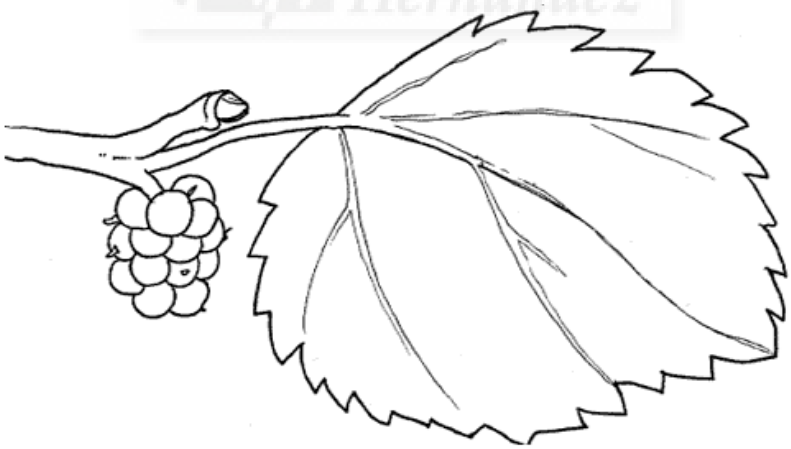
UNIVERSIDAD MIGUEL HERNÁNDEZ DE ELCHE

ESCUELA POLITÉCNICA SUPERIOR DE ORIHUELA

DPTO. PRODUCCIÓN VEGETAL Y MICROBIOLOGÍA

ESTUDIO DE LAS PROPIEDADES FÍSICAS Y FITOQUÍMICAS DE OCHO CLONES DE MORERA (*Morus spp.*)



EVA MARÍA SÁNCHEZ SALCEDO

TESIS DOCTORAL

2017

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ESCUELA POLITÉCNICA SUPERIOR DE ORIHUELA

PROGRAMA DE DOCTORADO EN RECURSOS Y TECNOLOGÍAS AGRARIAS, AGROAMBIENTALES Y ALIMENTARIAS

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CALIDAD DEL COMPENDIO DE CADA PUBLICACIÓN

Esta Tesis se presenta como compendio de las siguientes publicaciones:

Sánchez, E.M., Calín-Sánchez, A., Carbonell-Barrachina, A.A., Melgarejo, P., Hernández, F., & Martínez-Nicolás, J.J. (2014). Physicochemical characterisation of eight Spanish mulberry clones: processing and fresh market aptitudes. *International Journal of Food Science and Technology*, 49(2), 477-483.

Sánchez-Salcedo, E.M., Mena, P., García-Viguera, C., Martínez, J.J., & Hernández, F. (2015). Phytochemical evaluation of white (*Morus alba* L.) and black (*Morus nigra* L.) mulberry fruits, a starting point for the assessment of their beneficial properties. *Journal of Functional Foods*, *12*, 399-408.

Sánchez-Salcedo, E.M., Mena P., García-Viguera, C., Hernández, F., & Martínez, J.J. (2015). (Poly)phenolic compounds and antioxidant activity of white (*Morus alba*) and black (*Morus nigra*) mulberry leaves: Their potential for new products rich in phytochemicals. *Journal of Functional Foods*, *18*, 1039-1046.

Sánchez-Salcedo, E.M., Sendra, E., Carbonell-Barrachina, A.A., Martínez, J.J., & Hernández, F. (2016). Fatty acids composition of Spanish black (*Morus nigra* L.) and white (*Morus alba* L.) mulberries. *Food Chemistry*, 190, 566-571.

Sánchez-Salcedo, E.M., Tassotti, M., Del Rio, D., Hernández, F., Martínez, J.J., & Mena, P. (2016). (Poly)phenolic fingerprint and chemometric analysis of white (*Morus alba* L.) and black (*Morus nigra* L.) mulberry leaves by using a non-targeted UHPLC–MS approach. *Food Chemistry*, 212, 250-255.

Mena, P., **Sánchez-Salcedo, E.M**., Tassotti, M., Martínez, J.J., Hernández, F., & Del Rio, D. (2016). Phytochemical evaluation of eight white (*Morus alba* L.) and black (*Morus nigra* L.) mulberry clones grown in Spain based on UHPLC-ESI-MS^n metabolomic profiles. *Food Research International*, 89, 1116-1122.

International Journal of
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Physicochemical characterisation of eight Spanish mulberry clones: processing and fresh market aptitudes

Eva M. Sánchez,[1] Ángel Calín-Sánchez,[2] Ángel A. Carbonell-Barrachina,[2] Pablo Melgarejo,[1] Francisca Hernández[1] & Juan José Martínez-Nicolás[1]*

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Food Science and Technology	Q2	57/123	1,384 (2014)	1,669

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Phytochemical evaluation of white (*Morus alba* L.) and black (*Morus nigra* L.) mulberry fruits, a starting point for the assessment of their beneficial properties

Eva M. Sánchez-Salcedo [a], *Pedro Mena* [b,*], *Cristina García-Viguera* [c], *Juan José Martínez* [a], *Francisca Hernández* [a]

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Journal of Functional Foods 18 (2015) 1039–1046

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Food Chemistry 190 (2016) 566–571

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Fatty acids composition of Spanish black (*Morus nigra* L.) and white (*Morus alba* L.) mulberries

Eva M. Sánchez-Salcedo [a,*], Esther Sendra [b], Ángel A. Carbonell-Barrachina [c], Juan José Martínez [a], Francisca Hernández [a]

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(Poly)phenolic fingerprint and chemometric analysis of white (*Morus alba* L.) and black (*Morus nigra* L.) mulberry leaves by using a non-targeted UHPLC–MS approach

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Food Science and Technology	Q1	7/125	4,052 (2015)	4,232

Food Research International 89 (2016) 1116–1122

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Phytochemical evaluation of eight white (*Morus alba* L.) and black (*Morus nigra* L.) mulberry clones grown in Spain based on UHPLC-ESI-MS^n metabolomic profiles

Pedro Mena [a,*], Eva Mª Sánchez-Salcedo [b], Michele Tassotti [a], Juan José Martínez [b], Francisca Hernández [b], Daniele Del Rio [a]

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Dr. D. Ángel Antonio Carbonell Barrachina, Catedrático de Universidad y Coordinador del Programa de Doctorado Recursos y Tecnologías Agrarias, Agroambientales y Alimentarias (ReTos-AAA) de la Universidad Miguel Hernández de Elche (UMH),

CERTIFICA:

Que la Tesis Doctoral titulada **"Estudio de las propiedades físicas y fitoquímicas de ocho clones de morera (*Morus spp.*)"**, de la que es autora la Ingeniera Agrónomo **Dª. Eva María Sánchez Salcedo,** ha sido realizada bajo la dirección de los doctores **Dª. Francisca Hernández García** y **D. Juan José Martínez Nicolás**, profesores de la UMH, actuando como tutor el Dr. D. Ángel Antonio Carbonell Barrachina (UMH). Considero que la tesis es conforme en cuanto a forma y contenido a los requerimientos del Programa de Doctorado ReTos-AAA por tanto, es apta para su exposición y defensa pública.

Y para que conste a los efectos oportunos firmo el presente certificado en Orihuela a seis de diciembre de dos mil dieciséis.

Dr. Ángel A. Carbonell Barrachina

Coordinador Programa Doctorado ReTos-AAA

Programa de Doctorado
ReTos-AAA

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Esta memoria ha sido presentada por **Dña. Eva María Sánchez Salcedo**, Ingeniera Agrónoma y Máster en Técnicas Avanzadas para la Investigación y Producción en Fruticultura, para obtener el título de doctor.

Fdo. Eva María Sánchez Salcedo

Esta Tesis Doctoral ha sido dirigida por la **Dra. Francisca Hernández García**, Profesora Titular de Universidad del Departamento de Producción Vegetal y Microbiología, y del **Dr. Juan José Martínez Nicolás**, Catedrático de Escuela Universitaria del Departamento de Producción Vegetal y Microbiología, ambos de la Universidad Miguel Hernández de Elche.

Dra. Francisca Hernández García

Profesora Titular de Universidad
Dpto. Producción Vegetal y
Microbiología

Dr. Juan José Martínez Nicolás

Catedrático de Escuela Universitaria
Dpto. Producción Vegetal y
Microbiología

Orihuela, febrero de 2017

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AGRADECIMIENTOS

A la Dra. Francisca Hernández García y al Dr. Juan José Martínez Nicolás directores de esta Tesis Doctoral, por el asesoramiento y orientación prestada en la realización de la misma, por todo lo que me han enseñado, sus valiosos consejos y amistad.

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A mi tutor el Dr. Ángel Antonio Carbonell Barrachina.

A mis compañeros de doctorado en especial a Fuensanta, Juana, Fernando, Úrsula, Silvia, y Faten que me han acompañado durante este tiempo por lo buena gente que sois.

También me gustaría agradecer a las personas que durante esta Tesis Doctoral, me han echado una mano cuando lo he necesitado, a Nuria, Mamen, Lorena, Nallely, Diego, Monica, y Antonio.

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A mis hermanos y cuñados porque os habéis preocupado por mí y habéis estado ahí cuando os he necesitado. En especial a mí hermana Anabel, por animarme siempre y por leerse apasionadamente mis trabajos, y a mí hermano José Fulgencio por ser mi informático particular.

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En especial a mis padres que han hecho que esto sea posible, por la confianza que siempre me han ofrecido así como la oportunidad de estudiar y de formarme académicamente y como persona.

En definitiva a todas las personas que han colaborado de alguna manera en esta Tesis Doctoral. Muchas gracias a todos.

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A todas las personas que me quieren

Y en especial a mis abuelos

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ÍNDICE

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1. ABREVIACIONES Y SÍMBOLOS

1. ABREVIACIONES Y SÍMBOLOS

ABREVIACIONES

TSS	Sólidos solubles totales
TA	Acidez valorable total
MI	Índice de madurez
MA	*Morus alba*
MN	*Morus nigra*
HEc	Ángulo hue del color externo del fruto
HJc	Ángulo hue zumo
FW	Peso del fruto
FH	Longitud del fruto
Fhp	Longitud del fruto con pedúnculo
JD	Densidad del zumo
PL	Longitud del pedúnculo
aJc	Coordenada colorímetrica a*del zumo
aEc	Coordenada colorímetrica a* del color externo del fruto
SI	Índice de dulzor
CF	Fibra cruda
bjc	Coordenada colorímetrica b* del zumo
CEc	Croma del color externo del fruto
bEc	Coordenada colorímetrica b* del color externo del fruto
LEc	Luminosidad del color externo del fruto
Cjc	Croma del zumo
Ljc	Luminosidad del zumo
DE	Diámetro ecuatorial
FJY	Rendimiento del fruto en zumo
JD	Densidad del zumo
DW, dw	Peso seco
FW, fw	Peso fresco
M	Humedad
Cy-glc	Cyanidin 3-*O*-glucoside
Cy-rut	Cyanidin 3-*O*-rutinoside
Pg-glc	Pelargonidin 3-*O*-glucoside
Pg-rut	Pelargonidin 3-*O*-rutinoside
Tot_Anth	Total anthocyanins
Oth_flavonol	Other flavonol derivatives
Tot_Flavonol	Total flavonols
PCA	Protocatechuic acid
p-OHbenzA	*p*-Hydroxybenzoic acid
VA	Vanillic acid
Tot_benz	Total benzoic acid derivatives
CA	Caffeic acid
p-CoumA	*p*-Coumaric acid
FA	Ferulic acid
m-CoumA	m-Coumaric acid

Tot_ cinn	Total cinnamic acid derivatives
Q-rut	Quercetin 3-*O*-rutinoside
Q-glc	Quercetin 3-*O*-glucoside
K-rut	Kaempferol 3-*O*-rutinoside
3-CQA	Neo-chlorogenic acid
5-CQA	Chlorogenic acid
4-CQA	Crypto-Chlorogenic acid
1-CQA	Chlorogenic acid isomer
PUFA	Ácidos grasos poliinsaturados
MUFA	Ácidos grasos monoinsaturados
SFA	Ácidos grasos saturados
RDI	Ingestas dietéticas recomendadas

SÍMBOLOS

L*	Luminosidad
a*	Rojizo-verdoso
b*	Amarillento-azulado
C*	Croma
h*	Ángulo hue
$ABTS^{\cdot+}$	(Ácido 3-etil-benzotiazolina-6-sulfónico)
$DPPH^{\cdot}$	(2,2-difenil-1-picrilhidrazil)
TPC	Fenoles totales
C14:0	Ácido mirístico
C16:0	Ácido palmítico
C16:1	Ácido palmitoleico
C18:1	Ácido esteárico
C18:2	Ácido linoleico
C18:3	Ácido linolénico
C19:0	Ácido nonadecanoico
C20:0	Ácido araquídico
C20:1	Ácido eicosenoico
C20:4	Ácido araquidónico
C21:0	Ácido heneicosanoico
C22:0	Ácido behénico
C22:1	Ácido cetoleico

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2. PRODUCCIÓN CIENTÍFICA DURANTE EL PERIODO PREDOCTORAL

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2.1. ARTÍCULOS PUBLICADOS EN REVISTAS CIENTÍFICAS

Sánchez, E.M., Calín-Sánchez, A., Carbonell-Barrachina, A.A., Melgarejo, P., Hernández, F., & Martínez-Nicolás, J.J. (2014). Physicochemical characterisation of eight Spanish mulberry clones: processing and fresh market aptitudes. *International Journal of Food Science and Technology*, 49(2), 477-483.

