The total proanthocyanidin concentration ranged between 8.6 and 36.8 mg/100 g of FW. The results obtained by Buendía *et al*.[28] were higher (54−168 mg/100 g of fw), as expected, due to the fact that they used the phloroglucinolysis method. This approach generally leads to higher results compared with other methods. So, proanthocyanidins were the main strawberry phenolics found by these authors. In general, our results were lower than those previously found in the literature[27,28] and this could be explained by the analytical method used in addition to the differences between cultivars. Among the berries, the occurrence of galloyl esters was typical of strawberries.[29] Epicatechin gallate was found in the three surveyed strawberry cultivars (mean value 2.47 µg/g). Our results were lower than those reported by de Pascual *et al*.[26]

In general, the differences between results found in the literature and those shown here may be due to the variation in genotype, production area, soil and climatic conditions, and/or agricultural practices. Some of the differences may also stem from the variation in sample preparation and analysis procedures. Sample preservation and sample preparation parameters are critical for accurate estimation of phytochemicals present in foods. The freezing and thawing conditions can affect the content of strawberry phenolics. The extraction efficiency of phenolic compounds is influenced by the type of solvent, extraction procedure and conditions. Therefore, these factors must be taken into account when comparing data with literature.

The total content of phenolic acids of the three strawberry cultivars was similar among them, as can be seen in Table II.2. This group of compounds comprised 12.0% of the total content of phenolics. Ellagic and ellagic derivative acids are the major phenolic acids in the samples studied and represent approximately 50% of the total phenolic acids). This was in consonance with other authors findings.[30] The highest level of p-coumaric acid was found in cv.

Palomar (2.20 µg/g of fresh fruit, as mean value) and the lowest, in cv. Festival (1.55 µg/g of fresh fruit, as mean value). Other hydroxycinnamic acids such as m-coumaric acid, caffeic acid and caffeic acid derivative were also quantified. The last one was the most abundant (mean value 2.5µg/g). Regarding hydroxybenzoic acids, vanillic (mean value 3.23 µg/g) and gallic (mean value 4.64 µg/g) acids were found in all samples analyzed. Previous studies on strawberries have generally reported variable composition and contents of phenolic acids. Hydroxycinnamic acids occurred mainly as esters. Thus, p-coumaroyl-glucoside acid has been described as the major hydroxycinnamic acid in strawberries,[10,28,29,31,32] while p-coumaric acid has been described as the most abundant aglycone.[29,33] Padula *et al*.[10] reported relatively high contents of caffeic acid (from 0.7 to 11.40 mg/100 g fresh weight) and ferulic acid (from 0.76 to 13.99 mg/100 g fresh weight) in 20 strawberry cultivars grown in Italy. On the other hand, Mättila *et al*.[33] found caffeic, pcoumaric, p-hydroxybenzoic, cinnamic, and gallic acids in three strawberry cultivars grown in Finland (expressed as aglycones) but ferulic and vanillic acids were only detected in some of the strawberry cultivars. However, Määttä *et al*.[29] reported only the presence of p-coumarylester in cv. Jonsok.

The minor groups were flavonols and flavones, which accounted for 2.5 and 0.9%, of the total phenolic compounds, respectively. Regarding the family of flavonols, the mean content was 8.3µg/g. The highest levels were found in cv. Festival (9.3 µg/g of fresh fruit, as mean value) and the lowest in cv. Palomar (7.6 µg/g of fresh fruit, as mean value). Hyperoside (quercetin-3-O-galactoside) was the most important flavonol in terms of quantity (3.15µg/g) in the three cultivars. Other major flavonols were quercetin and isorhamnetin with mean values of 2.28 and 1.25 µg/g, respectively. Kaempferol and quercitrin were found in lower levels, whereas myricetin was not found at detectable levels in analyzed samples. Furthermore, two flavones, luteolin and apigenin, were detected in the three strawberry varieties. The mean content was 0.93 µg/g for luteolin and 2.16 µg/g for apigenin. As with the phenolic acids and other flavonoids, there are differences between results found in the literature. Glucosides and glucuronides of quercetin and kaempferol have been identified as the main flavonols in strawberry.[29,34] Our results for flavonol contents, averaging 8.4 µg/g, were low compared to those reported in the literature.[27–2]

The total antioxidant capacity was measured according to the ABTS method. Trolox (vitamin E analogue) was used as standard, and results, expressed as micromoles of Trolox equivalents per gram (TEAC), are displayed in Table II-2. It can be observed that TEAC values were quite similar in the three surveyed strawberry cultivars, with a mean value of 11.66 µmoles TE/g. These results were of the same magnitude as those previously reported.[8,15]

Josuttis *et al*.[14] reported higher values of antioxidant capacity for two strawberry cultivars (Everest and Elsanta) grown in Germany. The cultivars were grown in three different conditions. Two of them were cultivated in a tunnel covered with two films which vary in their UV transparency, and another one was cultivated under open-field conditions.

3. 2. Comparison of harvest time

The three varieties of strawberry were planted in September and harvested in December (extra-early production), January (early production), and March (early production) in order to assess differences in their profiles as a function of both variety and harvest time. Table II.3 shows the monthly weather parameters (outside the macrotunnel) during the experimentation period. These values are considered normal according to the climatological data recorded in the area (period 1971−2000).

Table II.3- Weather parameters recorded outside the macrotunnel during the harvest time.

Harvest time	Minimum Temperature (ºC)	Maximum Temperature (ºC)	Rainfall (mm)	Relative Humidity (%)	Global Radiation (MJ/m²)
December	8.2	16.1	156.9	86.4	197.78
January	6.6	15.9	55.2	88.9	246.45
March	7.5	18.8	124	82.4	504.37

The contents of average total sugars, total organic acids and five groups of polyphenols and TEAC as a function of strawberry variety and harvest time are displayed in Figure II.1. The comparison between three harvest times shows a similar trend in the three varieties. The amount of anthocyanins and flavan-3-ols (considering the sum of all individual phenolics) was higher in January (when rainfall, maximum and minimum temperatures were lower and relative humidity higher), whereas the total sugars content was more abundant in March (when maximum temperature and global radiation were higher). It has been previously observed that the content of many phenolic compounds and the antioxidant capacity increase in berry fruits as temperature increases.[32] On the other hand, low light intensity[35] and high temperatures[36] in day and night lead to decreased synthesis of sugar and ascorbic acid. Our results are apparently contradictory with these findings. However, Pereira *et al*.[37] observed it difficult to separate the effects of temperature from the photochemical effects. Moreover, the cultivation of strawberries under plastic tunnels decreases the dependency of quality on the climatic conditions.

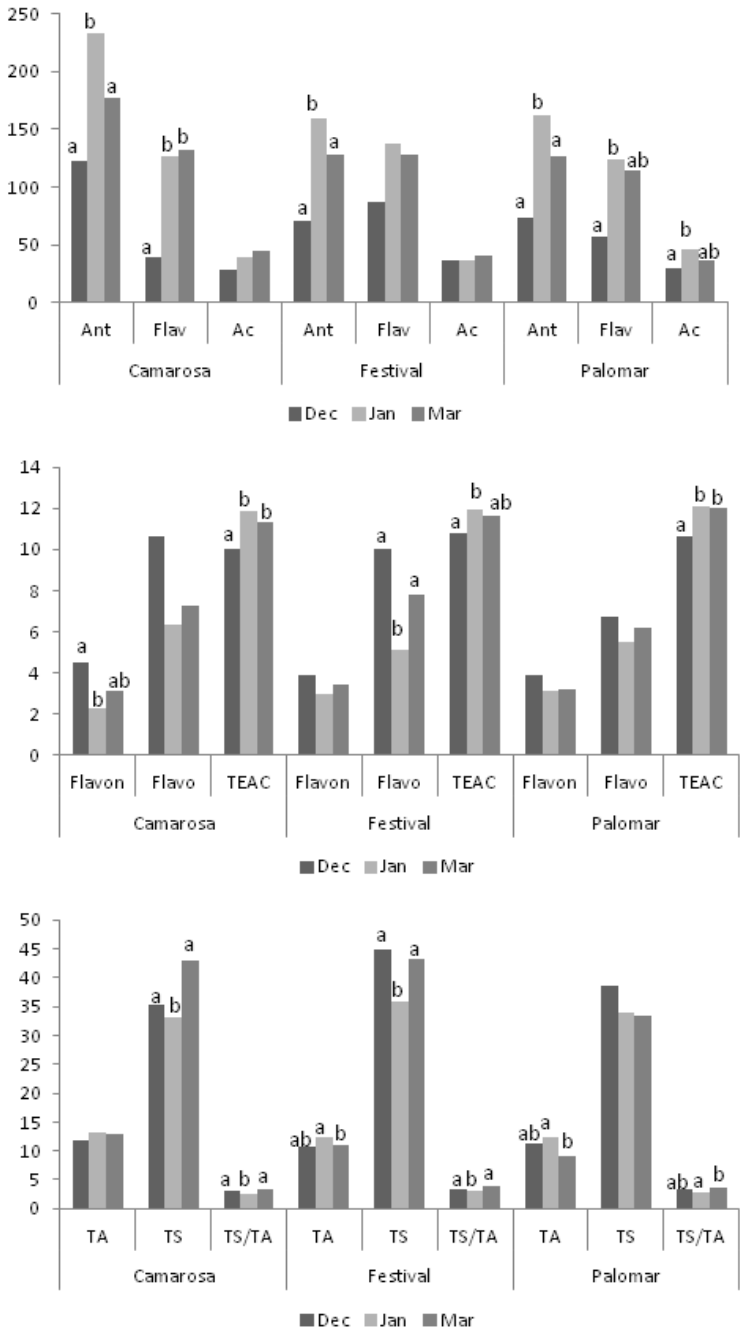

Figure II.1- Content of phenolic compounds (sum of anthocyanins (Ant), flavan-3-ols (Flav), acids and derivatives (Ac), flavones (Flavon) and flavonols (Flavo)), sugars (TS), organic acids (TA), total sugars/total organic acids ratio (TS/TA), and antioxidant activity (TEAC) in three strawberry cultivars harvested in December (Dec; n = 14), January (Jan; n = 73), and March (Mar; n = 50). Columns belonging to the same set of data with different letters are significantly different ($p < 0.05$) by Kruskal−Wallis test.

The higher levels for total anthocyanins and flavan-3-ols were verified in cv. Camarosa and Festival strawberries, respectively, both in January harvest; while the fruits of Palomar variety showed higher total sugar/total organic acids ratio in March harvest.

In order to find out significant differences of analyzed compounds between the three considered harvested times, the multiple comparison test of Kruskal−Wallis was applied. Results are also shown in Figure II.1. The harvests of December and January present statistical differences for anthocyanins and antioxidant activity in the three varieties. Additionally, Camarosa and Palomar cultivars also present differences for flavan-3-ols, and Festival cultivar for flavonols. Taste related parameters differentiate fruits harvested in January from March ones. The TS/TA ratio increased for the three varieties surveyed in March. Due to the importance of this parameter for strawberry flavor, seasonal changes in this ratio may influence consumer acceptance.

In order to confirm these results a principal component analysis (PCA) was carried out. PCA allows to visualize data trends and to provide a first evaluation for the discriminant power of the variables. PCA is applied to the data set to obtain linear combinations of variables, which are called principal components (PCs). It retains information about the variability of the data set, so that the first PC expresses the largest variability, and each successive PC represents as much of the residual variability as possible. In this case, five PCs, which account for 60% of the total variance, were extracted according to Kaiser Criterion (eigenvalues higher than 1.0). Figure II.2 shows samples distribution in the plane defined by two first components. As can be observed, samples harvested in January and December appear separated along of PC1, while fruits harvested in March are located in the negative part of PC2. The variables that contribute most to the first component are pelargonidin-3-glucoside and the sum of anthocyanins and flavan-3-ols, all with negative loadings. In the case of PC2, the most contributing variables are glucose, sum of sugars and total sugar/total organic acids ratio, also with negative loadings. Regarding the meteorological variables, PC1 is positively correlated to minimum temperature and rainfall. PC2 is positively correlated to relative humidity and negatively to maximum temperature and global radiation.