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Sánchez-Salcedo, E.M., Sendra, E., Carbonell-Barrachina, A.A., Martínez, J.J., & Hernández, F. (2016). Fatty acids composition of Spanish black (*Morus nigra* L.) and white (*Morus alba* L.) mulberries. *Food Chemistry*, 190, 566-571.

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Mena, P., **Sánchez-Salcedo, E.M**., Tassotti, M., Martínez, J.J., Hernández, F., & Del Rio, D. (2016). Phytochemical evaluation of eight white (*Morus alba* L.) and black (*Morus nigra* L.) mulberry clones grown in Spain based on UHPLC-ESI-MS^n metabolomic profiles. *Food Research International*, 89, 1116-1122.

2.2. PARTICIPACIÓN EN CONGRESOS NACIONALES

- **VII Congreso Ibérico de Agroingeniería y Ciencias Hortícolas**

E.M. Sánchez-Salcedo, Fca. Hernández, J.J. Martínez. 2013. Caracterización preliminar de los frutos de ocho clones de *Morus sp*. Madrid 26-29 de agosto.

- **XIV Congreso Nacional de Ciencias Hortícolas**

E.M. Sánchez-Salcedo, P. Mena, C. García-Viguera, F. García-Sánchez, Fca. Hernández, J.J. Martínez-Nicolás. 2015. Actividad antioxidante, contenido en fenoles totales y composición mineral de los frutos de cuatro variedades de morera. Orihuela 3-5 de junio.

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3. ESTRUCTURA DE LA TESIS

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Para la elaboración de la presente Tesis Doctoral se ha seguido la metodología basada en la publicación de artículos de investigación. Con esta Tesis Doctoral se pretende obtener el título de Doctor, para ello en la redacción de la misma, se ha seguido la normativa vigente de la Universidad Miguel Hernández de Elche.

La Tesis Doctoral se estructura en las siguientes partes:

A) **Introducción**

B) **Objetivos**

C) **Resumen de la Metodología**

D) **Publicaciones Científicas**

E) **Resumen de los Resultados, Discusión y Conclusiones**

F) **Conclusiones Generales**

G) **Investigaciones Futuras**

H) **Referencias Bibliográficas**

La **Introducción** contiene una breve revisión bibliográfica sobre la importancia del cultivo de la morera, debido a la producción de seda en el pasado en España. Además presenta una breve revisión bibliográfica sobre la importancia del cultivo actual, origen, distribución, descripción general, ubicación taxonómica, y usos de la morera. Y por último esta primera parte también incluye una breve revisión de las propiedades funcionales que presenta este frutal. En la segunda parte se describen los **Objetivos** estimados en la presente Tesis Doctoral. En la siguiente parte se detalla un **Resumen de la Metodología** utilizada, para la recopilación de los resultados y entender el diseño y la preparación de las muestras, además incluye los programas informáticos utilizados en los análisis estadísticos de los datos. A continuación, se recogen las **Publicaciones Científicas** publicadas que componen esta Tesis Doctoral.

- La **Primera Publicación** recoge los resultados de la caracterización morfológica y parte de la química de los frutos (moras). En ella se estudian las aptitudes que presenta esta fruta tanto para su consumo en fresco como para su industrialización. Se analizan propiedades de calidad, color, y composición en ácidos orgánicos y azúcares. Este artículo está publicado en la revista *International Journal of Food Science and Technology*.

- En la **Segunda Publicación** se aborda la evaluación fitoquímica de la mora: contenido e identificación de los compuestos fenólicos, actividad antioxidante, composición mineral, y contenido en proteína. Este artículo se publicó en la revista *Journal of Functional Foods*.
- La **Tercera Publicación** aborda parte de la caracterización química de las hojas de morera, estudiando el perfil polifenólico y la actividad antioxidante de las mismas. Este artículo está publicado en la revista *Journal of Functional Foods*.
- La **Cuarta Publicación** se centra en la identificación y cuantificación del perfil de ácidos grasos de las moras. Este artículo se publicó en la revista *Food Chemistry*.
- La **Quinta Publicación** hace referencia a la identificación y cuantificación de los compuestos bioactivos mediante UHPLC-MS en hojas. Este artículo está publicado en la revista *Food Chemistry*.
- La **Sexta Publicación** estudia la identificación y cuantificación de los compuestos bioactivos mediante UHPLC-ESI-MS^n en frutos. Este artículo está publicado en la revista *Food Research International*.

El **Resumen de los Resultados, Discusión y Conclusiones** muestra un resumen con los resultados más interesantes e importantes conseguidos en los estudios, una discusión general de los mismos y las conclusiones de cada publicación. A continuación se recogen las **Conclusiones Generales** obtenidas de los estudios realizados en esta Tesis Doctoral. En la siguiente parte se presentan las **Investigaciones Futuras**. Y en la última parte se recogen las **Referencias Bibliográficas** consultadas para la elaboración de esta memoria, sin tener en cuenta el apartado de Publicaciones Científicas.

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4. RESUMEN Y ABSTRACT

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4.1. RESUMEN

Actualmente, hay un creciente interés de los consumidores en aumentar el consumo de frutas y verduras en su dieta, debido a la presencia de compuestos bioactivos, que están vinculados con beneficios para la salud humana, como la disminución del riesgo de sufrir diferentes enfermedades.

Las moras poseen un sabor agradable y dulce, además algunas variedades presentan colores atractivos en la maduración, son populares y apreciados en el mercado mundial.

Los frutos de las moreras contienen una amplia gama de compuestos bioactivos y su uso como medicina natural es ancestral; en países asiáticos se les atribuye propiedades curativas a las hojas, frutos y corteza de las raíces. Debido a esto, en los últimos años ha aumentado su consumo.

La presente Tesis Doctoral tiene como objetivo principal la caracterización físico-química de frutos y hojas de ocho clones de morera (blancas y negras) cultivadas en el sureste español. Para alcanzar este objetivo general se plantean los siguientes objetivos específicos: caracterización morfológica de frutos y hojas, caracterización química de frutos y hojas, e identificación de compuestos bioactivos en hojas y frutos.

4.2. ABSTRACT

Nowadays, there is a growing interest of consumers in increasing the consumption of fruits and vegetables in their diet, due to the presence of bioactive compounds, which are linked with human health benefits, such as reducing the risk of suffering from different diseases.

Mulberries have a pleasant and sweet taste, in addition some varieties present attractive colors in maturation, they are popular and appreciated in the world market. The fruits of mulberries contain a wide range of bioactive compounds and their use as natural medicine is ancestral; In Asian countries curative properties are attributed to the leaves, fruits and bark of the roots. Due to this, in recent years has increased its consumption.

The main objective of this Doctoral Thesis is the physical-chemical characterization of fruits and leaves of eight mulberry clones (white and black) cultivated in the southeast from Spain. To achieve this general objective, the following specific objectives are proposed: morphological characterization of fruits and leaves, chemical characterization of fruits and leaves, and identification of bioactive compounds in leaves and fruits.

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5. INTRODUCCIÓN

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El cultivo de la morera en la vega murciana estaba fuertemente implantado en las primeras décadas del XVI, siendo la principal actividad económica murciana (Miralles, 2000). La sedería fue introducida en Murcia a través de Granada pasando por Lorca (Garzón, 1972). En esta influencia granadina tuvieron una actuación determinante los mercaderes genoveses (Olivares, 1986), fueron ellos los que introdujeron el cultivo de la morera en Murcia (Miralles, 2000).

El gusano de la seda (*Bombyx mori*) se alimenta exclusivamente con las hojas de morera (*Morus alba*) o de moral (*Morus nigra*) (Navarro, 2004).

El árbol que originariamente se utilizó fue el moral o morera negra (*Morus nigra*), igual que en Granada o en Segovia (Torres, 1977) era la especie autóctona del Mediterráneo (Navarro, 2004). La morera es una especie de origen asiático que no se conocía en el Mediterráneo antes del siglo V (Navarro, 2004). El moral se adaptaba a zonas más frías y requería menos cuidados y riesgos que la morera. La morera resultaba más adecuada en las zonas templadas, casi subtropicales del litoral. La morera no es tan fuerte y duradera, pero crece con mayor rapidez que el moral, y sus hojas, tiernas y jugosas, se pueden recoger con más facilidad (Navarro, 2004). Durante mucho tiempo convivieron ambas especies hasta que en los siglos XV-XVI el equilibrio se rompió en favor de la morera (*Morus alba*), a raíz de las exigencias crecientes de la demanda de fibra sérica en el mercado internacional (Navarro, 2004).

Desde Valencia, Navarro, 2004 detectó en la documentación la difusión del cultivo de la morera desde Murcia y Orihuela hacia comarcas valencianas a partir de finales del siglo XIV (Miralles, 2000).

Según el Catastro del Marqués de la Ensenada del siglo XVIII, sólo en la huerta de Murcia habían 684.000 moreras, de las cuales 558.600 se encontraban en linderos y 125.400 en cultivo intensivo, ocupando una superficie de 2.976,93 hectáreas, el 26,9% de la extensión de la Huerta de Murcia (Olivares, 1976).

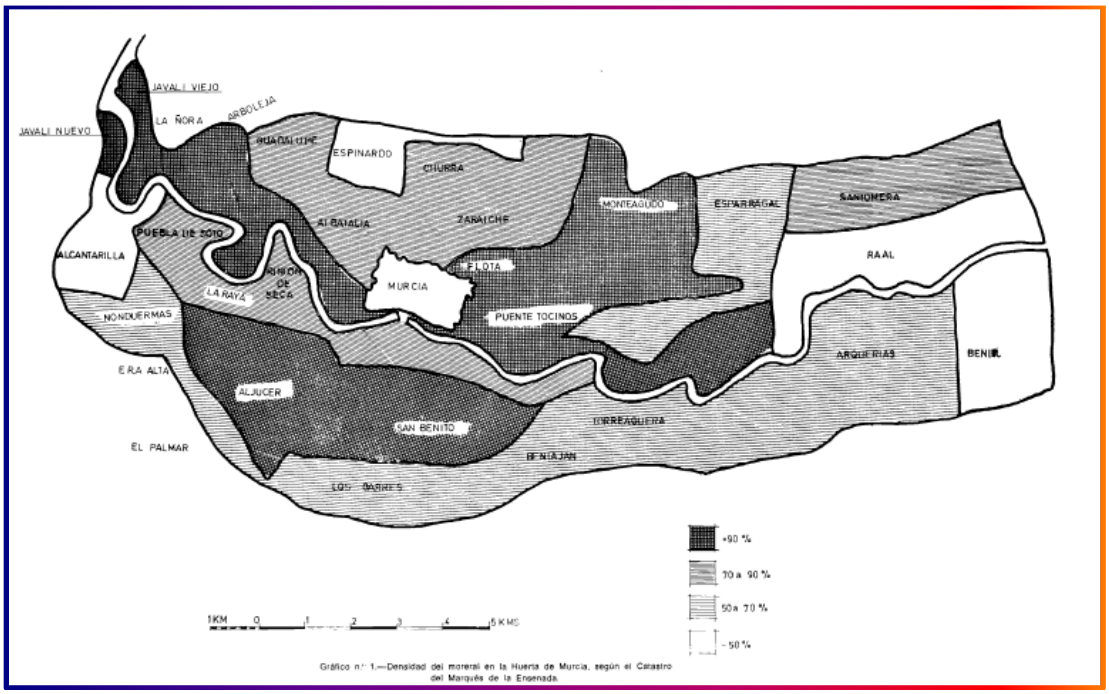

Figura 1: *Densidad del moreral en la huerta de Murcia, según el catastro del Marqués de la Ensenada.*
Fuente: *Olivares, 1972.*

Figura 2: *Pradera de moreras.*
Fuente: *González, 2001.*

Figura 3: *Compra oficial del capullo de seda.*
Fuente: *González, 2001.*

Durante los siglos XVI y XVII asistimos a la expansión y a la crisis de este monocultivo. El cultivo predominante en la huerta de Murcia durante estos siglos fue la morera (Miralles, 2000). La morera se plantaba de dos formas: abierto, en los límites que separaban las parcelas, o cerrado, cultivándolo a la vez con algunas hortalizas o frutales. Durante los siglos XVI y XVII el cultivo intensivo fue lo normal, en cambio durante el siglo XVIII predomina el sistema de lindero, síntoma de que la sericicultura

se ha convertido en una fuente de ingresos auxiliar, mientras que en los siglos anteriores era la principal fuente de ingresos (Chacon, 1979).

En el siglo XVII se dará el proceso de máximo esplendor del cultivo de la morera pero, también, el proceso de su complementariedad y sustitución parcial con el lino, el olivo, el trigo y, sobre todo, la vid (Miralles, 2000).

En los siglos XIX y XX se van a producir diversas circunstancias negativas que van a perjudicar la crianza de la seda. El primer problema grave que se presentará, vendrá ocasionado por la guerra de la independencia, y como consecuencia provocará la desaparición para la industria sedera murciana de las demandas que realizaban otras sederías españolas. Superado este periodo, la industria sedera murciana toma un nuevo impulso, era junto a la valenciana las que más sobresalían entre todas las españolas a mediados del siglo XIX. Mientras transcurre el siglo, las sederías españolas mantienen una actividad rutinaria y no incorporan los progresos científicos e innovaciones que en el cultivo e hilado de la seda se estaban produciendo en Europa. En 1848 aparece en Europa una grave enfermedad endémica del gusano de la seda, la pebrina. Los cosecheros, no sabiendo que hacer ante tanto problema y la falta de soluciones para detener tal enfermedad, empezaron a arrancar moreras, desapareciendo extensos morerales en toda España. Solamente en Murcia y Orihuela se conservaron la mayor parte de las moreras volviéndose a la faena con renovado ahínco. El 3 de mayo de 1892, se creó la Estación Sericícola, con emplazamiento en Murcia, con el propósito de ofrecer a los cosecheros la orientación técnica y científica necesaria en relación al cultivo de la morera, la selección e introducción de nuevas variedades de semillas más sanas y productivas, así como la adopción y puesta a punto de técnicas innovadoras en la crianza y el aprovechamiento del gusano de la seda. Cuando se empieza a recuperar la sericicultura en España, en Francia se implantaban medidas protectoras para su seda que eran muy perjudiciales para la producción española. Japón tenía en Norte América su principal mercado, el cual debido a la crisis económica y financiera de 1929 se vino abajo y se deshicieron los acuerdos comerciales con su proveedor de seda en Japón. Ante esta situación Japón decidió invadir el mercado europeo a precios muy bajos. Además la guerra civil española imposibilitó durante la contienda la producción de seda. En el año 1967 fue suprimido oficialmente el Servicio de Sericicultura (Zapata, 2001).

5.1. IMPORTANCIA DEL CULTIVO

La morera es una planta leñosa importante económicamente con un impacto significativo en la sociedad humana por su valor económico en la sericicultura, así como por sus beneficios nutricionales y propiedades medicinales (Priya, 2012).

El cultivo de forraje para la ganadería está ganando importancia en muchos países de Asia y Europa, debido a que sus hojas son muy ricas en proteínas, antioxidantes y minerales (Guha et al., 2010; Papanastasis et al., 2008).

La morera se cultiva principalmente por sus hojas para alimentar a los gusanos de seda (*Bombix mori* L.) en la mayoría de los países de Asia (Jiang y Nie, 2015). Sin embargo, las moreras son cultivadas principalmente para la producción de fruta en lugar de la sericicultura, en la mayoría de los países europeos; en donde los frutos se comen crudos, secos o procesados como zumos, vino y mermelada (Gerasopoulos y Stavroulakis, 1997; Yildiz, 2013).

La morera (*Morus spp*.) es un frutal importante en Turquía, con un tasa de producción de 74.600 toneladas en 2013 (Turkish Statistical Institute, 2013). No se han encontrado datos sobre producciones de moras a nivel mundial debido a que se incluyen con otros frutales (bayas). Países con producciones importantes son China, Turquía e India.

5.2. ORIGEN Y DISTRIBUCIÓN

Muchos autores coinciden en que los principales centros de origen de la morera (*Morus spp.*) se encuentran en algunas regiones de China, Japón y al pie del Himalaya (Datta, 2002). Esta planta tiene una larga historia de domesticación, las moreras crecen en una amplia gama de condiciones geográficas, climáticas y de suelo. En consecuencia, tiene una amplia distribución en todo el mundo (Jiang y Nie, 2015). Las moreras se encuentran ampliamente distribuidas en las zonas tropicales, subtropicales y templadas de Asia, Europa, América y África, que indican su gran capacidad de adaptación a diferentes condiciones ambientales (Ercisli y Orhan, 2007).

5.3. DESCRIPCIÓN GENERAL

La morera es un árbol de hoja caduca, que alcanza una altura de 10 a 13 m y es variable en forma, incluyendo formas colgantes y piramidales (Donno et al., 2015). Estos árboles se caracterizan por tener savia lechosa en los brotes (Łochyńska y

Oleszak, 2011). Las moreras crecen muy rápido durante 40-50 años alcanzando su tronco 60-80 cm de diámetro a un metro, entonces su tasa de crecimiento se reduce. Las moreras viven durante 200-300 años (Łochyńska y Oleszak, 2011). La corteza es de color gris oscuro-marrón con lenticelas horizontales (Chan et al., 2016). Las hojas miden de 10 a 20 cm de largo (Kostic et al., 2013; Lin y Lay, 2013). Son de color verde brillante, alternas, cordadas en la base y acuminadas en el ápice, los márgenes son dentados, y los peciolos son largos y delgados (Chan et al., 2016). Las hojas son muy variables en forma. Incluso en el mismo árbol algunas son enteras (no lobuladas) mientras que otras pueden ser casi palmeadas (Chan et al., 2016).

Figura 4: *Morus nigra (clon MN2).*

Figura 5: *Diferentes formas de hojas.*

Las moreras son monoicas y dioicas (Łochyńska y Oleszak, 2011). La mayoría de las flores de la morera son monosexuales; algunas son bisexuales. Las flores son pequeñas y sésiles formando un racimo alrededor de un eje, llamado amento (Figura 6-9) (Cifuentes y Kee-Wook, 1998). Los amentos pueden ser masculinos y femeninos y son colgantes, y verdosos (Chan et al., 2016). La polinización de la morera es anémofila (Awasthi et al., 2004).

Figura 6: *Flores masculinas.*

Figura 7: *Flores femeninas.*

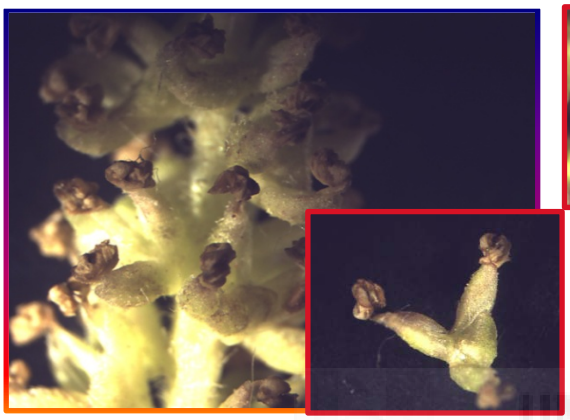

Figura 8: *Detalle flor masculina.*

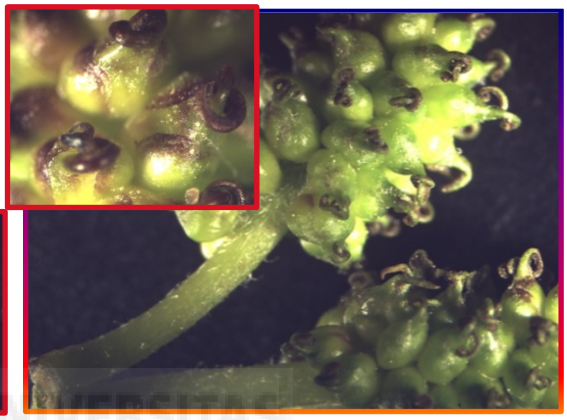

Figura 9: *Detalle flor femenina.*

El fruto de la morera se llama botánicamente sorosis, es jugoso y tiene un sabor dulce, con algo de acidez que es más prominente en los frutos menos maduros (Bajpai et al., 2015). La sorosis (Figura 10 y 11) es verde al principio, pero gradualmente se torna roja y a veces negro-violeta significando que ya están maduras. Los frutos maduros de algunas pocas variedades se tornan rojos o blancos, en lugar de negro-violeta (Olivera y Noda, 2010).

Figura 10: *Fruto clon MA2.*

Figura 11: *Fruto clon MN1.*

Las semillas de la mora son de color café amarillentas o amarillo brillantes y de forma carnosa y ovalada. Las semillas están compuestas de una cubierta, el embrión y el endospermo (Olivera y Noda, 2010).

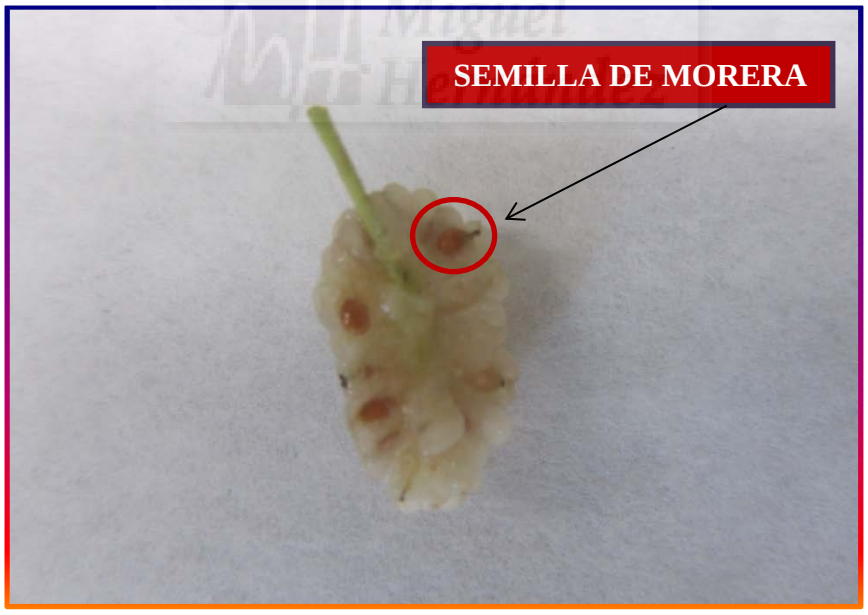

Figura 12: *Fruto partido con semillas clon MA1.*

5.4. UBICACIÓN TAXONÓMICA

La morera pertenece al orden Rosales, a la familia *Moraceae*, y al género *Morus* (Zhang et al., 2011). La taxonomía del género *Morus* es muy complicada (Datwyler y

Weiblen, 2004). Ha sido cuestionada (Vijayan et al., 2011a) debido a su amplia distribución geográfica, la plasticidad morfológica (Gray y Gray, 1987), la hibridación entre las especies (Burgess et al., 2005), la larga historia de la domesticación, la introducción y naturalización de las especies (Toyo, 1985). Hasta el momento, más de 150 especies de morera se han descrito, sin embargo, sólo 10-16 especies son ampliamente reconocidas por los botánicos (Datwyler y Weiblen, 2004).

Tabla 1. Ubicación Taxonómica de la morera.

Clasificación	Nombre científico y nombre común
Reino	Plantae - Plantas
Subreino	Tracheobionta - Plantas vasculares
Superdivisión	Spermatophyta - Fanerógamas
División	Magnoliophyta - Plantas con flores
Clase	Magnoliopsida - Dicotiledóneas
Subclase	Hamamelidae
Orden	Urticales
Familia	*Moraceae*
Género	*Morus* L.
Especies	*Morus alba* L. (morera blanca) *Morus nigra* L. (morera negra o moral)

Fuente: *USDA, 2016.*

5.5. USOS DE LA MORERA

La morera aunque está distribuida ampliamente alrededor del mundo, ha recibido poca atención con respecto a su potencial (Medina et al., 2004).