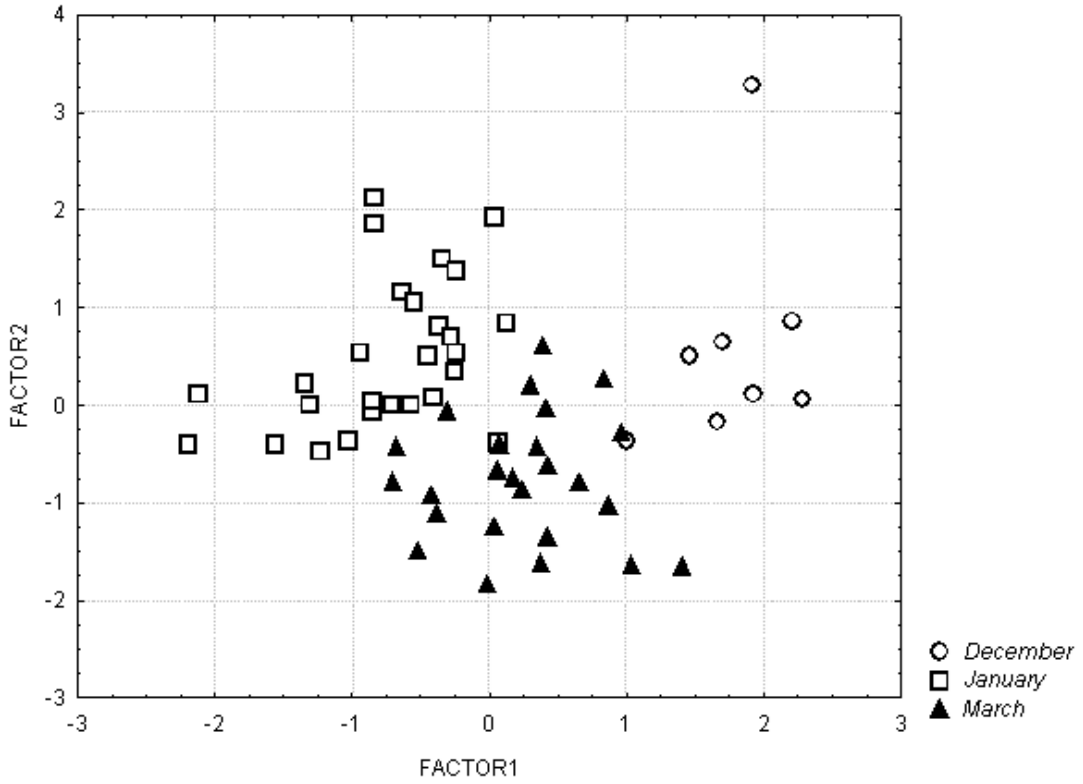

Figure II.2- Scores plot of first two principal components obtained by PCA of quality attributes of strawberry samples.

3. 3. Influence of agronomical practices

To meet the second overall objective of the study, that is, to examine changes in fruit quality depending on the cultivation practices and environmental conditions, a nested ANOVA was carried out. For this test, the expressions gathered in Table II.4 were applied. Nested ANOVA is used when the variation of the factor is subordinated to another variation factor. So, the survey had the following objectives: to explore whether chemical composition of strawberries varied in a purely random way across the two macrotunnels or whether it showed a variation due to different factors studied (electrical conductivity (B), variety (C), and substrate (D)). In the last case, the variance components associated with the levels of nested design is evaluated (Figure II.3). The effect significance of factors is tested by Fisher F test.

Table II.4- ANOVA table for the design proposed and calculation of variance component.

Source of variation	Mean squares	F-ratio	Variance component
Coverage (A)	$MS_A^2 = \frac{rdcb\sum_{i=1}^{a}(\overline{x_i} - \bar{x})^2}{a-1}$	MS_A^2/MS_B^2	$(MS_A^2 - MS_B^2)/rdc\,b$
Electrical conductivity (B(A))	$MS_B^2 = \frac{rdc\sum_{i=1}^{a}\sum_{j=1}^{b}(\overline{x_{ij}} - \overline{x_i})^2}{a(b-1)}$	MS_B^2/MS_C^2	$(MS_B^2 - MS_C^2)/rdc$
Variety (C(B(A)))	$MS_C^2 = \frac{rd\sum_{i=1}^{a}\sum_{j=1}^{b}\sum_{k=1}^{c}(\overline{x_{ijk}} - \overline{x_{ij}})^2}{ab(c-1)}$	MS_C^2/MS_D^2	$(MS_C^2 - MS_D^2)/rd$
Substrate (D(C(B(A))))	$MS_D^2 = \frac{r\sum_{i=1}^{a}\sum_{j=1}^{b}\sum_{k=1}^{c}\sum_{l=1}^{d}(\overline{x_{ijkl}} - \overline{x_{ijk}})^2}{abc(d-1)}$	MS_D^2/MS_R^2	$(MS_D^2 - MS_R^2)/r$
Residual	$MS_R^2 = \frac{\sum_{i=1}^{a}\sum_{j=1}^{b}\sum_{k=1}^{c}\sum_{l=1}^{d}\sum_{n=1}^{r}(\overline{x_{ijkln}} - \overline{x_{ijkl}})^2}{abcd(r-1)}$		MS_R^2

Table II.5 shows the F-test results (as p-value) from nested ANOVA for each variable. As can be observed, no statistically significant differences were found in the content of catechin, epicatechin gallate, and luteolin between two macrotunnels, electrical conductivity, variety or substrate. The type of coverage has a significant influence on the content of cyanidin-3-O-glucoside, pelargonidin-3-O-glucoside, pelargonidin-3-rutinoside, pelargonidin derivative 2, tyrosol, vanillic acid, m-coumaric acid, ellagic acid derivative, caffeic acid and caffeic acid derivative, apigenin, all flavonols, sugars and organic acids except for malic acid. So, the content of anthocyanins was higher in the cover macrotunnel, while the levels of flavan-3-ols, flavonols, ellagic acid and derivative, cinnamic acids and sugars were higher in the uncovered macrotunnel. These results were in agreement with those reported by Josuttis *et al*.[14] These authors found a higher content of flavonols and a lower total content of anthocyanins and pelargonidin related anthocyanins in open-field strawberry fruits compared to tunnel-grown fruits. The F-test also indicates significant differences between variety, mainly for the anthocyanins, sugars and organic acids. The electrical conductivity and type of substrate also influence the content of some studied compounds, mainly phenolic compounds. Keutgen and Pawelzik[38] observed that the NaCl stress significantly increased the concentration of most of organic acids in cv. Elsanta (sensitive), while remained

fairly constant in cv. Korona (less sensitive). They also observed that in both cultivars the individual sugars decreased progressively due to NaCl. On the other hand, these authors found that moderate salt stress significantly increased the antioxidant capacity, total phenolic compounds and anthocyanins in cv Korona.[39]

Table II.5- Results of the nested ANOVA as p-Value[a].

	Coverage	EC (coverage)	Variety (Coverage*EC)	Substrate (coverage*E* variety)
TEAC	**0.00024**	**0.00112**	**0.00199**	**0.00012**
Cyanidin-3-O-glucoside	**0.00000**	**0.00071**	**0.00000**	**0.03459**
Pelargonidin-3-O-glucoside	**0.00837**	**0.00025**	**0.00000**	**0.00049**
Pelargonidin-3-rutinoside	**0.00263**	0.06419	**0.00000**	0.08643
Pelargonidin derivative 1	0.32628	0.05872	**0.00000**	0.35070
Pelargonidin derivative 2	**0.00000**	0.15853	**0.00000**	0.15108
Procyanidin B1	**0.00024**	**0.00000**	**0.00000**	**0.00262**
Tyrosol	**0.03404**	0.95924	0.07552	0.12937
(+)-Catechin	0.41143	0.07256	0.35337	0.38687
Procyanidin B2	0.85775	0.44190	0.18871	**0.01656**
Vanillic acid	**0.02789**	**0.02499**	0.86120	0.23198
Epicatechin gallate	0.49857	0.51262	0.18343	0.35745
m-Coumaric acid	**0.00049**	**0.00000**	**0.00010**	**0.00003**
Gallic acid	0.84341	0.06758	**0.00053**	0.70332
Ellagic acid	0.12805	**0.01965**	**0.01767**	0.27533
Ellagic acid derivative	**0.00000**	**0.00000**	0.15149	**0.00709**
Caffeic acid	**0.00001**	**0.00317**	**0.00000**	0.06454
Caffeic acid derivative	**0.00009**	**0.02595**	**0.00000**	0.10699
p-Coumaric acid	0.95221	0.25085	**0.02924**	0.07407
Luteolin	0.95549	0.09715	0.15174	0.15031
Apigenin	**0.00309**	0.14195	**0.00385**	0.12573
Quercetin-3-O-galactoside	**0.00410**	0.28710	0.32153	0.30304
Quercetin	**0.00000**	0.80956	0.11525	0.40068
Kaempferol	**0.00350**	0.50823	**0.00002**	0.08555
Isorhamnetin	**0.00003**	**0.03369**	**0.00004**	**0.00495**
Quercitrin	**0.00002**	**0.00018**	**0.00572**	**0.00010**
Oxalic acid	**0.00042**	**0.00000**	**0.00000**	**0.00001**
Tartaric acid	**0.00000**	**0.01491**	**0.00042**	**0.00026**
Citric acid	**0.00025**	0.23353	**0.00001**	**0.00335**
Malic acid	0.27115	**0.00011**	0.43694	0.66901
Fructose	**0.00000**	0.28461	**0.00002**	0.12909
Glucose	**0.00000**	0.28617	**0.00000**	**0.00711**

[a]Figures in bold indicate significant F-tests at the level of significance showed in the table

Figure II.3 shows, for a selection of variables, the estimated contribution of the various levels of the nested design to the total variance. For most variables, the variance of the residual errors was the largest variance component. For phenolic compounds the variance components associated with the coverage

and variety contributed substantially to the total variance in addition to residual error. These findings are consistent with those found by other authors. They identify the genotype as the main source of variation for phenolic compounds[8,10,15] but they are also affected by crop conditions.[11,13,14] For sugars, the variance component due to coverage contributed noticeably to the total variance. Finally, the organic acids had different patterns of variation. The variance component associated with the substrate generally account for 1/5 of the total variance for citric, tartaric and oxalic acids. For oxalic and tartaric acids, the higher source of variability was due to variety and coverage, respectively.