Las hojas de morera se utilizan principalmente para la alimentación del gusano de la seda (*Bombix mori*) para la producción de seda (Chen et al., 2015). Además las hojas de morera se utilizan para alimentar a animales lecheros debido a que son nutritivas, agradables al paladar, no tóxicas y pueden mejorar la producción de leche (Arabshahi-Delouee y Urooj, 2007). El alto contenido en proteína cruda y la digestibilidad de la materia orgánica de las hojas de la morera, son superiores a la mayoría de los pastos tropicales usados comúnmente para el ganado (Vu et al., 2011). Las hojas de morera también son comidas por los vegetarianos (Łochyńska y Oleszak, 2011). Actualmente, las hojas de morera se consumen como alimento antidiabético en Corea y Japón (Katsube et al., 2006). En el Este y Sudeste Asiático el consumo del té de

morera está ganando popularidad. El té es rico en ácido γ-aminobutírico (2,7 mg/g de peso seco (dw)), el cual es diez veces mayor que en el té verde (Suttie, 2005).

La morera también es importante en la horticultura, alimentación y las industrias de cosméticos y produce fitoquímicos que tienen beneficios para la salud (Özgen et al., 2009; Vijayan et al., 2011b).

Las moras se consumen en fresco o procesadas en diferentes productos como, vino, zumo y mermelada debido a su bajo contenido en calorías, delicioso sabor y que son ricas en nutrientes. Además, se utilizan en la medicina popular tradicional para el tratamiento eficaz de la fiebre, anemia, dolor de garganta, la hipertensión en China y Corea. (Lee et al., 2013; Ma, 2002). Recientemente, debido a su atractivo aroma y beneficios para la salud, las moras se han procesado comercialmente en bebidas no alcohólicas (Butkhup et al., 2011), productos de panadería (Nayak et al., 2013), yogur (Celik y Bakirci 2003), etc. Las hojas y frutos después del secado se aplican como forraje para aves, reptiles y roedores (Łochyńska y Oleszak, 2011).

La morera se utiliza como planta ornamental, en parques, jardines y orillas de caminos, como árbol de sombra y embellecimiento de la ciudad, ya que soporta altos niveles de contaminación del aire (Natić et al., 2015). Las plantas jóvenes forman densos setos que protegen excelentemente los jardines contra el viento, el ruido, humos y roedores (Łochyńska y Oleszak, 2011). Además, también se utiliza para la estabilización de piedra arenisca (Natić et al., 2015).

Es una de las hierbas convencionales utilizadas en la medicina desde tiempos inmemoriales, debido a su composición química y su función farmacológica. La mayoría de las partes de las plantas de la morera se usan con fines medicinales en la medicina china e india (Yang et al., 2010).

La morera también muestra un gran potencial como combustible y producción de energía. En la India, todos los brotes anuales, disponibles después de la temporada de cría de gusanos de seda, se cortan, se secan y se utilizan como la principal fuente renovable de combustible (Chinnaswamy y Hariprasad, 1995). La madera de morera es muy valorada en la industria de equipamiento deportivo debido a su elasticidad y flexibilidad, ya que, es fácil de pulir y barnizar. Principalmente se realizan con madera de morera, palos de hockey, raquetas de tenis y bádminton; y también se utiliza en la fabricación de muebles y accesorios de madera (Łochyńska y Oleszak, 2011).

Otros usos de la morera son la producción de papel y hongos (Machii et al., 2002). Las astillas de morera se han utilizado como celulosa para la producción de papel y como substrato para el cultivo de setas (Chan et al., 2016).

5.6. PROPIEDADES FUNCIONALES

Recientemente, hay un creciente interés en las moras negras y blancas y los beneficios potenciales para la salud humana asociados con sus altas propiedades fitoquímicas y antioxidantes (Ercisli y Orhan, 2007; Ercisli y Orhan, 2008; Koyuncu et al., 2014; Natić et al., 2015; Sánchez et al., 2014; Sánchez-Salcedo et al., 2015). Según la medicina tradicional china, los frutos de morera pueden proteger del daño renal y hepático, mejorar la vista y fortalecer las articulaciones (Hamzaa et al., 2012; Wattanathorn et al., 2012). Además se ha comprobado que las moras mejoran el sistema inmune, presentan un excelente efecto antienvejecimiento, antioxidante, antiinflamatorio, hipolipemiante y propiedades hipoglumeciantes (Liu y Lin, 2012; Wang et al., 2010).

En los últimos años, varios trabajos de investigación han puesto de manifiesto que las moras contienen altas cantidades de compuestos polifenólicos (Juan et al., 2012; Natić et al., 2015; Pawlowska, et al., 2008; Sánchez-Salcedo et al., 2015), ácidos orgánicos, y azúcares (Ercisli y Orhan, 2007; Sánchez et al., 2014). Además, las moras son ricas en ácido linoleico, un ácido graso esencial (Gecgel et al., 2011; Sánchez-Salcedo et al., 2016).

Los ácidos orgánicos y los azúcares son otros componentes importantes de los frutos, caracterizando sus propiedades organolépticas. El sabor, que es un criterio importante en el mercado en fresco, se caracteriza generalmente por la relación de ácidos orgánicos y azúcares (Soyer et al., 2003).

La morera contiene una gran cantidad de compuestos bioactivos incluyendo polisacáridos, 1-desoxinojirimicina, moracina, ácido clorogénico, rutina, flavonoles en forma de glicósidos y antocianinas, los cuales son asociados con sus funciones biológicas, tales como antiobesidad, antidiabetes, antioxidación, antiinflamación y antiaterosclerosis (Harauma et al., 2007; Hunyadi et al., 2012; Katsube et al., 2009; Kimura et al., 2007; Li et al., 2011; Peng et al., 2011; Yang et al., 2012; Yatsunami, et al., 2008; Zhang et al., 2014).

Las principales antocianinas en la mora son: cianidina, pelargonidina, delfinidina, y petunidina, conjugadas con cualquiera de los gliclósidos o glicósidos acilados (Song et al., 2009; Natić et al., 2015; Veberic et al., 2015).

Dentro del grupo de los flavonoles los principales derivados glicósidos son quercetina y kaempferol.

En cuanto a los ácidos fenólicos los mayoritarios son el ácido clorógenico y el ácido protocatéquico (Zhang et al., 2008; Gundogdu et al., 2011; Peng et al., 2011).

Debido a su alto contenido en polifenoles los frutos de morera poseen una amplia gama de actividades bioquímicas tales como: la actividad de eliminación de radicales libres (Du et al., 2008), efectos antidiabéticos (Wang et al., 2013), neuroprotectores (Song et al., 2014), antifatiga (Jiang et al., 2013), antihiperlipidémicos y antiateroscleróticos (Chen et al., 2005).

La investigación fitoquímica ha puesto de manifiesto que hay muchos componentes activos, tales como flavonoides, alcaloides, polisacáridos, compuestos fenólicos y esteroides en las hojas de morera (Doi et al., 2001; Thabti et al., 2012).

Morus alba posee numerosas propiedades farmacológicas como por ejemplo, antioxidante, antimicrobial, blanqueamiento de la piel, citotóxicas, antiinflamatoria, antidiabética, antihiperlipidémicas, antiaterosclerótica, contra la obesidad, hepaprotectora, y actividades cardioprotectoras (Chan et al., 2016). Además la morera contiene otras propiedades farmacológicas las cuales son antiplaquetaria, ansiolítica, antiasmática, antihelmíntica, antidepresiva, inmunomoduladora y cardioprotectora (Chan et al., 2016).

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6. OBJETIVOS

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El objetivo principal de esta Tesis Doctoral fue la caracterización físico-química de frutos y hojas de ocho clones de morera, 4 de *Morus alba* y 4 de *Morus nigra* cultivadas en el sureste español.

Para alcanzar dicho objetivo general se han planteado los siguientes objetivos específicos:

1. Caracterización morfológica de frutos y hojas.
2. Caracterización química de frutos y hojas.
3. Identificación de compuestos bioactivos en hojas y frutos.

Con la finalidad de: (i) conocer el potencial de la morera, un frutal subutilizado como productor de fruta, y (ii) seleccionar aquellos clones que presenten mejores propiedades para su consumo y/o uso industrial.

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7. RESUMEN DE LA METODOLOGÍA

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7. RESUMEN DE LA METODOLOGÍA

El **material vegetal** empleado fueron ocho clones de morera, cuatro de morera blanca: MA1, MA2, MA3 y MA4, y cuatro de morera negra: MN1, MN2, MN3 y MN4. Los 8 clones se encuentran en condiciones in situ, Orihuela (latitud 38 º 04'08" N, longitud 0 º 58'58" W, 27 m sobre el nivel del mar) Alicante (sudeste de España).

En cuanto a la preparación de las muestras, los frutos se recolectaron en su punto óptimo de madurez; e inmediatamente se trasladaron al laboratorio para su análisis. Se cogieron al azar 200 **frutos** por cada clon y año. Una parte de los frutos se destinaron a la caracterización morfológica y química (básica), y otra parte de ellos fueron congelados a -80ºC y posteriormente liofilizados para los análisis de compuestos bioactivos.

Las hojas se recolectaron de todas las orientaciones de los árboles y partes medias de los brotes, y sólo se seleccionaron hojas adultas sanas y sin dañar. Después de la recogida de **hojas**, las muestras se lavaron con agua destilada y se liofilizaron. A continuación se molieron en un polvo fino con un molinillo de café doméstico (Moulinex, Écully, Francia) (partícula tamaño de <0,4 mm) y se almacenaron en un recipiente hermético a 20°C.

Los **parámetros morfológicos** estudiados en el **fruto** fueron los siguientes: peso del fruto (FW), expresado en g, diámetro ecuatorial; (DE) expresado en mm, altura de fruto (FH), expresada en mm, altura de fruto con pedúnculo (FHp) expresado en mm, altura del pedúnculo (PL) expresado en mm. El diámetro, la altura de los frutos y longitud del pedúnculo se midieron con un calibre digital marca Mitutoyo (modelo CD-15 DC, con 0,01 mm de precisión). Las determinaciones de **color** se hicieron en cada **fruto** en tres puntos de la zona ecuatorial, se evaluaron de acuerdo con la Comisión Internationale de l'Éclairage (CIE) y se expresaron como L* (brillo y ligereza; 0 = negro, 100 = blanco), a* (-a* = verdor, +a* = enrojecimiento), y b* (-b* = azulado, + b* = amarillez). Estos valores se utilizaron para calcular el ángulo Hue [Hº* = arctang (b*/a*)], donde 0º = rojo-púrpura, 90º = amarillo, 180º = color verde azulado y 270º = azul, y el croma [C* = (a* 2 + b* 2) $^{1/2}$], lo cual es indicativo de la intensidad o de la saturación de color. Como sugiere McGuire (1992), el matiz (hº*) y el croma (C*) han sido aceptados como formas intuitivas y comprensibles de variables de color. Se

midieron las variables de color utilizando un colorímetro Minolta Chroma modelo C-300 acoplado a un procesador de Minolta DP-301 de datos.

Los **parámetros químicos** estudiados en el **fruto** fueron: sólidos solubles totales, pH, acidez valorable total, humedad (M), fibra cruda (CF), el perfil de ácidos orgánicos, azúcares, elementos minerales y el perfil de ácidos grasos. En el **fruto** y la **hoja** fueron fenoles totales, compuestos fenólicos y actividad antioxidante.

Para las determinaciones analíticas de los **sólidos solubles totales, pH y acidez valorable total** 60 frutos fueron seleccionados, tres submuestras por clon (cada uno compuesto por 20 frutos) se escogieron al azar, y los frutos de cada submuestra se exprimieron a mano con una malla. Para evitar cualquier reacción de oxidación al zumo se añadió 150 mL/kg de $CaCl_2$ (Alonso et al., 2009). Los zumos recién exprimidos fueron centrifugados a 15.000 rpm durante 20 min en una centrífuga (Sigma 3-18K) y se mantuvieron en el congelador a una temperatura de -18ºC hasta su análisis.

Los sólidos solubles totales (TSS) fueron medidos con un refractómetro digital (Atago N1) a 20°C, y los resultados se expresaron en °Brix. La acidez total valorable (TA) se determinó con un valorador de acidez automático (877 Titrino plus, CH9101) con NaOH 0,1 N hasta pH 8,1 usando 1 mL de zumo diluido en 25 mL de H_2O destilada, y los resultados se expresaron como gramos de ácido cítrico por litro de zumo. A continuación, se determinó el MI (Índice de Madurez) que se calculó como la relación entre TSS/TA. Todas las determinaciones se realizaron por triplicado.

El porcentaje de **humedad (M)** del **fruto** se determinó mediante el secado del material en un horno de aire caliente a 50ºC hasta alcanzar peso constante. El contenido de **fibra cruda (CF)** se determinó con un analizador de fibra Ankon (modelo A220), siguiendo la metodología oficial establecida por el Ministerio de Agricultura, Pesca y Alimentación (MAPA, 1993). Los resultados se expresaron en g/100 g de peso seco (dw). Los análisis se realizaron por triplicado.

La determinación del perfil de **ácidos orgánicos** en los **frutos**, se determinó mediante un cromatógrafo Hewlett-Packard 1100. El sistema de elución consistió en ácido fosfórico al 0,1% con un caudal de 0,5 mL/min. Los ácidos orgánicos se separaron en una columna Supelcogel TM C-610H (30 cm x 7,8 mm id, Supelco) acoplada a una precolumna Supelguard (5 cm x 4,6 mm, Supelco), y se detectó mediante un detector de diodos a 210 nm.

Para los **análisis de azúcares**, en los **frutos** se utilizó el mismo equipo de HPLC, sistema de elución, velocidad de flujo, y las columnas. La detección de los azúcares se llevó a cabo utilizando un detector de índice de refracción (HP 1100, G1362A). Los resultados se expresaron en % de peso fresco (fw). Los resultados se valoraron por triplicado.

El índice de dulzor (SI) del fruto, es una estimación de la percepción del total de la dulzura, se calculó basándose en la cantidad relativa y las propiedades de dulzor de cada carbohidrato individual (Keutgen y Pawelzik, 2007). El índice de dulzor utilizado para estimar la percepción total de dulzor fue descrito por Baldwin et al. (1998) y Obando-Ulloa et al. (2009) como equivalentes de sacarosa (Suceq) según la fórmula:
Suceq = 1 x [Sacarosa] + 0,74 x [Glucosa] + 1,73 x [Fructosa].

En lo que se refiere a los **fenoles totales**, la extracción de los compuestos fenólicos en los **frutos** y las **hojas** se hizo de acuerdo a la metodología propuesta por González-Barrio et al. (2010) y; Pérez-Gregorio et al. (2011). Brevemente, tanto en **fruto** como en **hoja**, se realizó una mezcla con 200 mg de polvo liofilizado, 1 mL de metanol acuoso al 80%, acidificado con ácido fórmico 1% y se sonicó durante 25 min. La mezcla se centrifugó a 10.480 g durante 5 min. (model EBA 21, Hettich Zentrifugen, Tuttlingen, Germany) a temperatura ambiente. Se realizaron dos extracciones adicionales para cada muestra con 0,5 mL adicionales del mismo disolvente, después de lo cual se centrifugaron. Los tres sobrenadantes se combinaron y se filtraron a través de un filtro 0,45 μm de fluoruro de polivinilideno (PVDF) (Millex HV13, Millipore, Bedford, MA, USA) previo a los análisis por HPLC.

El contenido de **fenoles totales (TPC)** se determinó por el método del reactivo *Folin-Ciocalteau* adaptado a microescala en los **frutos** y en las **hojas**, de acuerdo al protocolo descrito por Mena et al. (2013). Los **TPC** se evaluaron con la medición de la variación de la absorbancia a 765 nm después de 1 hora de reacción. Los ensayos se midieron mediante el uso de microplacas de 96 pocillos (Nunc, Roskilde, Denmark) y un lector de placas Infinite® M200 (Tecan, Grödig, Austria). Los resultados se expresaron como mg de ácido gálico equivalente (GAE) por gramo de material seco.

La identificación y cuantificación de **compuestos fenólicos** mediante **análisis HPLC** en **frutos** y **hojas,** se llevó a cabo en una columna Luna C18 (250 × 4,6 mm, 5 mm tamaño de partícula; Phenomenex, Macclesfield, UK) con pre-columna C18-ODS (4,0 × 3,0 mm) cartridge system (Phenomenex, Macclesfield, UK). Se utilizaron como

fases móviles A: Agua/ácido fórmico (95:5, v/v) y B: acetonitrilo, con una velocidad de flujo de 1 mL/min. El gradiente se inició en **frutos** con 1% de disolvente B, manteniendo condiciones isocráticas durante 5 min, alcanzando el 22% del disolvente B en 55 min, y el 40% a los 60 min, manteniéndolo hasta el minuto 70. Mientras que en **hojas** el gradiente se inició con 8% de disolvente B, alcanzando el 15% del disolvente B en 25 min, el 22% a los 55 min, y 40% a los 60 min, manteniéndolo hasta el minuto 70. El volumen de inyección fue de 20 µL en **frutos** y **hojas.** Las muestras se procesaron en un sistema de análisis cromatográfico Merck-Hitachi D-7000 HSM. Los cromatogramas se registraron en **frutos** a 280, 320, 360, y 520 nm y en **hojas** 280, 320 y 360 nm. La comparación cromatográfica se realizó con patrones analíticos y, espectros de absorbancia, utilizando espectros de masas de experimentos MS^n para confirmar la identificación de algunos de los compuestos y en base a metodologías anteriores en **frutos** (Juan et al., 2012; Memon et al., 2010; Wang et al., 2013) y en **hojas** (Dugo et al., 2009; Memon et al., 2010; Wang et al., 2013). Los compuestos se cuantificaron por las absorbancias de sus respectivos picos. Los derivados del ácido benzoico se cuantificaron como ácido gálico (se detectaron a 280 nm), los derivados de ácido cinámico como el ácido clorogénico (detectado a 320 nm), los flavonoles como la rutina (a 360 nm), y las antocianinas como cyanidin-3-glucoside (a 520 nm).

La **actividad antioxidante** tanto en **frutos** como en **hojas** se determinó utilizando los métodos *$ABTS^{\bullet+}$ y $DPPH^{\bullet}$* según la metodología desarrollada por Mena et al. (2011). Los ensayos se llevaron a cabo mediante el uso de microplacas de 96 pocillos (Nunc, Roskilde, Denmark) y un lector de microplacas Infinite® M200 (Tecan, Grödig, Austria). Los resultados se expresaron en equivalentes de TEAC en mg/g de materia seca.

La determinación de **elementos minerales** en los **frutos** se llevó a cabo después de una digestión ácida HNO_3—$HClO_4$ (2:1, v/v) del material liofilizado, por espectroscopia de emisión con plasma de acoplamiento inductivo (ICP) (OES Thermo ICAP 6000 SERIES®; ThermoElectron Corp., Franklin, MA, USA), diluyendo el extracto de partes alícuotas con $LaCl_3$ + CsCl, según la metodología descrita por Domínguez-Perles et al. (2010). El total de C y N en las muestras se determinó empleando un autoanalizador Thermo FlashEA 1112 (Thermo Fisher Scientific SA, Madrid, Spain).

En los **frutos** el contenido de **proteína** se calculó a partir del contenido de nitrógeno (%N × 6,25) el cual se analizó por el método de Kjeldahl.

Los **ácidos grasos** en los **frutos** se metilaron *in situ* siguiendo la metodología desarrollada por Trigueros y Sendra (2015) con algunas modificaciones. La composición de ácidos grasos FAMEs se analizó en un cromatógrafo de gases Agilent (model 6890, Palo alto, CA, USA) equipado con un detector de ionización de llama (FID) y una columna capilar DB-23 (30 m de largo, 0,25 μm de espesor de película, 0,25 mm de diámetro interno; J&W Scientific, Agilent Technologies). Los FAMEs fueron identificados por comparación con los tiempos de retención de los estándares de FAMEs, y cuantificados a partir del área y su comparación con el área del patrón interno C17:0. Las concentraciones de ácidos grasos en frutos de morera fueron expresadas como porcentaje del total de ácidos grasos y también se cuantificaron unitariamente en mg/100 g de peso seco (dw). Los análisis se realizaron por triplicado.

En la **determinación del perfil polifenólico,** los compuestos fenólicos en los **frutos** y las **hojas** de morera se extrajeron según el método descrito por Sánchez-Salcedo et al. (2015). Brevemente, se mezcló 200 mg de polvo liofilizado del **fruto** y 1 mL de metanol acuoso al 80% y acidificado con ácido fórmico al 1% y se sonicó durante 25 min. En las **hojas** se mezcló 150 mg de polvo liofilizado de morera y un 1 mL de metanol acuoso al 80% y se acidificó con ácido fórmico (1%) se sonicó durante 60 min. En ambos casos la mezcla se centrifugó a 10.480 *g* durante 5 min a temperatura ambiente y el sobrenadante se recogió. Se realizaron dos extracciones adicionales con 0,5 mL del mismo disolvente, tal y como se describió anteriormente. En los **frutos** después se centrifugaron. En las **hojas** la última extracción se mantuvo a 4°C durante la noche y se sonicó de nuevo durante 25 min, después de lo cual se centrifugó. Tanto en **frutos** como en **hojas** los tres sobrenadantes se combinaron antes del análisis UHPLC-MS. Cada muestra se extrajo por triplicado.

En la **caracterización por cromatografía líquida acoplada a espectrometría de masas** en **frutos** y en **hojas,** los extractos metanólicos de polvo del **fruto** y la **hoja** de morera se analizaron utilizando un cromatógrafo líquido a ultra-alta presión Accela UHPLC 1250 equipado con un espectrómetro de masas (MS) de trampa iónica lineal (MS) (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) equipado con fuente de ionización por electrospray (H-ESI-II; Thermo Fisher Scientific Inc., San Jose, CA, USA). Las separaciones se realizaron usando una columna BlueOrchid C18

(50x2 mm), de 1,8 μm de tamaño de partícula (Knauer, Berlin, Germany). El volumen de inyección fue de 5 μL y la temperatura del horno de la columna se fijó a 30 ºC. En los **frutos** se realizaron tres experimentos de masas, dos en modo negativo y uno en modo positivo, y en las **hojas** dos experimentos de masas: en modo de iones negativos (Mena et al., 2012) (publicación 5 y 6). Cada muestra se analizó por duplicado.

Respecto al **tratamiento estadístico**, los datos se analizaron mediante los programas estadísticos SPSS 20.0 para Windows (SPSS Science, Chicago, IL, USA) y Statgraphics Plus 5.0 (Manugistics, Inc., Rockville, MD). Se realizó un análisis estadístico descriptivo básico. A continuación se efectuó un análisis de la varianza (ANOVA) para las comparaciones de medias. El método utilizado para discriminar medias fue el Test de Rango Múltiple de Fisher de las diferencias mínimas significativas (LSD) a un nivel de confianza del 95,0%. Además se realizaron análisis multivariantes, en concreto análisis de componentes principales (PCA) y factoriales, realizando cuando fue preciso, una rotación Varimax. También se efectuó como método de agrupación y clasificación el análisis de Conglomerados (CA), realizando estandarización de datos en los casos que se precisaba, utilizando la distancia euclídea al cuadrado y el método de Ward como medida de disimilitud.

8. PUBLICACIONES

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8.1. PUBLICACIÓN 1

Physicochemical characterisation of eight Spanish mulberry clones: processing and fresh market aptitudes

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Original article

Physicochemical characterisation of eight Spanish mulberry clones: processing and fresh market aptitudes

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Summary The objective of this study was to evaluate and compare, for the first time, white and black mulberry species in terms of their main physicochemical characteristics in eight Spanish clones. The results showed significantly different characteristics between the black and white mulberry species. Fruit weight of mulberry species ranged from 2.10 to 4.15 g, and fruit juice yield, from 41% to 62%. Fructose (~61%) and glucose (~39%) were the predominant sugars in all mulberries. The MN1 clone displayed the highest total acidity (>2.6 g L^{-1}), and malic acid was the most abundant organic acid (6.65 g kg^{-1}). Cluster analysis has allowed grouping of the clones into three groups (i) MN1 and MN2; (ii) MN3 and MN4; and (iii) MA1, MA2, MA3 and MA4. Experimental results proved that Spanish mulberries have high potential for fresh consumption (attractive dark colour in *Morus nigra* clones, high sugars content and intense sweetness) and industrialisation (~50% juice yield, attractive juice colour, high content of crude fibre and intense sweetness). This study is also a step towards identification of this fruit as a potential healthy food, which may also be used in food industry and also have pharmaceutical interest.

Keywords Colour, crude fibre, *Morus alba*, *Morus nigra*, organic acids, sugars.