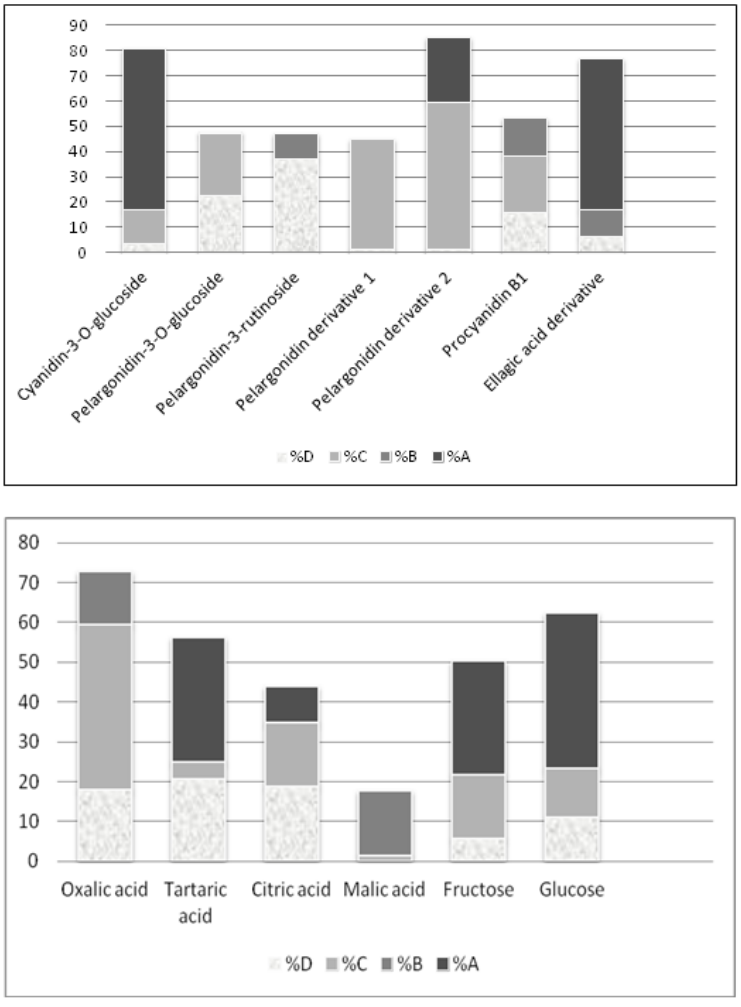

Figure II.3- Variance component (%) corresponding to the different sources of variability coverage (A), electrical conductivity (B), variety (C), and substrate (D).

The influence of agronomical practices was also investigated by multivariate chemometric method. Data were subjected to supervised

chemometrics using partial least-squares discriminant analysis (PLS-DA). PLS-DA is a supervised method that explains the maximum variation in the data set (X) using a dummy matrix (Y), which can be used to sharpen the separation between groups or observations. Four models were constructed to assess the influence of strawberry cultivars and the growing conditions (macrotunnel type, electrical conductivity and substrate). In the first case, a two-component model was obtained with good quality of fit and validity (R^2 = 0.202) and prediction (Q^2 = 0.529). Figure II.4a shows the scores plot obtained for the three strawberry cultivars, grouped accordingly to the first two components. As it can be observed, the separation between cultivars was satisfactory and they were clearly discriminated. PLS models also provided the possibility of obtaining a quantitative measure of the discriminating power of each variable by means of variable importance for the projection (VIP). This is a weighted sum of squares of the PLS loadings which takes into account the amount of explained Y variance of each PLS component. X-variables, characterized by VIP values which are larger than 1, had major importance for modeling the responses, whereas variables with VIP values smaller than 0.5 had little or no influence on the model. The VIP scores indicated that anthocyanins were the most important parameters for cv. Camarosa, whereas glucose, fructose, total sugars and TS/TA ratio were more relevant for cv. Festival and phenolics acids and flavonols for cv. Palomar.

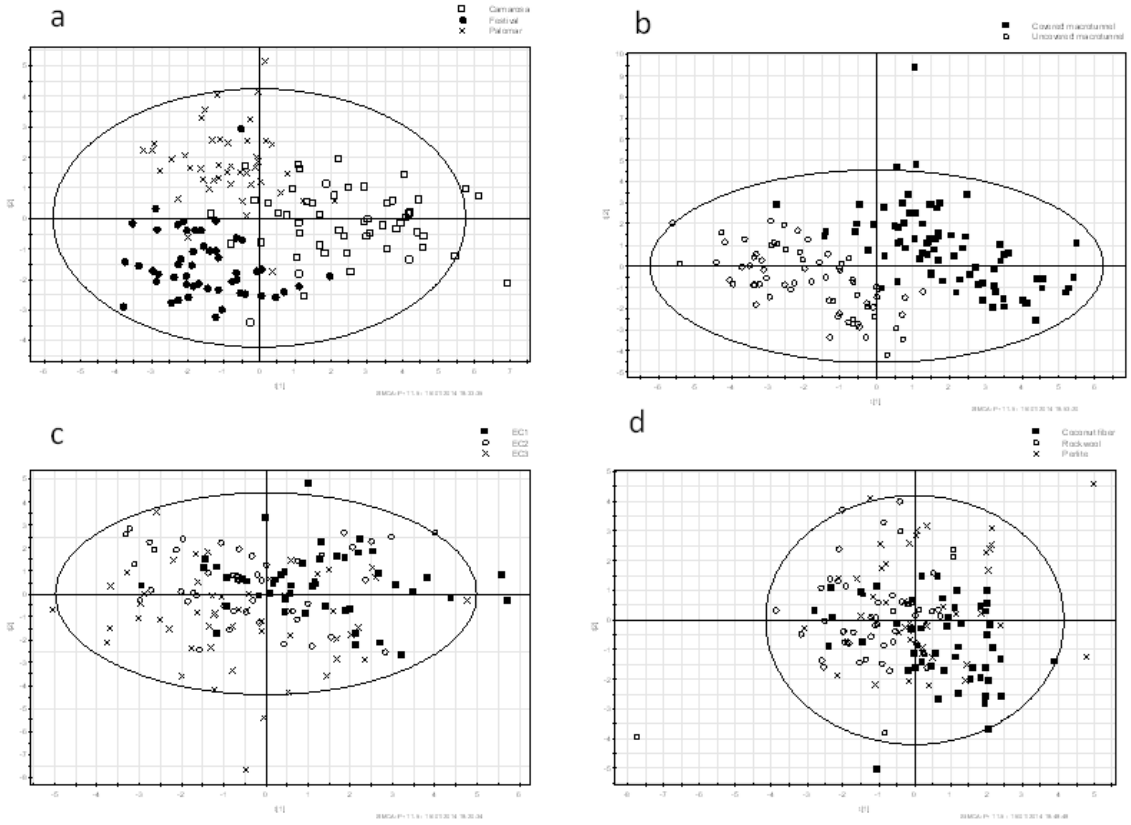

Figure II.4- PLS-DA scores plots based on chemical composition of strawberry samples (n = 162) according to cultivars (a), covered or uncovered macrotunnel (b), electrical conductivity (c), and substrate (d).

A second model was obtained with data from strawberries grown in two macrotunnels, covered and uncovered. This three-component model explained 35.5% of the variance (R^2 = 0.355), and the t1 and t2 components allowed discrimination between two sets of samples (Figure II.4b): strawberries grown in covered macrotunnel (positioned on the right side of the plot) and strawberries grown in uncovered macrotunnel (positioned on the left side of the plot). The variables which contribute the most to the discrimination between samples were anthocyanins, ellagic acid derivative, glucose and fructose contents and total sugars. All these variables had VIP values higher than 1.0. The loading plot showed how the first component (t1) was related to glucose, fructose, total sugars and ellagic acid derivative on the negative side where the uncovered grown samples were situated (data not shown), and with pelargonidin and their derivatives on the positive side where covered grown samples were situated, confirming the results discussed above.

The effect of the other two factors, electrical conductivity and substrate, was also evaluated. In this case, the PLS-DA scores plots showed a poor separation between samples (Figure II.4c, d), resulting in low predictability of

the models as indicated by a Q^2 of 0.0878 and 0.0188, for electrical conductivity and substrate, respectively.

In summary, we have shown that the content of several metabolites related to strawberry quality, including sugars, organic acids, phenolic phenolic compounds and TEAC, depend on cultivar and growing conditions. Festival was the sweetest genotype and also had the highest total sugars/total acids ratio, whereas Camarosa was richest in anthocyanins. Considering the harvest time and after applying pattern recognition techniques such as PCA, it was concluded that strawberry harvested in January and March (early production) had higher quality. Fruits harvested in January were richer in phenolic compounds whereas fruits harvested in March were richer in taste-related compounds. The influence of agronomical conditions was investigated by nested ANOVA and PLS-DA. The type of coverage and the variety were the main source of variation for most of the variables studied mainly phenols and sugars. A good discrimination between three cultivars and macrotunnel type was also achieved by PLS-DA, confirming the results from Nested ANOVA. The VIP scores indicated that anthocyanins were the most important parameters for cv. Camarosa, whereas glucose, fructose, total sugars and TS/TA ratio were more relevant for cv. Festival and phenolics acids and flavonols for cv. Palomar. Strawberries grown in covered macrotunnel were richer in pelargonidin and their derivatives. Uncovered grown samples were richer in glucose, fructose, total sugars, and ellagic acid derivative. Other growing conditions, such as electrical conductivity and substrate did not provide a clear discrimination between samples.

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CAPÍTULO III

GC-MS metabolomic fingerprinting of selected strawberry cultivars with different sensitivity to environmental stress

Abstract

In this paper, an untargeted metabolomic strategy was used to characterize the primary metabolome of different strawberry cultivars and to study metabolite alterations in response to different crop conditions and environmental stress. For this purpose, we analyzed three varieties of strawberries with different sensitivity to environmental factors (Camarosa, Festival and Palomar) grown in soilless systems, and using various agronomic conditions, including electrical conductivity, type of coverage and substrate. Primary metabolites were profiled by gas chromatography mass spectrometry (GC-MS), and then multivariate statistical techniques were applied in order to discriminate strawberry samples according to the cultivar and the growing conditions. In this way, different discriminant compounds could be identified, such as L-alanine, L-norvaline, L-threonine, L-aspartic acid, malic acid, citric acid, D-fructose, D-glucose, glycerol and inositol.

Keywords: Food metabolomics, gas chromatography mass spectrometry, strawberry, quality, cultivars, crop conditions

1. Introduction

Strawberries (Fragaria x ananassa Duch.) are one of the most economically important and widely cultivated fruit crops across the world, with an annual output of about four million tons worldwide, of which 7% is produced in Spain.[1] Strawberries are cultivated in a broad range of climatic zones, although most of the commercial production is limited to areas with seasonal temperate temperatures such as Huelva (southwest Spain), where strawberry is one of the most important crops because of its characteristic climatic conditions, as well as soil and water quality. This region is one of the main strawberry providers of EU, whose main destination is fresh consumption. In this sense, 289.398 tons were produced in the 2013-14 campaign, predominantly early strawberry cultivars to the detriment of the Camarosa variety, mostly cultivated until recent dates.[2] In addition to their economic importance, strawberries are one of the most popular crops and commonly consumed berry fruits due to its pleasant flavor, as well as the high content of health-promoting/beneficial bioactive compounds, including both micronutrients and phytochemicals.[3-4] In particular, strawberries are a very rich source of phenolic compounds, which are well known for their antioxidant, cardioprotective, anti-inflammatory and anti-cancer activities.[5] Besides these beneficial-to-health compounds, strawberry plants contain numerous primary metabolites common to all or most plant species, which play essential roles in plant growth, development and reproduction. These primary metabolites are intermediates and products of central metabolism involved in photosynthesis and other biosynthetic processes, which may belong to different compound families including carbohydrates, organic and amino acids, nucleotides or lipids.[6-8] Sugars are considered as primary photosynthetic products, participating in a wide variety of metabolic pathways such as glycolysis, oxidative pentose phosphate pathway and the tricarboxylic acid cycle, responsible for energy production and synthesis of precursors for obtaining primary and secondary metabolites such as organic acids, amino acids, flavonoids and aroma compounds. Furthermore, all these metabolites also contribute to fruit quality and organoleptic characteristics. On the one hand, sugars and some free amino acids determine the sweetness and taste of the fruit, respectively, while organic acids are involved in the sourness, the regulation of pH value and the stabilization of anthocyanins, thus influencing strawberry color.[9-11] On the other hand, some of these compounds are also involved in the tolerance mechanisms in plants. L-Proline is well known for participating in the plant response to several biotic or abiotic stresses.[12] Inositol and related molecules contribute to plant protection against salt stress,[13] so that the accumulation of inositols and methylated

derivatives, like ononitol and pinitol, has been reported as an indicator of tolerance to salinity, desiccation and extremely high temperatures.[14-16]