Introduction

Mulberry is a fast-growing deciduous woody tree, which grows worldwide under different climatic conditions such as tropical, subtropical and temperate (Vijayan, 2010; Butkhup *et al*., 2013). Mulberry tree is present in almost all the continents and has been traditionally cultivated for their leaves [food for silkworms (*Bombyx mori* L.) and animal fodder] and as ornamental trees (Vijayan, 2010); however, today and due to its nutritive value, the mulberry fruit is consumed in both fresh and processed forms (Gundogdu *et al*., 2011). In recent years, mulberry fruit has been used in the production of juices, jams, syrups, beverages, natural dyes and cosmetics (Sánchez, 2000a,b; Ercisli & Orhan, 2005). Besides, consumption of mulberry leaves as infusions is widespread in Asian countries (Thabti *et al*., 2012).

Although more than 68 species have been widely recognised (Datta, 2002), the taxonomy of mulberry is still a matter of great dispute and intense research due the high rate of natural hybridisation among the species (Tikader & Dandin, 2007). Worldwide, of these 68 species, only a few are, mostly white mulberry (*Morus alba* L.), used for sericulture, while a few other species such as red mulberry (*Morus rubra* L.) and black mulberry (*Morus nigra* L.) are used for fresh consumption of fruits (Vijayana *et al*., 2011). However, in Spain, only white and black mulberries are cultivated and therefore will be evaluated in this study.

Recent studies have proved that mulberry fruits produce positive effects on human diet and health due to the presence of healthy or bioactive compounds, such as organic acids, phenolic compounds and sugars (Ercisli & Orhan, 2007; Özgen *et al*., 2009; Gundogdu *et al*., 2011). In Spain, mulberry trees are planted all over the country, but the physicochemical properties of their fruits have not been previously studied. Because mulberry fruit consumption is driven by both fresh market and processing industry requirements, it is crucial to fully characterise the fruits characteristics not only to classify varieties from a botanical point of

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view, but also to check whether they meet current market demands for high-quality products (good sensory properties and high contents of bioactive compounds). Therefore, the aim of this study was to describe and compare, for the first time, the main physicochemical parameters of white and black mulberry species grown in Spain.

Materials and methods

Plant material

Eight mulberry clones, four white ones (*M. alba* L.): MA1, MA2, MA3 and MA4 and four black ones (*M. nigra* L.): MN1, MN2, MN3 and MN4, were collected in Orihuela (latitude 38°04′08″N, longitude 0°58′58″W, 27 m above sea level), Alicante (south-eastern Spain) in 2011 and 2012. Fully matured fruits were manually harvested and transferred to the laboratory for physicochemical analysis. Five trees were selected for each clone, and 60 fruits per clone (12 fruits per tree) were randomly picked every year.

Physicochemical analyses

Once in the laboratory, 60 mulberries were selected per clone for analytical determinations. The mulberries were not stored and directly hand-squeezed. Three subsamples by clone (each one composed of 20 fruits) were randomly prepared, and then, the fruits from each subsample were cut in halves and carefully hand-squeezed using a commercial kitchen juicer; $CaCl_2$ at a rate of 150 mL kg^{-1} was added to prevent any oxidation reactions in the juice (Alonso *et al.*, 2009). The freshly squeezed juices were centrifuged at 10 000 *g* for 20 min (Sigma 3-18K, Osterode, Germany), and they were kept in freezer at temperature at −18 °C until analysis. The following physical fruit parameters were studied: fruit weight (FW) expressed in g, equatorial diameter (DE) expressed in mm, fruit height (FH) expressed in mm, fruit height with peduncle (FHp) expressed in mm, peduncle height (PL) expressed in mm. The diameter, fruit height and peduncle length were measured with an electronic digital slide gauge Mitutoyo (model CD-15 DC, Telford, UK, 0.01 mm accuracy). The fruit weights were taken using a Mettler scale (Mettler AJ50, 0.0001 g accuracy).

The colour determinations were made in each fruit on three opposite sides at the equatorial zone, and they were assessed according to the *Commission Internationale de l'Éclairage* (CIE) and expressed as L^*(brightness or lightness; 0 = black, 100 = white), a^* ($-a^*$ = greenness, $+a^*$ = redness) and b^* ($-b^*$ = blueness, $+b^*$ = yellowness). These values were then used to calculate Hue angle in degree [$H°^*$ = arctang (b^*/a^*)], where 0° = red purple; 90° = yellow, 180° = bluish green and 270° = blue, and chroma [$C^* = (a^{*2} + b^{*2})^{1/2}$], which is indicative of the intensity or colour saturation. As suggested by McGuire (1992), hue angle ($h°^*$) and chroma (C^*) have been accepted as intuitive and understandable colour variables. Colour variables were measured using a Minolta C-300 Chroma Meter (Minolta Corp., Osaka, Japan) coupled to a Minolta DP-301 data processor. The fruit juice yield was determined by AOAC (1984).

Total soluble solids (TSS) were assessed by triplicate with a digital refractometer (Atago N1; Atago Co. Ltd., Tokyo, Japan) at 20 °C and expressed as °Brix. Total titratable acidity (TA) was also determined by triplicate using an automatic titration device (877 Titrino plus, Metrohm ion analyses CH9101, Herisau, Switzerland) with 0.1 N NaOH up to pH 8.1, using 1 mL diluted juice in 25 mL distilled H_2O, and the results were expressed as g citric acid L^{-1}. Once the TSS and TA contents were assessed, the MIs (maturity index) (TSS/TA) of the evaluated clones were determined. All results were shown as mean values ± SE (standard error). The moisture (M) percentage of pulp was determined by drying the material in a hot air oven at 50 °C until reaching constant weight. The crude fibre (CF) contents were determined by a digester , an Ankon fiber analyzer (model A220, Macedon, NY, USA), following the official methodology established by the Spanish Ministry of Agriculture, Fisheries and Food (MAPA, 1993). Results were expressed as g 100^{-1} g DW (dry weight); the DW was determined using the fresh weight and the moisture content previously described. Analyses were conducted in triplicate.

Analysis of organic acids and sugars

A 1-mL aliquot of centrifuged juice was passed through a 0.45-μm Millipore filter, and then, 20 μL was injected into a Hewlett-Packard series 1100 (Wilmington Del.) high-performance liquid chromatography (HPLC). The elution system consisted of 0.1% phosphoric acid with a flow rate of 0.5 mL min^{-1}. Organic acids were separated on a Supelcogel TM C-610H column (30 cm × 7.8 mm i.d., Supelco, Bellefonte, PA, USA) and Supelguard column (5 cm × 4.6 mm, Supelco, Inc.) and detected using a diode array detector set-up at 210 nm. For sugar analyses, the same HPLC equipment, elution system, flow rate and columns were used. The detection of sugars was performed using a refractive index detector (HP 1100, G1362A). Standard curves for pure standards of organic acids (oxalic, citric, malic, succinic, tartaric, fumaric, formic, quinic, lactic, acetic and ascorbic acids) and for sugars (glucose, fructose, sorbitol and sucrose) (Sigma, Poole, Dorset, UK) were used for quantification. Results for both organic acids and sugars were expressed as concentrations g (100 g^{-1}) of fresh weight (FW). Sugars and organic acids were determined in triplicate.

Sweetness index (SI) of the fruit, an estimate of the total sweetness perception, was calculated based on the relative amount and sweetness properties of each individual carbohydrate (Keutgen & Pawelzik, 2007). The sweetness index used to estimate the total sweetness perception was first described by Baldwin *et al.* (1998) and more recently by Obando-Ulloa *et al.* (2009) as sucrose equivalents (Suceq) according to the formula:

$$\text{Suceq} = 1 \times [\text{Sucrose}] + 0.74 \times [\text{Glucose}] + 1.73 \times [\text{Fructose}] \quad (1)$$

Statistical treatment of the data

Statistical analyses were performed using SPSS 20.0 for Windows (SPSS Science, Chicago, IL, USA). A basic descriptive statistical analysis was followed by an analysis of variance (ANOVA) test for mean comparisons. The method used to discriminate among the means (multiple range test) was Fisher's least significant difference (LSD) procedure at a 95.0% confidence level. Principal component analysis (PCA) and cluster analysis (CA) were also performed.

Results and discussion

Physical and chemical determinations

Table 1 shows the results obtained for the characterisation of fruit weight, diameter and height; peduncle length; moisture; and fruit juice yield and juice density. Fruit weight of mulberry species ranged between 2.10 g (MA4) and 4.15 g (MN2). This range agreed quite well with the previous data provided by Turkish researchers (Ercisli & Orhan, 2007), range of 2.14–5.05 g. The variation of fruit weight and size in mulberry could be attributed to different species, cultivars, rootstocks, environmental conditions and the nutritional status of the orchards (Ercisli & Orhan, 2007, 2008). Among the analysed clones, the smallest fruits were found in MA4 (12.2 mm $ø_{equatorial}$ and 23.2 mm fruit height) and MN4 (12.9 mm $ø_{equatorial}$ and 21.5 mm fruit height) (Table 1). Moisture content of fresh mulberry samples ranged from 61.9% (MN3) to 78.5% (MN1). The highest moisture contents were observed in MN1, MA2 and MA3, while the lowest value was found in MN3. The values differed significantly ($P < 0.05$) from each other, except clones MA1, MA4 and MN2, which were statistically similar. According to Ercisli & Orhan (2007), the common mulberry species grown in Turkey presented moisture contents from 71.5% to 74.6%, and these values were within the range found in the Spanish mulberries. However, Imran *et al.* (2010) reported higher values of moisture up to 82.40%.

The fruit juice yield of mulberry clones ranged between 41.0% (MA4) and 62.0% (MA2). Ercisli &

Table 1 Morphological and physical properties of mulberry fruits

	Cultivars							
	White mulberry (*Morus alba*)				Black mulberry (*Morus nigra*)			
Parameters	MA1	MA2	MA3	MA4	MN1	MN2	MN3	MN4
Fruit weight (g)	3.19 ± 0.11^{c}*	3.03 ± 0.10^{bc}	2.8 ± 10.12^{b}	2.10 ± 0.06^{a}	3.49 ± 0.09^{d}	4.15 ± 0.11e	2.89 ± 0.10^{b}	2.26 ± 0.05^{a}
Ø Equatorial (mm)	13.3 ± 0.2^{b}	13.3 ± 0.2^{b}	15.6 ± 0.2^{e}	12.2 ± 0.1^{a}	13.9 ± 0.1^{c}	14.4 ± 0.2d	14.3 ± 0.2d	12.9 ± 0.1^{b}
Fruit height (mm)	25.9 ± 0.5^{e}	24.5 ± 0.5^{d}	20.5 ± 0.5^{a}	23.2 ± 0.4^{c}	27.7 ± 0.4^{f}	30.3 ± 0.4g	22.6 ± 0.5^{bc}	21.6 ± 0.3^{ab}
Fruit height with peduncle (mm)	31.1 ± 0.6^{c}	30.3 ± 0.5^{c}	25.0 ± 0.6^{a}	28.2 ± 0.5^{b}	33.3 ± 0.4d	36.4 ± 0.4^{e}	28.5 ± 0.7^{b}	28.5 ± 0.5^{b}
Peduncle length (mm)	5.16 ± 0.22^{abc}	5.80 ± 0.27^{cd}	4.55 ± 0.23^{a}	5.03 ± 0.18^{ab}	5.68 ± 0.24^{bcd}	6.09 ± 0.20^{d}	5.99 ± 0.32^{b}	6.97 ± 0.30^{e}
Moisture (%)	73.2 ± 0.6^{c}	76.5 ± 0.2^{d}	76.4 ± 0.4^{d}	72.6 ± 0.3^{c}	78.5 ± 0.9^{d}	71.0 ± 1.6^{c}	61.9 ± 1.4^{a}	66.9 ± 0.1^{b}
Fruit juice yield (%)	55.8 ± 0.4^{b}	62.0 ± 0.6^{c}	44.0 ± 2.9^{a}	41.0 ± 1.5^{a}	58.5 ± 0.3^{bc}	59.2 ± 0.6^{bc}	41.3 ± 1.5^{a}	44.7 ± 1.5^{a}
Juice density (kg L^{-1})	0.92 ± 0.01^{a}	1.02 ± 0.01^{b}	1.06 ± 0.01^{c}	1.08 ± 0.01^{c}	1.21 ± 0.01^{e}	1.06 ± 0.01^{c}	1.12 ± 0.02^{d}	1.09 ± 0.01^{cd}

*Values (means ± SE) ($n = 60$) followed by the same letter, within the same row, are not significantly different according to Fisher's least significant difference (LSD) procedure at 5% significance level.

Table 2 Fruit and juice CIE*L*a*b** colour coordinates of mulberries

	Cultivars							
	White mulberry (*Morus alba*)				**Black mulberry (*Morus nigra*)**			
Parameters	**MA1**	**MA2**	**MA3**	**MA4**	**MN1**	**MN2**	**MN3**	**MN4**
Fruit								
*L**	57.3 ± 0.9^{e}*	51.6 ± 1.8^{d}	60.1 ± 0.9 ^{ef}	62.5 ± 1.4 f	23.9 ± 0.1^{a}	27.3 ± 0.8^{ab}	38.9 ± 1.2^{c}	29.1 ± 1.2^{b}
*a**	2.13 ± 0.47^{bc}	4.39 ± 0.38^{d}	−1.78 ± 0.41^{a}	2.84 ± 0.35^{cd}	0.89 ± 0.21^{b}	3.29 ± 0.56^{cd}	9.50 ± 0.31^{f}	7.44 ± 0.71^{e}
*b**	18.5 ± 1.1^{d}	19.2 ± 1.1^{d}	27.4 ± 0.9^{f}	23.6 ± 0.6 e	−0.88 ± 0.08^{a}	1.56 ± 0.60^{ab}	11.2 ± 0.9^{c}	3.59 ± 0.68^{b}
*C**	18.7 ± 1.1^{d}	19.7 ± 0.9^{d}	27.4 ± 0.9^{f}	23.8 ± 0.6 e	1.34 ± 0.15^{a}	3.91 ± 0.68^{a}	14.9 ± 0.7^{c}	8.39 ± 0.93^{b}
*h**	84.4 ± 1.8^{c}	76.4 ± 1.78^{bc}	93.5 ± 0.8^{c}	83.1 ± 0.9^{c}	310 ± 7^{d}	107 ± 43^{c}	47.4 ± 2.5 a,b	22.2 ± 2.1^{a}
Juice								
*L**	37.8 ± 0.4^{f}	36.8 ± 0.5^{e}	36.5 ± 0.1^{e}	35.5 ± 0.4^{d}	31.9 ± 0.1^{b}	32.7 ± 0.1^{b}	33.7 ± 0.1^{c}	31.1 ± 0.1^{a}
*a**	0.58 ± 0.05^{c}	0.30 ± 0.16^{b}	−1.04 ± 0.04^{a}	1.00 ± 0.06^{e}	0.66 ± 0.02^{c}	0.56 ± 0.04^{c}	0.91 ± 0.04 de	0.71 ± 0.04^{cd}
*b**	4.04 ± 0.32^{d}	2.68 ± 0.35^{c}	5.21 ± 0.16^{e}	5.26 ± 0.45^{e}	−1.82 ± 0.05^{a}	−1.38 ± 0.10^{a}	2.62 ± 0.13^{c}	0.34 ± 0.06^{b}
*C**	4.08 ± 0.31^{d}	2.71 ± 0.33^{c}	5.31 ± 0.17^{e}	5.36 ± 0.43^{e}	1.94 ± 0.05^{b}	1.49 ± 0.09^{ab}	2.77 ± 0.11^{c}	0.79 ± 0.06^{a}
*h**	81.6 ± 1.1^{c}	82.4 ± 5.0^{c}	101 ± 1^{d}	78.9 ± 1.6^{c}	289 ± 1^{e}	292 ± 2^{e}	70.7 ± 1.4^{b}	25.0 ± 2.9^{a}

*Values (means ± SE) (n = 180) followed by the same letter, within the same row, are not significantly different according to Fisher's least significant difference (LSD) procedure at 5% significance level.

Orhan (2008) reported higher levels of juice yield up to 68.6%. This information is quite relevant if mulberries are aimed for juice manufacturing.

The colour of food has always been an important quality attribute. The values of both the fruit and juice colour are shown in Table 2. The fluctuations of the fruit colour coordinates were as follows: *L** values, from 23.9 (MN1) to 62.5 (MA4), *a** values from −1.78 (MN3) to 9.50 (MN3), *b** values from −0.88 (MN1) to 27.4 (MA3), *C** values from 1.34 (MN1) to 27.4 (MA3) and *h** values from 22.2 (MN4) to 311 (MN1). Ercisli & Orhan (2007) and Özgen *et al.* (2009) obtained lower values of *L** in Turkish clones of *Morus nigra*. The instrumental colour coordinates of the juice were significantly different from those of the fresh fruits (Table 2). MN4 clone had the darkest juice, while MA1 had the brightest one among all studied mulberry clones. The *a** and *b** coordinates, which indicate redness and yellowness, respectively, showed significant differences ($P < 0.05$). MA4 had the highest redness value, while MA3 had the lowest one. For yellowness (*b**), MA3 and MA4 clones showed the highest values, whereas MN1 and MN2 clones presented the lowest values (Table 2). Regarding chrome (*C**), which represents the 'purity' or colour intensity, the clones MN4 and MN2 showed the lowest colour intensity, while the clones MA3 and MA4 were characterised by the highest *C** values. Large significant differences ($P < 0.05$) among the clones were reported in the hue angle (*h**) of the juices, which ranged from 25.0° (MN4) to 292° (MN2).

pH values of the mulberry juice were clone dependent ($P < 0.05$) and ranged from 5.95 (MN1) to 7.39 (MN3). However, slightly lower pH values (3.35–5.60) were previously reported (Ercisli & Orhan, 2007; Imran *et al.*, 2010). Total soluble solids (TSS) were significantly affected by clone ($P < 0.05$) and ranged between 12.0 °Brix (MN1) and 25.8° Brix (MN3). These results are in agreement with those previously reported in the scientific literature (Ercisli & Orhan, 2007, 2008); the only exception were MN3 and MN4 clones, which juices were extremely sweet and had very high values of total soluble solids. Even though statistically significant differences were found in the titratable acidity (TA), the differences among extreme value were not too high; the values ranged from 0.93 to 2.65 g citric acid *per* litre. The MN1 clone had significantly higher titratable acidity (>2.6 g citric acid L^{-1}) than other clones (Table 3). The values of the titratable acidity found in the present study agreed well with previously reported data (Elmaci & Altug, 2002; Ercisli & Orhan, 2007; Imran *et al.*, 2010); however, Darias-Martin *et al.* (2003) obtained higher values, 16.2–28.1 g citric acid L^{-1}.

The maturity index (MI = TSS/TA) has an evident influence on the mulberry taste and flavour. MI varied considerably from 46.6 in MN1 clone to 278 in MN3 clone. According to the results, all clones under study were sweet enough and may be recommended for fresh consumption.

Sugar and organic acid content

Sugars are an important food constituent and an instant source of energy for the body activities. A high sugar level in a fruit also serves as an index of maturity. Recent studies (Ercisli & Orhan, 2007; Özgen *et al.*, 2009) have proved that mulberries had beneficial effects on human health due to their elevated contents of bioactive compounds, including

Table 3 Chemical properties of mulberry fruits

	Cultivars							
	White mulberry (*Morus alba*)				Black mulberry (*Morus nigra*)			
Parameters	MA1	MA2	MA3	MA4	MN1	MN2	MN3	MN4
pH	7.20 ± 0.08 ^{bcd}*	7.05 ± 0.07^{bcd}	6.99 ± 0.06^{bc}	7.26 ± 0.09^{bcd}	5.95 ± 0.10 a	6.89 ± 0.10^{b}	7.39 ± 0.17^{d}	7.29 ± 0.23^{cd}
Total soluble solids (°Brix)	16.9 ± 0.2^{c}	15.6 ± 0.4^{b}	15.1 ± 0.2^{b}	17.4 ± 0.2^{cd}	12.0 ± 0.3 a	18.0 ± 0.2^{d}	25.8 ± 0.1 f	24.1 ± 0.2 e
Total acidity (g citric acid L^{-1})	0.97 ± 0.02^{a}	1.17 ± 0.08^{a}	1.08 ± 0.01^{a}	0.97 ± 0.04^{a}	2.65 ± 0.31^{c}	1.81 ± 0.09^{b}	0.93 ± 0.03^{a}	0.94 ± 0.02^{a}
Maturity index	175 ± 2 d	135 ± 9^{c}	139 ± 3^{c}	179 ± 8 d	46.6 ± 4.9^{a}	100 ± 6^{b}	278 ± 11^{f}	256 ± 4^{e}
Glucose (g 100 g^{-1})	4.88 ± 0.02^{c}	4.22 ± 0.12^{b}	5.05 ± 0.12^{cd}	5.37 ± 0.18^{d}	3.19 ± 0.12^{a}	4.91 ± 0.07^{c}	7.45 ± 0.13^{e}	7.21 ± 0.03^{e}
Fructose (g 100 g^{-1})	7.41 ± 0.06^{c}	6.53 ± 0.19^{b}	7.68 ± 0.19^{c}	8.55 ± 0.27^{d}	4.82 ± 0.13^{a}	7.59 ± 0.10^{c}	11.7 ± 0.2^{e}	11.4 ± 0.1^{e}
Quinic acid (g 100 g^{-1})	0.40 ± 0.01^{a}	0.46 ± 0.03^{ab}	0.71 ± 0.03^{c}	0.83 ± 0.04^{d}	0.37 ± 0.02^{a}	0.51 ± 0.01^{b}	0.84 ± 0.04^{d}	0.83 ± 0.04^{d}
Formic acid (g 100 g^{-1})	0.02 ± 0.01^{a}	0.02 ± 0.01^{a}	0.02 ± 0.01^{a}	0.06 ± 0.01^{c}	0.02 ± 0.01^{a}	0.03 ± 0.01^{ab}	0.03 ± 0.01^{ab}	0.04 ± 0.02^{bc}
Fumaric acid (g 100 g^{-1})	0.06 ± 0.01^{cd}	0.05 ± 0.01^{c}	0.03 ± 0.01^{b}	0.07 ± 0.01^{d}	0.01 ± 0.01^{a}	0.06 ± 0.01^{cd}	0.05 ± 0.01^{c}	0.07 ± 0.01^{d}
Citric acid (g 100 g^{-1})	0.04 ± 0.01^{a}	0.14 ± 0.01^{c}	0.07 ± 0.01^{b}	0.18 ± 0.01^{d}	0.66 ± 0.02^{f}	0.25 ± 0.01^{e}	0.14 ± 0.01^{c}	0.18 ± 0.01^{d}
Malic acid (g 100 g^{-1})	0.66 ± 0.01 ^{ab}	0.58 ± 0.03^{ab}	0.68 ± 0.02^{ab}	0.79 ± 0.03^{b}	0.41 ± 0.01^{a}	0.69 ± 0.01^{ab}	0.72 ± 0.36^{ab}	0.79 ± 0.03^{b}
Tartaric acid (g 100 g^{-1})	0.02 ± 0.01^{a}	0.03 ± 0.01^{bc}	0.03 ± 0.01^{ab}	0.03 ± 0.01^{ab}	0.04 ± 0.01^{bc}	0.04 ± 0.01^{bc}	0.04 ± 0.01^{c}	0.03 ± 0.01^{ab}
Crude fibre (g 100 g^{-1} DW)	1.95 ± 0.15 ^{abc}	1.84 ± 0.15^{ab}	1.72 ± 0.12^{ab}	2.32 ± 0.16^{d}	1.68 ± 0.28^{ab}	2.10 ± 0.06^{cd}	1.95 ± 0.04^{abc}	1.56 ± 0.10^{a}
Sweetness index	21.9 ± 0.2^{c}	19.2 ± 0.6^{b}	22.7 ± 0.5^{c}	25.0 ± 0.8^{d}	14.3 ± 0.4^{a}	22.4 ± 0.3^{c}	34.4 ± 0.7^{e}	33.5 ± 0.1^{e}

*Values (means ± SE) (n = 3) followed by the same letter, within the same row, are not significantly different according to Fisher's least significant difference (LSD) procedure at 5% significance level.

sugars and organic acids. Individual sugar and organic acid contents of mulberries are presented in Table 3. The predominant sugar was fructose (~61%) followed by glucose (~39%), while sucrose was presented only at trace level. Fructose and glucose were significantly higher in fruits of clones MN3 and MN4 than in fruits of other clones. Experimental results showed that the sugar contents of Spanish *Morus* species were higher than those reported for *Morus* species from Pakistan and Turkey (Elmaci & Altug, 2002; Imran *et al*., 2010; Gundogdu *et al*., 2011); these differences may be due to the different genotypes and environmental and geological conditions. However, the presence of important amounts of sugars in mulberries should encourage their use as natural sugar sources in different food recipes. The presence of sucrose at trace levels may be due to the state of maturity of the berries, because the concentration of sucrose decreases regularly in mulberries cultivars as the maturity progresses (Mahmood *et al*., 2012).