Many factors may contribute to this chemical complexity of strawberry, from plant growth conditions (e.g. soil composition and texture, climatic conditions, growth altitude and agricultural practices)[17-19] and post-harvest factors (e.g. storage in controlled atmosphere),[20] to seasonal and genetic factors.[4] Moreover, the synthesis of primary and secondary metabolites can change in response to biotic and abiotic stress.[21,11] Therefore, the characterization of the metabolite profile of strawberries could have multiple applications in food control, such as authentication of variety and origin, as well as the assessment of agricultural and processing practices.[22-24] In this context, metabolomics has become a very important tool in the science of food and agriculture in recent years, providing valuable information on the genotypic and phenotypic biodiversity, biochemical changes associated with vegetative and reproductive cycles during plant growth and fruit ripening, comparison of different species and varieties, as well as the characterization of the response of plants to a wide range of biotic and abiotic stressors and their impact on productivity.[6,25-26] Metabolomics can be defined as the field of science that deals with the comprehensive measurement of low molecular weight metabolites in biological samples. Thus, metabolomic profiling is emerging as a powerful tool for the characterization of complex phenotypes affected by both genetic and environmental factors. Multiple analytical platforms can be used for high-throughput analysis of plant metabolites. Currently, the main analytical techniques used for the analysis of the metabolome are nuclear magnetic resonance spectroscopy (NMR) and hybrid techniques such as gas chromatography and liquid chromatography coupled to mass spectrometry (MS). LC–MS and GC–MS are very sensitive and selective techniques that combined with suitable chemometric methods can be useful tools in metabolomic studies in order to evaluate the relationships between the plant metabolome and their geographical origin, growing treatments, as well as nutritional and nutraceutical quality. In this way, numerous metabolomic studies have been previously performed to understand the biochemical basis of plant physiology and to identify biomarkers related to fruit growth, ripening and/or storage, as well as for studying their geographical origin or to assess food traceability and authenticity.[27] In combination with different multivariate data analysis tools, such as principal components analysis (PCA) and partial least squares discriminant analyses (PLS-DA), NMR has been widely used as a fingerprinting tool for the characterization and quality assessment of natural and industrial products such as tea,[28] grapes and wines,[29] tomato fruits[30] and Arabidopsis leaves.[31] On the other hand, gas chromatography-mass

spectrometry is a robust platform, more suitable for comprehensive metabolomic analysis given that combines high separation efficiency with versatile, selective and sensitive mass detection. For example, a GC–MS approach has been applied for analyzing potato tuber metabolites[32] and following time-dependent metabolic changes in the course of the malting process of barley.[33] Other metabolic profiling studies of fruits have focused on measuring changes associated with the fruit development and maturation. In this sense, Oms-Oliu *et al*.[30] used a metabolomic approach based on GC-MS to characterize compositional changes of tomato during pre-harvest fruit development, ripening and post-harvest shelf-life. Hurtado-Fernández *et al*.[34] investigated the effect of ripening process on the metabolic profiling of[13] avocado varieties by untargeted GC-APCI-TOF-MS combined with different statistical tools. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were employed by Zhang *et al*.[6] to explore the non-polar and polar metabolite profiles from strawberry fruit samples at seven developmental stages. GC-MS based metabolic profiling has also been used to differentiate citrus varieties with different sensitivity to citrus huanglongbing, also known as the citrus greening disease,[21] and to characterize metabolic changes in plants after biotic and abiotic stresses.[26]

The aim of this study was to obtain a metabolic characterization of three varieties of strawberries (Camarosa, Festival and Palomar) grown in soilless systems, characterized by their different sensitivity to environmental conditions. To highlight these differences, gas chromatography coupled to mass spectrometry technique combined with suitable chemometric methods such as principal component analysis (PCA) and partial least-squares-discriminant analysis (PLS-DA) was applied. This approach was also used to assess the influence of agronomic conditions (electrical conductivity, coverage and substrates) on primary metabolites profile of each of the three cultivars.

2. Material and methods

2. 2. Reagents and samples

Methanol and pyridine (HPLC grade), as well as reagents for derivatization N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) and methoxylamine hydrochloride, were purchased from Sigma-Aldrich (Steinheim, Germany). Standards of fructose, glucose, malic acid, citric acid, oxalic acid, tartaric acid and ascorbic acid were obtained from Merck (Darmstadt, Germany), sucrose from Panreac (Barcelona, Spain), and ribitol (internal standard) and the amino acids lysine, tyrosine, threonine, serine, alanine, aspartic acid, proline and valine were purchased from Sigma-Aldrich

(Steinheim, Germany). Water was purified with a Milli-Q Gradient system (Millipore, Watford, UK).

2. 2. Plant material, growth conditions and sampling

The study was conducted in experimental plantations managed by the University of Huelva, in southwest Spain (latitude 37°14'N, longitude 6°53'W, and altitude 23 m) where strawberry plants (Fragaria x ananassa Duch.) were grown in soilless systems. The assay is a replicate (second year) of a recently published study on strawberry quality in relation to harvest time and crops conditions,[4] so detailed description of samples can be found in this paper. Briefly, the experimental design consisted of two macrotunnels (covered and uncovered) each containing three breeding lines of plants grown under different conductivities (EC = 1, 2 and 3 dS / m). Each line of breeding plants was composed of three strawberry cultivars (Palomar, Festival and Camarosa). Finally, each cultivar was grown in three different commercial substrates (coconut fiber, perlite and rockwool). The three varieties were chosen basing on their sensitivity to environmental conditions: Palomar (PAL, very sensitive), Festival (FES, sensitive) and Camarosa (CAM, resistant).

Immediately after harvesting, fruits were sorted, frozen in situ in a deep freezer and shipped to our laboratory in polystyrene punnets. Fruits were washed, sepals were dissected and finally, fruits were gently homogenized by means of a kitchen mixer. The pastes obtained were subsequently stored at -21 °C for further analysis.

2. 3. Sample preparation

For the extraction of metabolites, homogenized fruits (0.5 g) were mixed with 10 mL of methanol/water (1:1, v/v), and then the mixture was vortexed for 10 minutes at room temperature and centrifuged at 10000 rpm for 10 min at 4 ^{o}C. Then, 20 µl of the extract were mixed with 30 µL of ribitol (0.2 mg mL^{-1} in water), used as internal standard, and dried under nitrogen stream.[6] Later, derivatization was carried out according to the two step methodology proposed by Begley *et al*.[35] For protection of carbonyl groups by methoxymation, dried extracts were redissolved in 50 µL of 20 mg mL^{-1} methoxyamine in pyridine, and after briefly vortexing were incubated at 80°C for 15 min in a water bath. Then, silylation was performed by adding 50 µL of MSTFA and incubating again at 80 °C for 15 min. Finally, derivatized samples were equilibrated to room temperature and centrifuged at 4000 rpm for 5 minutes before injection. Three analytical replicates were prepared for each sample.

2. 4. GC-MS analysis

Chromatographic analysis was performed following the described procedure by Zhang *et al*.[6] with a few modifications. The analysis was carried out in a Varian CP 3800 gas chromatograph coupled to an ion trap mass spectrometer detector Saturn 2200, using a Factor Four fused-silica capillary column (VF-5MS 30m×0.25mm i.d., 0.25 μm film thickness stationary phase, Varian). The GC was programmed at an initial temperature of 100 ^{o}C for 2 min; increased to 140 ^{o}C at 5 ^{o}C min^{-1} for 3 min; increased to 180 ^{o}C at 5 ^{o}C min^{-1} for 6 min; increased to 320 ^{o}C at 5 ^{o}C min^{-1}; and finally held for 5 min. The injector temperature was kept at 250 ^{o}C, and samples were injected in splitless mode (1 μL), using helium as carrier gas at a constant flow rate of 1 mL min^{-1}. The significant MS operating parameters were as follows: ion source temperature was set at 200 ^{o}C, ionization was carried out by electronic impact using a voltage of 70 eV. Data were obtained acquiring full scan spectra in the m/z range 40-600.

2. 5. Data processing

Prior to statistical analysis, the acquired analytical data needs to be processed in order to assign the equal identity to the same variable in each sample. For this, raw data was processed following the pipeline described by Katajamaa and Oresic,[36] which proceeds through multiple stages including feature detection, alignment of peaks and normalization. For this purpose, we employed the freely available software XCMS, included in the R platform (http://www.r-project.org). GC-MS files were converted into netCDF using the Varian SMS Import Converter tool (OpenChrom) and subsequently, data were extracted using the matchedFilter method. This algorithm slices data into extracted ion chromatograms (XIC) on a fixed step size (default 0.1 m/z), and then each slice is filtered with matched filtration using a second-derivative Gaussian as the model peak shape. The XCMS parameters were optimized according to the characteristics of data sets obtained in order to extract the maximum information as possible, as previously described by González-Domínguez *et al*.[37] Settings applied for GC-MS data were S/N threshold^{2} and full width at half-maximum (fwhm)^{3}. After peak extraction, grouping and retention time correction of peaks (alignment) was accomplished in three iterative cycles with descending bandwidth (bw) from 5 to 1 seconds. Then, imputation of missing values was performed by returning to the raw spectral data and integrating the areas of the missing peaks which are below the applied signal-to-noise ratio threshold, using the fillPeaks algorithm. For data normalization, the locally weighted scatter plot smoothing (LOESS) normalization method was used, which adjusts the local median of log fold changes of peak intensities between samples in the data set to be approximately

zero across the whole peak intensity range.[38] Finally, data were submitted to logarithmic transformation, in order to stabilize the variance of results. The preprocessed data were then exported as a .csv file for further data analysis by multivariate procedures.

2. 6. Data analysis

Data were subjected to multivariate analysis by principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) in order to compare metabolomic profiles obtained, using the SIMCA-P™ software (version 11.5, UMetrics AB, Umeå, Sweden). Before performing statistical analysis, data are usually scaled and transformed in order to minimize the technical variability between individual samples to extract the relevant biological information from these data sets.[39] For this, data was submitted to Pareto scaling (the squared root of standard deviation is used as the scaling factor) for reducing the relative importance of larger values, and logarithmic transformation in order to approximate a normal distribution. The Hotelling T2 region defines the 95% confidence interval of the modeled variation and it is displayed as an ellipse in score plots of the models. Quality of the models was assessed by the R^2 and Q^2 values, which provide information about the class separation and predictive power of the model, respectively. These parameters are ranged between 0 and 1, and they indicate the variance explained by the model for all the data analyzed (R^2) and this variance in a test set by cross-validation (Q^2). Finally, the most influential variables were selected according to the Variable Importance in the Projection, or VIP (a weighted sum of squares of the PLS weight, which indicates the importance of the variable in the model), considering only variables with VIP values higher than 1.5, indicative of significant differences among groups. In addition, group comparison was conducted by one-way analysis of variance and non-parametric test using the STATISTICA 8.0 software (StatSoft, Tulsa, USA). Only p-values below 0.05 were regarded as statistically significant.

2. 7. Identification of potential biomarkers

Discriminant metabolites detected by GC-MS were tentatively identified using the NIST Mass Spectral Library, considering only those variables with a similarity index (SI) greater than 70%. Then, the identity of these potential markers was confirmed by injecting commercial standards.

3. Results and discussion

3. 1. Metabolomic differentiation of strawberry cultivars

The metabolomic approach based on GC-MS profiling previously described was applied to strawberry samples from three cultivars grown in soilless systems in order to determine differences in primary metabolites. After raw data processing, the data matrix consisting of 54 rows (samples) and 254 columns (variables with the format of intensity at different m/z ratio and retention time) was Pareto scaled, which led to most adequate model, and then was subjected to chemometric analysis.

As a first exploratory step, principal component analysis (PCA) was applied for a preliminary evaluation of the quality of data before the application of any supervised statistical analysis. This unsupervised method allows to get an overview of the data, and to identify possible outliers and trends towards the grouping of samples. When PCA was applied to the data,[40] latent variables (principal components) were obtained with eigenvalues higher than 1 (Kaiser criterion). However, the ANOVA test showed that only the first three components were significant ($p<0.001$), which explained 24.9, 17.3 and 6.3% of the original variance, respectively. Figure III.1 shows the distribution of the samples in the space defined by the first three components, where a clear distinction among the strawberry cultivars can be observed suggesting metabolic differences among these three varieties. Moreover, an interesting feature can be pointed out: the three cultivars are arranged along the direction of PC1, in a way that reflects their sensitivity to environmental stress, that is, samples of cv. Camarosa (resistant) have higher scores than samples of cv. Festival (sensitive) and cv. Palomar (very sensitive), these latter having the lowest scores on PC1. Moreover, Festival and Palomar samples can be clearly separated by PC2, with negative and positive PC2 scores, respectively.

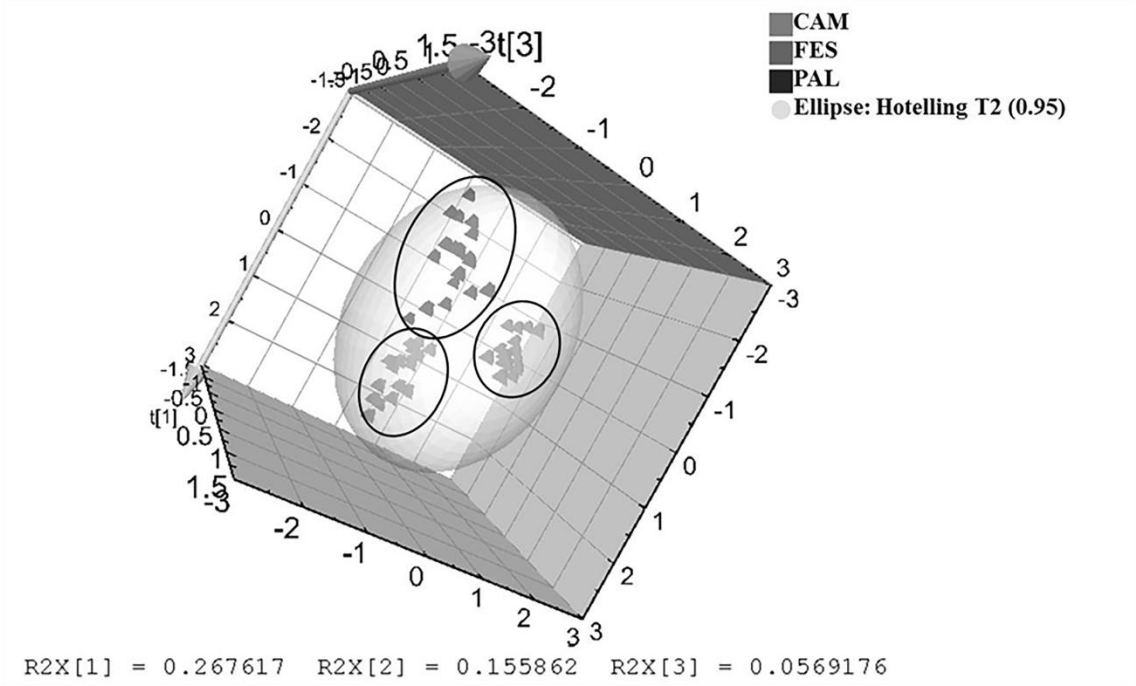

Figure III.1- Tri-dimensional scores plot of the PCA model for all samples. Camarosa: light grey, Festival: dark grey, Palomar: black.

Taking into account these results, partial least squares discriminant analysis (PLS-DA) was applied in order to obtain adequate classification models. PLS-DA is a supervised method that explains the maximum variation in the dataset (X) using a dummy matrix (Y), which can be used to sharpen the separation between groups or observations. To understand the interclass separation and identify potential characteristic markers for each cultivar, three binary models were developed. The first two models compare cv. Camarosa with the other two cultivars, Festival and Palomar. In both cases, satisfactory values for the quality parameters R^2 and Q^2 were obtained, with a variance explained above 95% and variance predicted around 80%. A third model was developed with data from Festival and Palomar strawberries. In this two-component model, the R^2 and Q^2 values obtained by 7-fold cross validation were 0.95 and 0.739, respectively.

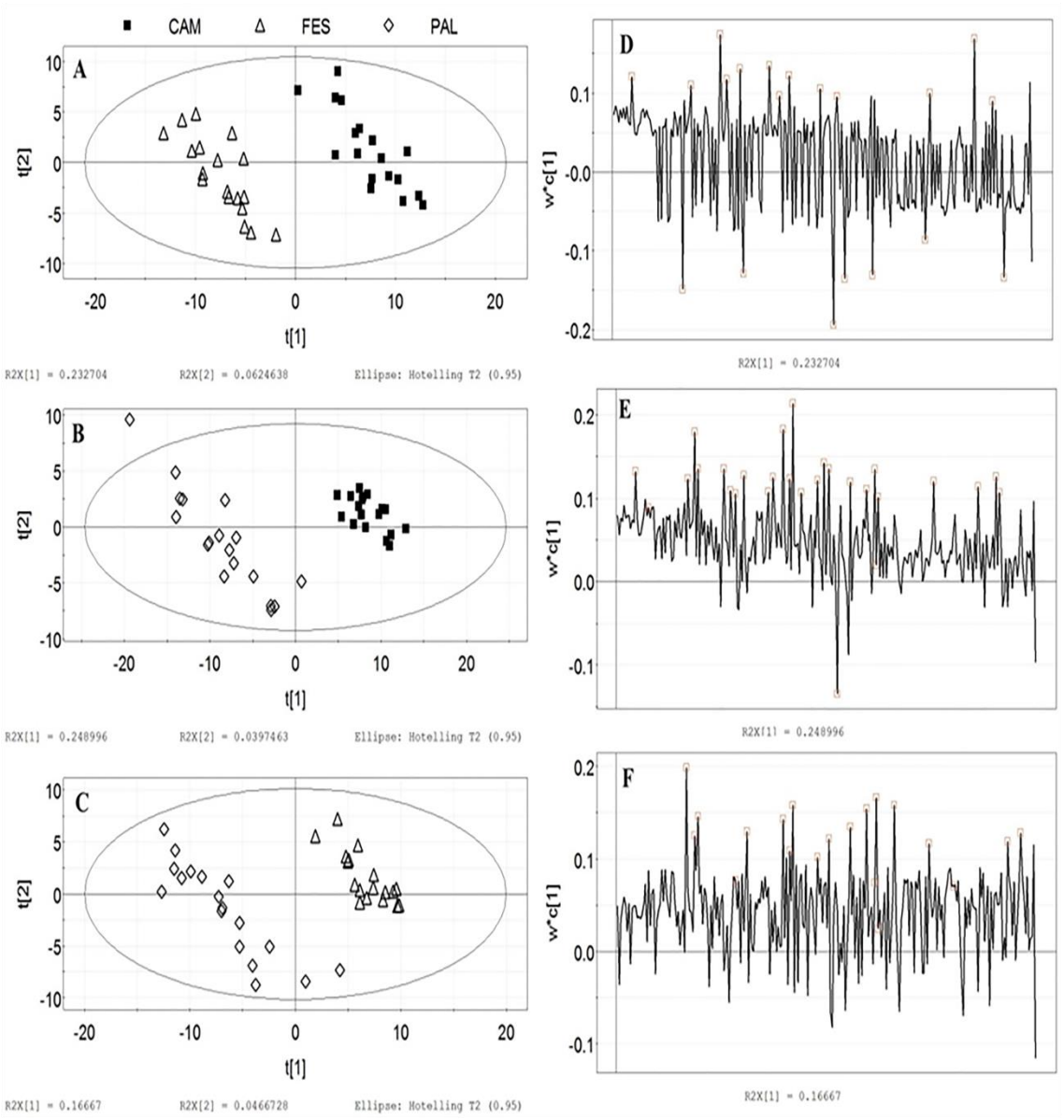

Figure III.2- Scores and loadings plots of PLS-DA models for two-class comparisons. (A) Scores plot for the comparison Festival vs. Camarosa, (B) scores plot for the comparison Palomar vs. Camarosa, (C) scores plot for the comparison Palomar vs. Festival, (D) loadings plot for the comparison Festival vs. Camarosa, (E) loadings plot for the comparison Palomar vs. Camarosa, (F) loadings plot for the comparison Palomar vs. Festival.

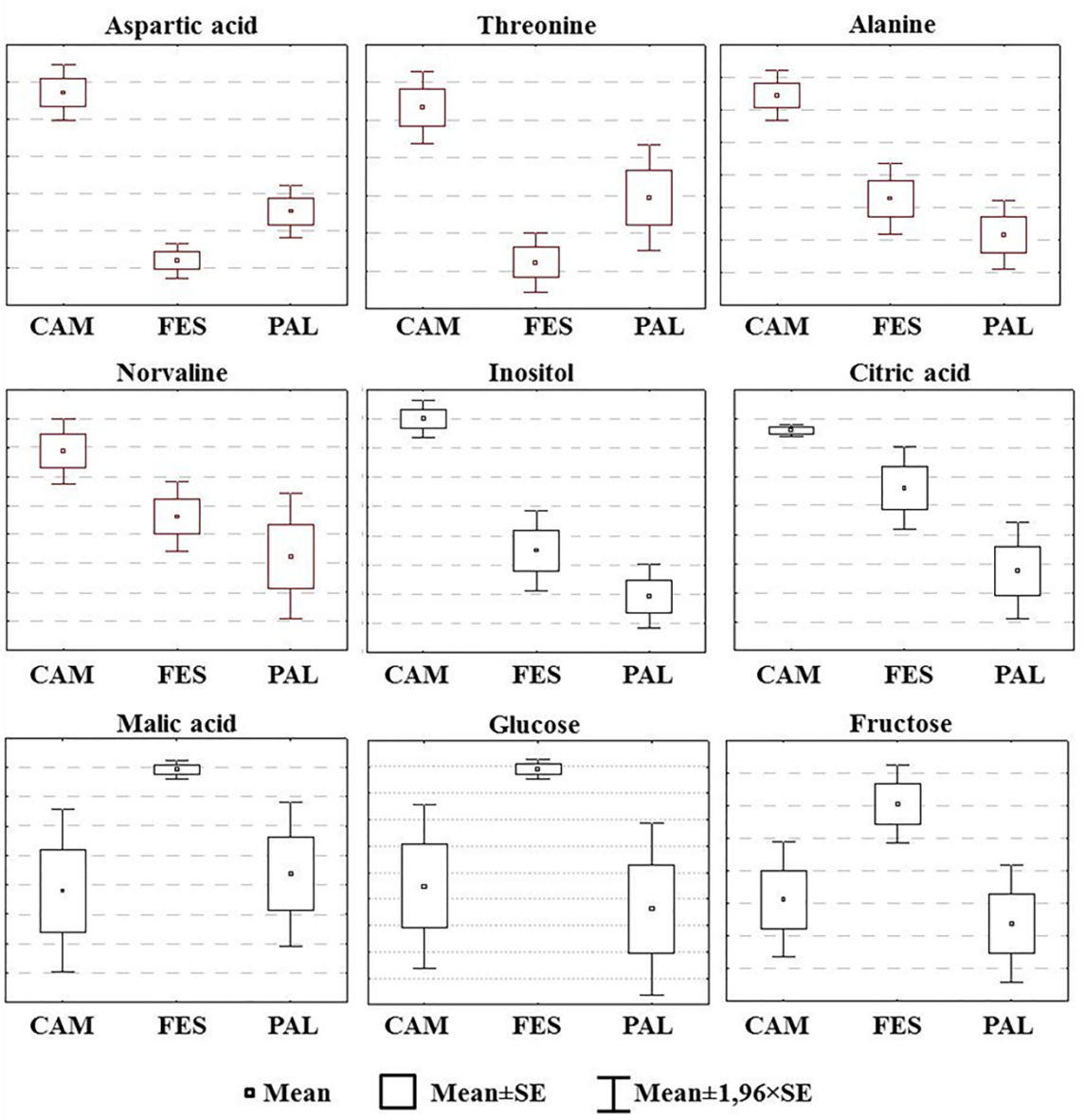

Figure III.3- Box plots with whiskers for discriminant metabolites identified by GC-MS profiling for differentiation of strawberry cultivars.

Figure III.2 (a-c) shows the scores plots obtained for the three strawberry cultivars, grouped accordingly to the first two components. As it can be observed, the separation between cultivars was satisfactory in all cases and they were clearly discriminated. On the other hand, the evaluation of PLS-DA loadings plots (Figure III.2 d-f) allows a good understanding of the variables that contribute to the discrimination of classes. Furthermore, PLS models also provide the possibility of obtaining a quantitative measure of the discriminating power of each variable by means of the variable importance for the projection (VIP) parameter. Only variables with VIP values higher than 1.5 were selected

and used to identify metabolites that contribute to the separation between cultivars. Thereby, the examination of these loadings line plots demonstrated that metabolites such as inositol, citric acid, alanine, norvaline, threonine and aspartic acid were increased in strawberries of Camarosa cultivar compared to those of Festival and Palomar cultivars (Figure III.2d and e). The loadings plot of Camarosa and Festival cultivars (Figure III.2d) also showed that Camarosa strawberries contain less sugars (glucose and fructose) and malic acid that Festival variety. On the other hand, the loadings plot displayed in Figure III.2f showed relatively high levels of glucose, fructose and malic acid in strawberries of Festival cultivar compared to those of Palomar. Finally, these metabolites were validated by using non parametric univariate analysis methods such as the Mann-Whitney U test ($p \leq 0.05$), given that most of the variables showed a skewed distribution (checked by normal probability plots) and variances were not homogeneous (checked by Levene's test). Discriminant metabolites identified in this study are listed in Table III.1, along with the experimental retention time, the percentage of change and the p-value for each comparison (Festival vs. Camarosa, Palomar vs. Camarosa, Palomar vs. Festival). Furthermore, box-plots representing the mean values with standard deviation intervals for these selected metabolites are shown in Figure III.3 in order to highlight metabolomic differences found between the three cultivars. Although statistically significant, these metabolic changes between cultivars were not very pronounced, especially for amino acids, probably due to the controlled conditions in which strawberries were cultivated. In this sense, it is noteworthy that fold changes calculated in this study were very similar to those provided in previous works focused on the biochemical differentiation of strawberry varieties.[4,40]

Table III.1- Discriminant metabolites identified by GC-MS profiling for differentiation of strawberry cultivars.

Metabolite	RT	FES *vs.* CAM		PAL *vs.* CAM		PAL *vs.* FES	
	(min)	fold change	p value	fold change	p value	fold change	p value
Alanine	4.05	-1%	0,000350	-2%	0,000004	-1%	0,173685
Norvaline	5.50	-1%	0,017650	-2%	0,002387	-1%	0,775838
Glycerol	5.95	+2%	0,026781	+1%	0,466805	-1%	0,358871
Threonine	8.60	-2%	0,000002	-1%	0,003988	+1%	0,076435
Malic acid	12.21	+38%	0,000497	+5%	0,703175	-24%	0,000273
Aspartic acid	13.15	-4%	0,000001	-3%	0,000011	+1%	0,004865
Fructose	20.65	+71%	0,000988	-18%	0,773753	-52%	0,003909
Glucose	21.03	+42%	0,017497	-8%	0,630111	-36%	0,003531
Citric acid	21.51	-26%	0,002134	-64%	0,000008	-51%	0,012543
Myoinositol	24.90	-13%	0,000005	-18%	0,000000	-5%	0,121073

3. 2. Interpretation of metabolic alterations between cultivars

Metabolomic analysis by GC-MS revealed significant alterations in primary metabolites between the three strawberry cultivars studied in this work, including sugars (fructose, glucose), organic acids (malic acid, citric acid) and amino acids (alanine, threonine, aspartic acid), among others. These metabolites are important compounds for normal plant growth and development because they participate in a wide range of physiological reactions, as well in the resistance of plants to biotic and abiotic environmental factors. Among them, sugars and organic acids are the most interesting metabolites in strawberry, because they determine the organoleptic quality of fruit. In most plants, sucrose is the principal carbohydrate synthesized during photosynthesis in leaves, which is then transported to the fruit via phloem. Then, sucrose can be hydrolyzed in the cytosol through enzymatic reactions or transported to the vacuole for storage. Thereby, the most common sugars found in strawberry fruits are glucose and fructose,[4,9,19] products from enzymatic breakdown of sucrose. They are responsible for providing energy in form of ATP via glycolysis, and serve as precursors for the synthesis of organic acids and amino acids. The two main organic acids in strawberry are citric acid and

malic acid, which are mainly produced in mitochondria through the tricarboxylic acid (TCA) cycle, although malic acid can also be synthesized by carboxylation of phosphoenolpyruvate. In turn, these TCA intermediates can serve as precursors for the biosynthesis of amino acids.

In this work, GC-MS profiles evidenced that cv. Camarosa, the most resistant cultivar, presents significant higher levels of citric acid compared with more sensitive strawberries, which could suggest the up-regulation of the TCA cycle in cultivars adapted or tolerant to environmental stress. Moreover, levels of sugars (fructose and glucose) and malic acid were also higher in cv. Festival, indicative of a different energy metabolism in this cultivar. On the other hand, it was been observed that several amino acids also play an important role on discrimination between cultivars, mainly those derived from oxalo acetic and pyruvic acid, such as threonine, aspartic acid and alanine. In this sense, many researchers have previously described that increased levels of amino acids can be related to tolerance mechanisms in plants in response to abiotic stress, generally attributed to osmotic adjustment leading to water retention and/or protection of biochemical pathways.[15] Plants are exposed to several abiotic factors under field conditions, such as drought, salinity, low temperatures or heavy metals, which affect plant growth and yield. Salt, drought and heavy metals exert their negative impact by disrupting the ionic and osmotic equilibrium of the cell, whereas cold temperatures cause mechanical constraint to the membrane. Finally, these adverse environmental conditions can affect to water availability, thus depleting plant transpiration. In order to avoid this water loss, plants can develop several physiological and metabolic adjustments, including a decrease of photosynthesis rate, alterations in the translocation and distribution of photo as similates, as well as the accumulation of osmotically active metabolites such amino acids, quaternary amines, soluble sugars, polyols, or polyamines, among others. In general, these compounds are naturally occurring plant constituents that contribute to osmotic regulation. Among these metabolites, free amino acids such as L-proline are well known for participating in osmotic adjustment[10-12], while other amino acids such as glutamine and asparagine have also been reported to accumulate in plants in response to salt stress.[10] Cevallos-Cevallos *et al*.[21] found an altered content of amino acids in several citrus varieties with different sensitivity to citrus huanglongbing. So, higher levels of the amino acids L-proline, L-serine, and L-aspartic acid, were characteristic of the most sensitive variety, Madam vinous sweet orange, while tolerant varieties showed higher levels of L-glycine. In the present study, higher levels of alanine, norvaline, threonine and aspartic acid were found in the most resistant cultivar (Camarosa) compared to sensitive cultivars Festival and Palomar. Another interesting metabolic alteration found

in this work was the significant increase of inositol, a well-known osmolyte, in cv. Camarosa. Inositol is an essential component in the biosynthesis of an array of derivatives ranging from simple inositol phosphates to complex membrane-associated products with important cellular functions. This compound and its derivatives are implicated in membrane biogenesis, phosphate storage, cell signaling, salinity stress tolerance and storage and transport of plant hormones.[13-14] In this context, a number of these compounds have been recognized as osmoprotectant metabolites, which accumulate in osmotically challenged plants. Thereby, the accumulation of inositols and their O-methyl ethers in response to salinity has been studied extensively in the ice plant Mesmbryanthemum crystallinum[15] and different crop species[14]. It has been demonstrated that the protective function of inositol and related molecules in relation to salt tolerance occurs in two major ways: (1) protecting cellular structures from reactive oxygen species, such as hydrogen peroxide, and (2) controlling water pressure inside cells. Under salt stress conditions, the ice plant undergoes shock and wilts for a short period of time, followed by the production and accumulation of inositol, D-ononitol and D-pinitol. After this increase of osmolytic molecules, water pressure is restored and inositol is detected in the phloem.[15] Therefore, the accumulation of these osmolytes could therefore reflect the adaptation to environmental stress.

Table III.2-Statistical parameters of PLS-DA models for classification of strawberries according to growing conditions (type of coverage, electrical conductivity and substrate).

Cultivar	Factor	R^2_X	R^2_Y	Q^2
Camarosa	Coverage	0.225	0.91	0.19
	EC	0.232	0.688	-0.13
	Substrate	0.218	0.716	-0.21
Festival	Coverage	0.228	0.943	0.18
	EC	0.16	0.727	-0.21
	Substrate	0.166	0.796	-0.21
Palomar	Coverage	0.149	0.982	0.095
	EC	0.183	0.802	0.021
	Substrate	0.168	0.836	-0.038

3. 3. Metabolomic changes associated with environmental and agronomic conditions

PLS-DA was also applied to previous GC-MS data sets in order to evaluate metabolic alterations associated with growing conditions of strawberries, i.e. macrotunnel type, electrical conductivity and substrate. For this purpose, three statistical models were constructed for each cultivar, and the performance of these models was evaluated through the parameters R^2 and Q^2, listed in Table III.2. Furthermore discriminant metabolites, selected following the pipeline previously described, are shown in Table III.3 along with the experimental retention time, the percentage of change and the p-value for each comparison (covered vs. uncovered, EC3 vs. EC2 vs. EC1, perlite vs. coconut fiber, rockwool vs. coconut fiber, perlite vs. rockwool).

Table III.3- Discriminant metabolites identified by GC-MS profiling associated with different growing conditions of strawberries cultivated in soilless systems.

Metabolite	RT (min)	Fold change (p value)								
		Coverage			EC			Substrate		
		Camarosa	Festival	Palomar	Camarosa	Festival	Palomar	Camarosa	Festival	Palomar
Alanine	4.05	N.S.	N.S.	N.S.	-4.4% (0.042640)	-1.8% (0.003730)	-1.3% (0.022396)	N.S.	N.S.	N.S.
Norvaline	5.50	N.S.	-1.5% (0.015384)	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Glycerol	5.95	N.S.	N.S.	N.S.	N.S.	N.S.	+5% (0.044061)	N.S.	N.S.	+6.4%*** (0.013077)
Threonine	8.60	+1.3% (0,013577)	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	+1.2%** (0.047610)	N.S.
Malic acid	12.21	N.S.	N.S.	N.S.	N.S.	N.S.	-4% (0.036234)	N.S.	N.S.	N.S.
Aspartic acid	13.15	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	+1.2%** (0.017621)	N.S.
Fructose	20.65	-73% (0.003836)	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	-68%** (0.017965)
Glucose	21.03	N.S.	N.S.	N.S.	N.S.	N.S.	-14% (0.017446)	N.S.	-10%** (0.002230)	-2%** (0.023159)
Citric acid	21.51	N.S.	N.S.	N.S.	+6.3% (0.012958)	-64% (0.039544)	-67% (0.019861)	-5.3%* (0.038386)	-65%* (0.003400)	-5%* (0.027005)
Myoinositol	24.90	N.S.	N.S.	N.S.	+6.3% (0.006085)	N.S.	N.S.	N.S.	N.S.	N.S.

*perlite vs. coconut fiber; **rockwool vs. coconut fiber; ***perlite vs. rockwool; N.S. non significant.

In relation to the macrotunnel type (i.e. covered and uncovered), PLS-DA models obtained for each cultivar explained 22.5, 22.8 and 14.9% of the variance for Camarosa, Festival and Palomar, respectively. Furthermore, the t1 and t2 components allowed a clear discrimination between the two sets of samples on scores plots (figures not shown in the text): strawberries grown in covered macrotunnels were positioned on the right side of the plot, while strawberries grown in uncovered macrotunnels were located on the left one, regardless of the variety. The VIP scores indicated that sugars (fructose and glucose), organic acids (citric and malic), glycerol, threonine and norvaline were the most important parameters for discrimination between covered and uncovered samples. For the three cultivars, the loadings plot showed that citric acid was higher in strawberries from uncovered macrotunel and malic acid in covered macrotunel. For cv. Camarosa, higher content of fructose and lower content of threonine were found in strawberries grown in the uncovered macrotunel

compared to those grown in covered macrotunel. On the other hand, norvaline levels were higher in the uncovered macrotunel for cv. Festival, while no significant metabolites were found for cv. Palomar. These alterations in primary metabolites between strawberries grown in the two macrotunel types could be explained by differences in climatic conditions, such as light, temperature and rainfall, in addition to the differences between cultivars. In covered macrotunel, where strawberries are grown under controlled conditions, light intensity is reduced by plastic covers; temperature is higher under plastic than in the outside, mainly in coldest months, and moisture is controlled by regular irrigation. In these conditions strawberries were richer in malic acid, in agreement with our previous study findings[4] and with results reported by other authors about the relation between environmental factors and grape berry metabolome.[41-42]They found that malic acid content can be related to light exposure, so that its production is increased under low sun exposure and humid climate. On the other hand, Son *et al*.[41] observed that amino acid profile was also characterized by climatic conditions. Thus, grapes with low sun exposure contained more alanine, arginine, leucine and GABA but less proline, while grapes with more sun exposure contained less leucine, theronine, alanine and arginine but more proline, in line with our results. Finally, it is also noteworthy that warmer and drier climatic conditions can be responsible for the increase of sugars synthesis.[42]

Considering electrical conductivity as the categorical factor, the PLS-DA scores plots showed a poor separation between samples, resulting in low predictability of the models as indicated by Q^2 values of -0.13, -0.21 and 0.021, for Camarosa, Festival and Palomar, respectively, as it is shown in Table III.2. The most discriminant variables between samples grown under different conductivities were citric acid, alanine and inositol for cv. Camarosa, citric acid and alanine for cv. Festival and citric acid, malic acid, glucose, glycerol and alanine for cv. Palomar. For the three cultivars, a decrease of citric acid content can be observed when the conductivity increases from EC1 to EC2. However, if conductivity is increased to EC3, citric acid only suffers a slightly increase for cv. Camarosa, while it is significantly decreased (approximately 65%) for cvs. Festival and Palomar. Furthermore, it also is noteworthy that an increase in the electrical conductivity leads to decreased levels of alanine in the three cultivars investigated, increased inositol in the cv. Camarosa, as well as increased glycerol and decreased malic acid in cv. Palomar, while glucose stayed almost constant. These results are in agreement with those reported by Keutgen and Pawelzik.[10-11] These authors found that the content of essential free amino acids, mainly threonine, rise significantly in cv. Korona (less salt-sensitive) at the highest salt level, remaining fairly stable in cv. Elsanta (more salt-sensitive).

Other amino acids such as alanine, glycine, isoleucine and valine can also vary between cultivars in response to salinity. In cv. Elsanta, a significant decrease in concentrations of these amino acids was observed at the highest salt level.[10] On the other hand, these authors also noticed that cultivars exposed to different salinities differed in their contents of soluble sugars and organic acid, in line with our findings. In cv. Korona, a decrease of fructose and sucrose content stress was observed due to salt, while levels of glucose and total organic acid remained fairly constant. In the more sensitive cv. Elsanta, a significantly increased of glucose, fructose and citric acid was observed at the highest salt level.[11] Finally, our results also showed higher levels of inositol and glycerol when salinity is increased in cv. Camarosa and Palomar, respectively, indicative of osmotic stress.

To conclude, strawberry samples were also modelled according the substrate employed for growing (i.e. coconut fiber, perlite and rockwool). These two-component models explained 21.8, 16.6 and 16.8% of the variance for Camarosa, Festival and Palomar, respectively, but the PLS-DA scores plots showed a poor separation between substrates (figures not shown in the text), resulting in low predictability as indicated by low values of Q^2 (Table III.2). In this case, significant metabolites for discrimination between groups were citric acid for cv. Camarosa; citric acid, glucose, threonine and aspartic acid for cv. Festival; and citric acid, fructose, glucose and glycerol for cv. Palomar, in agreement with our previous results[17]. The higher citric acid proportion was observed in strawberries grown in coconut fiber, regardless of the cultivar. For Festival variety, strawberries grown in coconut fiber were also richer in glucose, while strawberries cultivated in perlite were richer in threonine and aspartic acid. Finally, Palomar strawberries grown in coconut fiber presented increased levels of glucose and fructose, while strawberries grown in perlite were richer in glycerol. In this context, it should be emphasized that differences in strawberry quality as a function of the soilless cultivation system or the substrate type have been studied in depth, and it could be concluded that the coconut fiber is the best substrate for the production of strawberry in soilless culture.

4. Abbreviations

CAM: Camarosa; cv.: cultivar; EC: electrical conductivity; FES: Festival; GC: gas chromatography; MS: mass spectrometry; PAL: Palomar; PC: principal component; PCA: principal component analysis, PLS-DA: partial least squares discriminant analysis; VIP: variable importance in the projection.

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CONCLUSIONES GENERALES

Conclusiones generales

Se ha llevado a cabo un estudio de las características de calidad de fresas cultivadas en sistemas sin suelo, evaluando la influencia de distintas condiciones de cultivo y ambientales sobre las propiedades organolépticas, nutricionales y nutracéuticas. De los datos experimentales obtenidos y de su interpretación se pueden deducir las siguientes conclusiones:

I.1. La comparación de diferentes variedades de fresas cultivadas en sistemas sin suelo y convencionalmente en suelo, revelan diferencias significativas en la calidad organoléptica y nutricional, principalmente en la dulzura. Tamar y Camarosa fueron las variedades más dulces entre las variedades cultivadas en sistema sin suelo y sistema convencional, respectivamente. Ambas variedades también mostraron mayor valor para la relación azúcares totales/ácidos totales. Por lo tanto, estas dos variedades fueron aparentemente más palatables y equilibradas.

I.2. Las variedades de fresas estudiadas también mostraron diferencias en su contenido mineral. En las fresas cultivadas en sistemas sin suelo, el contenido de N y Sr fueron las variables significativamente diferentes. En cambio, las fresas analizadas no mostraron diferencias en relación a su acidez en ninguno de los sistemas de cultivo.

I.3. La aplicación de técnicas estadísticas multivariantes tales como PLS-DA a cada sistema de cultivo, permitió observar una buena discriminación entre variedades cultivadas convencionalmente en suelo, siendo el pH y el contenido en glucosa, fructosa, ácido succínico, Ca, Mg, Ba, Fe, Al y Cu las variables que contribuyeron a la discriminación entre las variedades. Por otra parte, la separación entre cultivares procedentes de sistemas sin suelo no fue tan clara.

I.4. Se ha estudiado el efecto del sistema de cultivo sobre los parámetros nutricionales de las variedades Camarosa y Candonga, siendo la influencia del tipo de cultivo más significativa en la variedad Candonga. La variedad Camarosa cultivada en sistema convencional mostró mayor valor de acidez y de N, pero los contenidos en Ca, Ba, Na y K fueron prácticamente los mismos para ambos sistemas. Por otro lado, la concentración de magnesio y el valor de pH fueron más altos en el sistema convencional independientemente de la variedad. La técnica de PLS-DA también ha permitido diferenciar las muestras según el tipo de cultivo.

II.1. El contenido de diversos metabolitos relacionados con la calidad de la fresa, incluyendo azúcares, ácidos orgánicos, compuestos fenólicos y actividad antioxidante depende de la variedad y de las condiciones de cultivo. La variedad Festival fue, de las tres variedades estudiadas, la más dulce y la que presentaba una mayor relación azúcares totales/ácidos totales, mientras que Camarosa fue más rica en antocianos.

II.2. Considerando el tiempo de cosecha y aplicando técnicas de reconocimiento de pautas como ACP, podemos concluir que las fresas cosechadas en enero y marzo (producción temprana) fueron de mayor calidad. Las primeras fueron más ricas en compuestos fenólicos y las segundas más ricas en compuestos relacionados con el sabor.

II.3. La influencia de las condiciones agronómicas fue investigada por ANOVA anidado y PLS-DA. El tipo de cobertura y la variedad fueron la principal fuente de variación para la mayoría de las variables estudiadas principalmente fenoles y azúcares. La aplicación del PLS-DA mostró una buena discriminación entre los tres cultivares y el tipo macrotúnel. Los valores VIP pusieron de manifiesto que las antocianinas fueron los parámetros más importantes para cv Camarosa, mientras glucosa, fructosa, azúcares totales y relación TS / TA fueron más relevantes para cv Festival y ácidos fenólicos y flavonoles para cv Palomar. Las fresas cultivadas en macrotúnel cubierto fueron más ricas en pelargonidina y sus derivados y las cultivadas en macrotúnel descubierto fueron más ricas en glucosa, fructosa, azúcares totales y derivado del ácido elágico. Otras condiciones de cultivo, tales como la conductividad eléctrica y el tipo de sustrato no proporcionaron una clara discriminación entre muestras.

III.1. El estudio metabolómico llevado a cabo en tres variedades de fresas ha revelado alteraciones significativas en los metabolitos primarios, incluyendo azúcares (fructosa, glucosa), ácidos orgánicos (ácido málico, ácido cítrico) y aminoácidos (alanina, treonina, ácido aspártico), entre otros.

III.2. Los perfiles metabolómicos obtenidos por GC-MS evidenciaron que la variedad Camarosa (más resistente), presentó niveles significativos de ácido cítrico en comparación con las variedades Festival y Palomar(más sensibles) lo que podría sugerir una sobrerregulación del ciclo de los ácidos tricarboxílicos en cultivares adaptados o tolerantes al estrés ambiental.

III.3. Niveles elevados de aminoácidos, alanina, norvalina, treonina y ácido aspártico y de inositol se encontraron en la variedad más resistente (Camarosa)

en comparación con las variedades sensibles (Festival y Palomar). Estos compuestos podrían estar relacionados con los mecanismos de tolerancia de las plantas en respuesta al estrés abiótico, generalmente atribuido a ajustes osmóticos.

III.4. La respuesta de la planta a los cambios en las condiciones de cultivo ha sido evaluada mediante PLS-DA. Los azúcares (fructosa y glucosa), ácidos orgánicos (cítrico y málico), glicerol, treonina y norvalina fueron los parámetros más importantes para la discriminación entre muestras cultivadas en los dos macrotúneles. Para las tres variedades, se encontró que el valor de ácido cítrico fue mayor en las fresas cultivadas en macrotúnel descubierto y el contenido de ácido málico mayor en macrotúnel cubierto. Estas alteraciones en los metabolitos primarios podrían ser explicadas por cambios en las condiciones climáticas.

III.5. Para las tres variedades, se observó una disminución en el contenido de ácido cítrico y de alanina cuando aumenta la conductividad eléctrica. Asimismo, se observó un aumento en los niveles de inositol y glicerol para Camarosa y Palomar, respectivamente, cuando aumentó la salinidad. Esto fue indicativo de un estado de estrés osmótico. También se encontraron alteraciones de metabolitos primarios en función del tipo de sustrato empleado, pudiéndose concluir que la fibra de coco es el mejor sustrato para la producción de fresas en sistemas sin suelo.

OVERALL CONCLUSIONS

Overall conclusions

Quality characteristics study of strawberries grown in soilless systems, evaluating the influence of different environmental and growing conditions on the organoleptic, nutritional and nutraceutical properties has been carried out. From the experimental data obtained and their interpretation can be drawn the following conclusions:

I.1. The comparison of different strawberry cultivars grown in two crop systems revealed differences in the nutritional and organoleptic quality, mainly in the sweetness. Tamar and Camarosa were the sweetest genotypes among those grown in soilless and soil system, respectively, as it was determined by the sweetness index. Both varieties also had the highest total sugars/total acids ratio; so these cultivars were seemingly more palatable and balanced.

I.2. Differences among strawberry cultivars were observed based on the mineral composition. In soilless system only the contents of N and Sr were significantly different. However, no significant differences in sourness between varieties were detected in either culture systems.

I.3. A good discrimination was observed between cultivars of conventional crop when multivariate statistical techniques such as PLS-DA were applied. The pH and the content of glucose, fructose, succinic acid, Ca, Mg, Ba, Fe Al and Cu were the most discriminant variables. Moreover, no clear separations between cultivars grown in soilless was achieved.

I.4. The effect of culture system (soil and soilless) on nutritional quality was investigated in Camarosa and Candonga cultivars. This effect was more significant in Candonga variety than in Camarosa. Strawberries of cv. Camarosa, cultivated traditionally in soil, present slightly higher acidity and N concentration, but the content of Ca, Ba, Na and K, are practically the same for both systems. On the other hand, magnesium concentration and pH value were higher in soil system independently from the cultivar. In addition, a good discrimination between strawberries cultivated in soilless systems and conventional crop system was also achieved by PLS-DA.

II.1. The content of several metabolites related to strawberry quality, including sugars, organic acids, phenolic compoundas and TEAC, depend on cultivar and growing conditions. Festival was the sweetest genotype and also had the highest total sugars/total acids ratio, whereas Camarosa was richest in anthocyanins.

II.2. When pattern recognition techniques such as PCA, considering the harvest time, were applied it was concluded that strawberry harvested in January and March (early production) had higher quality. Fruits harvested in January were richer in phenolic compounds whereas fruits harvested in March were richer in taste-related compounds.

II.3. The influence of agronomical conditions was investigated by Nested ANOVA and PLS-DA. The type of coverage and the variety were the main source of variation for most of the variables studied, mainly phenols and sugars. A good discrimination between three cultivars and macrotunnel type was also achieved by PLS-DA, confirming the results from Nested ANOVA. The VIP scores indicated that anthocyanins were the most important parameters for cv Camarosa, whereas glucose, fructose, total sugars and TS/TA ratio were more relevant for cv Festival and phenolics acids and flavonols for cv Palomar. Strawberries grown in covered macrotunnel were richer in pelargonidin and their derivatives. Uncovered grown samples were richer in glucose, fructose, total sugars and ellagic acid derivative. Other growing conditions, such as electrical conductivity and substrate did not provide a clear discrimination between samples.

III.1. Metabolomic analysis revealed significant alterations in primary metabolites between the three strawberry cultivars studied, including sugars (fructose, glucose), organic acids (malic acid, citric acid) and amino acids (alanine, threonine, aspartic acid), among others.

III.2. GC-MS profiles evidenced that cv. Camarosa, the most resistant cultivar, presents significant higher levels of citric acid compared with cvs Festival and Palomar (more sensitive), which could suggest the up-regulation of the TCA cycle in cultivars adapted or tolerant to environmental stress.

III.3. Higher levels of aminoacids, alanine, norvaline, threonine and aspartic acid and inositol were found in the most resistant cultivar (Camarosa) compared to sensitive cultivars (Festival and Palomar). These compounds can be related to tolerance mechanisms in plants in response to abiotic stress, generally attributed to osmotic adjustment.

III.4. Metabolic alterations associated with growing conditions of strawberries were evaluated by PLS-DA. Sugars (fructose and glucose), organic acids (citric and malic), glycerol, threonine and norvaline were the most important parameters for discrimination between covered and uncovered samples. The content of citric acid was higher in strawberries from uncovered macrotunel and malic acid in covered macrotunel, for the three cultivars. These alterations in primary metabolites between strawberries grown in the two macrotunel types could be explained by differences in climatic conditions.

III.5. For the three cultivars, a decrease of citric acid content can be observed when the conductivity increases. Also, an increase in the levels of inositol and glycerol to Camarosa and Palomar, respectively, is observed when increased salinity, which is indicative of osmotic stress. Alterations of primary metabolites were also found depending on the type of substrate used, it could

be concluded that the coconut fiber is the best substrate for the production of strawberry in soilless culture.

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ANEXO III

1. Research Article

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Influence of cultivar and culture system on nutritional and organoleptic quality of strawberry

Ikram Akhatou and Ángeles Fernández Recamales*

Abstract

BACKGROUND: Strawberries are widely appreciated and consumed as a pleasant-tasting fruit either in fresh form or as processed product. Of the many factors which can affect the quality of fruit (sensorial and nutritional), the cultivar is especially important, but it is also affected by crop conditions. The main characteristics related to the fruit quality and nutritional attributes were assessed in several varieties of strawberry grown in soilless and soil culture. The effect of different cultivars and cropping systems on selected quality parameters, were assessed by applying multivariate statistical methods, such as principal component analysis and partial least square discriminant analysis (PLS-DA).

RESULTS: Differences among cultivars were observed based on the mineral composition and glucose and fructose concentrations in both cultivation systems. However, no significant differences between cultivars were detected in sourness. Tamar and Camarosa were the sweetest cultivars among those grown in soilless and soil system, respectively. Both varieties also had the highest total sugars/total acids ratio. The comparison between strawberries cultivated in soil and soilless systems also revealed significant differences in the mineral composition, content of sugars and related parameters.

CONCLUSION: Good discrimination was observed between cultivars of conventional crops, whereas no clear separation between cultivars grown in the soilless system was achieved by PLS-DA. PLS-DA also allowed differentiation of samples by type of crop (soilless vs. conventional crop). This information could be useful for selection of growing conditions and high-quality cultivars.
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Keywords: strawberry; quality; cultivars; soilless system

INTRODUCTION

Strawberry (*Fragaria ananassa* Duch.) is a very important crop in Europe and the USA owing to its nutritional and sensorial characteristics and its profitability. Modern production methods allow strawberry cultivation under a wide range of environmental conditions, making the fruit available on the market almost independent of the season. However, this could affect its final price. For this reason strawberry and other small fruits could represent an important and valuable portion of daily fresh food consumption.

Strawberry is widely cultivated in several areas under different cultivation conditions, and every year new varieties come into trade and cultivation. In Spain, strawberry is one of the most important crops, especially in the south of Huelva (southwest of Spain), because of soil and climatic conditions and water quality. Strawberry production in this region accounts for 35% of strawberry production in the European Union (EU) and 90% of national production, Camarosa being the most used variety, followed by Candonga, Festival and Ventana.[1] Nowadays, the largest production of strawberries is in tunnels on soil beds. Nevertheless, they can also be produced in soilless growing systems,[2] which allow cultivation to be developed independently from environmental conditions.

Soilless culture has been strongly developed in the southern regions of Spain, France and Italy as a potential alternative to methyl bromide (MB). This soil fumigant was definitively phased out for strawberry in the EU in 2007. Since then, several chemical and non-chemical alternatives to MB have been developed.[3] The replacement of soil crops with soilless crops may have important advantages: reduction in water requirements, prevention from loss of nutrients and pesticides if recirculation is done, and product quality improvement.[4] The differences in strawberry quality as a function of the soilless cultivation system or substrate type have been studied in depth in previous papers, in which it was concluded that coconut fiber was the best substrate for the production of strawberry in soilless culture.[2,5,6]

The final goal of modern agriculture is production of fruit with high organoleptic, nutritional and nutraceutical qualities, free from biotic and xenobiotic contaminants. On the other hand, consumers highly value quality fresh foods, so the influence of factors such as cultivation method and genotype on organoleptic and nutritional characteristics should be investigated.

Quality, safety and sensorial attributes such as color, flavor and taste are the most important characteristics that determine

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Nutritional and Nutraceutical Quality of Strawberries in Relation to Harvest Time and Crop Conditions

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ABSTRACT: Three strawberry varieties cultivated in soilless systems were studied for their content of primary and secondary metabolites in relation to harvest time and crop conditions. The three varieties were chosen based on their sensitivity level to environmental stress: Palomar (very sensitive), Festival (sensitive), and Camarosa (resistant). Throughout the campaign, three samplings were performed: December (extra-early production), January, and March (early production). Difference among cultivars and harvest times were observed based on the contents of sugars, organic acids, phenolic compounds, and antioxidant capacity. The higher levels for total anthocyanins and flavan-3-ols were found in Camarosa and Festival strawberries, both in the January harvest. The Palomar variety showed higher total sugar/total organic acids ratio in the March harvest. The influence of cultivation practices and environmental conditions was assessed by nested ANOVA and PLS-DA. Differences in the sugar and phenolic content were observed depending upon variety and coverage type. TEAC was most influenced by the substrate type.

KEYWORDS: strawberry, quality, metabolites, cultivars, soilless system, crop conditions

INTRODUCTION

Strawberries (Fragaria x ananassa Duch.) are one of the most popular berry fruits. They are widely used for industrial food processing but mainly consumed as fresh fruit. Its consumption has been increasing over the years due to the fact that epidemiological studies have revealed the relationship between diets which are rich in fruits and vegetables, and a lower incidence of some major human chronic diseases. Besides its proven health benefits,[1−3] strawberries have an attractive red color and flavor, and these attributes influence the choice of consumers and strawberries marketability.

The sugar content is very important from a nutritional point of view and, in combination with organic acids, contributes to Strawberry taste and flavor. So, good taste is the result of a balance between sweetness provided by sugars and the sourness of organic acid. It depends not only on the total sugar and organic acid contents but also on the type and quantity of individual compounds. Different sugars differ from sweetness level percepted by human tongue. For this reason, the relative proportion of each sugar is important for sweetness perception and consumer acceptability. The strawberry color is related to the presence of pigments, among which, anthocyanins play an essential role. Moreover, anthocyanins and other phenolic compounds have attracted a great deal of attention due to their properties as antioxidants, which can protect the human body against cellular oxidation reactions through scavenging of free radicals. These antioxidants have been shown to reduce the risk of cardio- and cerebrovascular diseases, cancer and other age-related diseases[4,5] and also contribute to the high nutritional quality of the fruit. However, it should also be considered that antioxidants protective effect can be dose-dependent and some of them may be harmful at high doses.[6]

Recent studies postulated that the antioxidant potential of anthocyanins and other phenolic compounds, as a basic mechanism of biological activity, is compromised by their low level of bioavailability especially when compared to the concentrations of endogenous antioxidants. Consequently, further research in this area is required in order to target other mechanisms of action that could be involved in the health-promoting effects beyond antioxidant activity.[2,3]

Strawberry is widely cultivated in several areas under different cultivation conditions and every year new varieties come into trade and cultivation. In Spain, strawberry is one of the most important crops, especially in the south of Huelva (southwest of Spain) due to soil and climatic conditions and water quality. Strawberry production in this region is accounting for 35% of strawberry production in the EU and 90% of national production. The economic importance of this crop and its great competitivity in the strawberry market drive strawberry producers to develop new growing methods such as soilless culture,[7] and to use early varieties. These varieties come into market in winter with enhanced fruit quality, higher resistance to stresses (biotic and abiotic) as well as higher productivity.

The chemical composition of berry fruits is affected by a number of preharvest and postharvest factors. Among the preharvest factors, the variety is considered the main source of variation in composition. Their effects on the nutritional and sensorial quality are well-known[8−10] and several breeding and biotechnological programs are focused on them.[3] In addition, nutritional and nutraceutical quality is also influenced by crop conditions (environmental and cultivation techniques), sampling time and the degree of ripeness, among other factors.[11−17] Ripeness and maturity are the key factors that influence the taste of the fruit. In wild strawberry, climatic conditions, such as

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