A proper equilibrium between organic acids and sugars is essential for the attractive flavour of fruits and vegetables (Cemeroglu *et al*., 2004). Organic acids have an important influence on the taste because of their presence reduces the excessive sweetness of some mulberry cultivars, favouring their sourness. The predominant organic acid was malic acid, generally followed by quinic, citric, tartaric, fumaric and formic acids. Considerable different contents of organic acids have been reported by various authors (Koyuncu, 2004; Özgen *et al*., 2009; Gundogdu *et al*., 2011; Mahmood *et al*., 2012). The differences between clones in terms of sugars and organic acids might be caused by genetic factors as well as farming practices and ecological factors (temperature, light, humidity, etc.) (Gundogdu *et al*., 2011).

The crude fibre content ranged in a narrow 1.56 (MN4) and 2.32 (MA4) g 100 g^{-1} DW. These values are slightly higher than those reported by Imran *et al*. (2010). These results showed that Spanish mulberries are also a good source of fibre.

Finally, a total sweetness index concept was used to assess fruit sweetness as sucrose equivalent (Suceq) (Table 3). *Morus nigra* L. clones MN4 and MN3 had the highest total sweetness index (>33.5 Suceq) followed by *M. alba* L. clone MA4. Mahmood *et al*. (2012) studied the total sweetness index of Pakistani mulberries and obtained lowest values (11.0 for *M. alba* L. and 11.2 for *M. nigra* L.). These results showed that some Spanish mulberries could be an important source of carbohydrates and hence energy.

Principal component analysis

To achieve a better understanding of the trends and relationships among the many studied variables (32) for the different mulberry samples (8 clones), principal

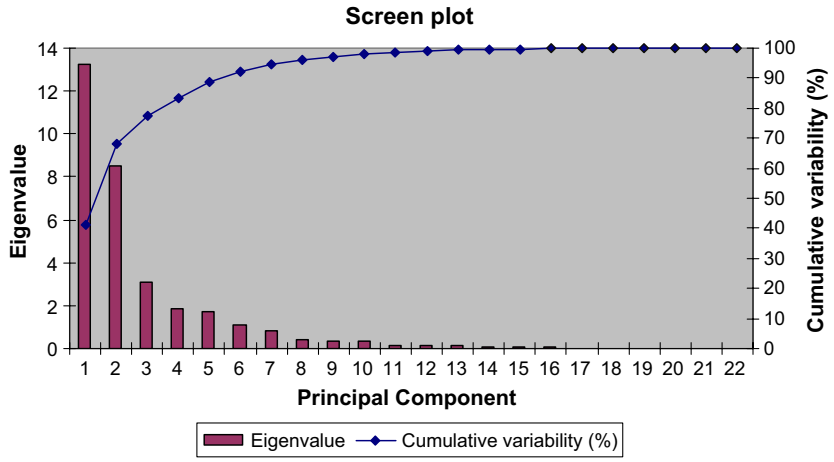

Figure 1 Principal component screen plot.

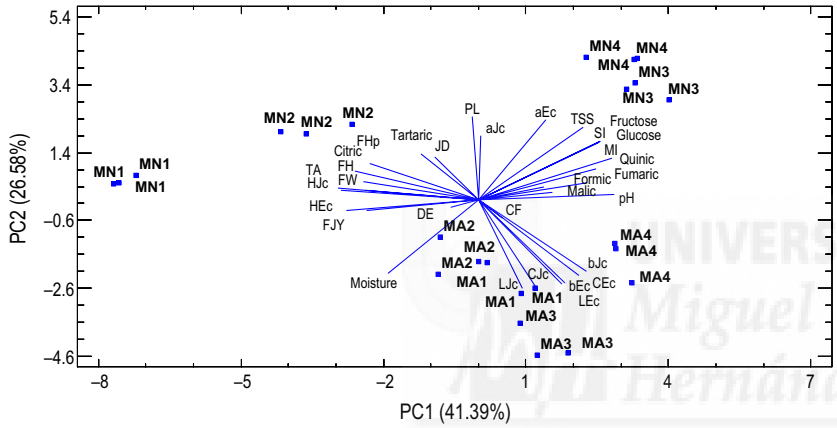

Figure 2 Principal component analysis (PCA) of physical and chemical parameters of mulberry fruits.

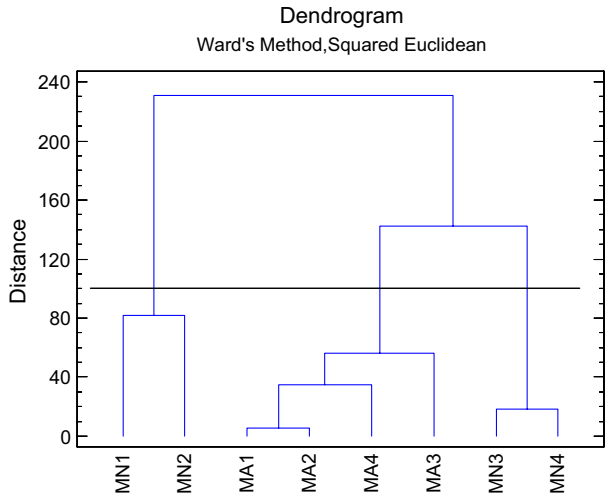

Figure 3 Dendrogram of the eight mulberry fruit clones using Ward's method based on squared Euclidean distance from physical and chemical parameters.

component analysis (PCA) was initially applied to the results obtained in the current research.

The first six principal components explained >90% of the total variation (Fig. 1). Nearly a 77.6% of the observed variability (Fig. 1) was explained by the first three components regarding physical and chemical parameters. The first component, PC1 representing 41.39% of total variance, was positively linked to pH, maturity index, sugars and sweetness index and negatively to total acidity, hue angle and citric acid (Fig. 2). PC2 accounted for 26.58% of the total variance. It was positively correlated with total soluble solids and colour coordinates of both fruit and juice, but inversely with the moisture content (Fig. 2). PC3 accounted for 9.63% of the total variance.

The biplot (Fig. 2) showed that some clones as MN4 and MN3 were relatively close along the *x*-axis (PC1). Clones MN1 and MN2 had large negative scores on the PC1, and they were separated from the other clones across PC1. They were characterised by high acidity and large fruit size. MA1, MA2, MA3 and MA4 clones had large negative scores on the PC2

axis and were linked with moisture, fruit colour and juice colour (Fig. 2).

The results obtained from hierarchical CA, using linkage method between groups, are shown as a dendrogram (Fig. 3), where three main groups were clustered. The first group consisted of two clones (MN1 and MN2), the second one also included two clones MN3 and MN4, and the third and last group included all *M. alba* L. clones (MA1, MA2, MA3 and MA4). Within the group of the *M. alba* L. clones, MA1 and MA2 were close, showing very similar characteristics.

Conclusions

This study is likely to provide the first set of data on the physicochemical properties of Spanish mulberry fruits. Black and white mulberry species displayed significantly different characteristics. The variation observed in the eight clones of mulberry fruits was mainly due to genotype-specific differences because trees were grown in the same soil and under the same weather conditions. Cluster analysis has allowed classifying the eight clones into three different groups (i) MN1 and MN2; (ii) MN3 and MN4; and (iii) MA1, MA2, MA3 and MA4. All mulberry clones studied showed very promising and interesting physicochemical properties for both fresh consumption and industrialisation. The final classification of the mulberry fruits for fresh consumption or industrialisation would be different according to the classification parameter. For instance, if the appropriateness for fresh consumption is based on 'appearance' (heavy fruit weight and attractive colour), the best clones would be MN1, MN2, MA1 and MA2; however, if classification is based on flavour (high intensity of sweetness), the best cultivars would be MN3, MN4, MA3 and MA4. Consequently, it is crucial to have information about the requirements and needs of consumers on this particular fruit. This study is the first step towards the identification of these fruits as potential healthy foods, which may also be used in food and pharmaceutical industries. However, further pomological studies are required for selecting which mulberry cultivars/clones are best suited for fresh consumption or industrialisation; those studies should include hedonic tests to link physicochemical properties with consumer acceptance.

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8.2. PUBLICACIÓN 2

Phytochemical evaluation of white (*Morus alba* L.) and black (*Morus nigra* L.) mulberry fruits, a starting point for the assessment of their beneficial properties

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Phytochemical evaluation of white (*Morus alba* L.) and black (*Morus nigra* L.) mulberry fruits, a starting point for the assessment of their beneficial properties

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ABSTRACT

This study evaluated, for the first time, the phenolic content of white (*Morus alba*) and black mulberry (*Morus nigra*) fruits with proven market aptitudes and grown in Spain, one of the main European producers. The antioxidant activity and mineral composition of these promising berry fruits were also assessed. Black mulberry clones showed higher antioxidant activity and amounts of phenolic compounds than white mulberry clones, although a wide intra-species variability was noted, according to principal component analysis. The total anthocyanins varied significantly among clones of *M. nigra*. These results are keys for the design of future dietary intervention studies examining the role of mulberry fruits in disease risk reduction. They can also be used for the development of mulberry derived-products rich in phenolic compounds.

1. Introduction

Over the last years, berry fruits have moved under the spotlight of nutritional research since an overwhelming body of research has highlighted their ability to impact human health and disease prevention (Nile & Park, 2014). Berry fruits comprise a varied range of small edible fleshy fruits (not true berries by the botanical description of their fruit morphology) that are commonly consumed in all Europe and North America not only in fresh and frozen forms but also as processed products. Major berries include the *Rubus* (blackberry, red and black raspberries, cloudberry, loganberry, Arctic bramble, boysenberries, and marionberry), *Ribes* (red and black

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currants), and *Vaccinium* (blueberry, different cranberries, bilberry, and lingonberries) genera. Other important berries are strawberry (*Fragaria* × *ananassa*), elderberries (*Sambucus spp.*), and chokecherry (*Aronia melanocarpa*) (Seeram, 2008). From a phytochemical point of view, a shared characteristic of most of these berries is their high content of phenolic substances (Nile & Park, 2014).

Mulberry belongs to the genus *Morus* of the family Moraceae and it is found in East, West and South-East Asia, South Europe, South of North America, Northwest of South America and some areas of Africa (Watson & Dallwitz, 2007). There are 24 species of *Morus* and one subspecies, with at least 100 known varieties (Ercisli & Orhan, 2007). The most commonly known species in the *Morus* genus are white mulberry (*Morus alba* L.), black mulberry (*Morus nigra* L.) and red mulberry (*Morus rubra* L.) (Gundogdu, Muradoglu, Gazioglu-Sensoy, & Yilmaz, 2011). Mulberry tree has been cultivated traditionally for their leaves [food for silkworms (*Bombyx mori* L.) and animal fodder] and as ornamental trees (Vijayan, 2010); however, due to its nutritive value, the mulberry fruit is nowadays consumed in both fresh and processed forms, such as juice, jams, syrups, beverages, natural dyes, or dried fruits (Ercisli & Orhan, 2007; Gundogdu et al., 2011; Sánchez, 2000). Several studies have shown that mulberries may have positive effects on human health, especially in people with type 2 diabetes mellitus, being these effects mainly linked to their phenolic composition (Wang, Xiang, Wang, Tang, & He, 2013). Similarly, mulberry leaves have also shown promising biological effects (Jeszka-Skowron et al., 2014; Wu et al., 2013). Actually, mulberries are a rich source of phenolic compounds including flavonols and phenolic acids, as well as anthocyanins in the case of black and red mulberry fruits (Juan, Jianquan, Junni, Zijian, & Ji, 2012; Özgen, Serçe, & Kaya, 2009; Pawlowska, Oleszek, & Braca, 2008). Attending to the growing demand on high-quality berry fruits and the health-promoting features of mulberries, the identification and quantification of phenolic substances in mulberry fruits is a critical starting point for assessing their biological and nutritional properties. Therefore, the aim of this study was to evaluate for the first time the phenolic content of white and black mulberry fruits grown in Spain, one of the main European producers, and with proven market aptitudes (Sánchez et al., 2014). In addition, the antioxidant activities of these promising berry fruits together with their mineral composition were assessed.

2. Material and methods

2.1. Chemicals

The compounds 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulphonic acid) diammonium salt (ABTS), Folin–Ciocalteau's reagent, chlorogenic acid, and rutin (quercetin 3-β-D-rutinoside) were purchased from Sigma-Aldrich (Steinheim, Germany); cyanidin 3-glucoside from Polyphenols (Sandnes, Norway); formic acid, methanol, acetonitrile, and anhydrous sodium carbonate, all of analytical grade, from Panreac Química S.A (Barcelona, Spain). The compounds 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) and gallic acid were from Fluka Chemika (Neu-Ulm, Switzerland). Milli-Q water used was produced using an Elix®3 Millipore water purification system coupled to a Milli-Q module (model Adventage10) (Molsheim, France).

2.2. Plant materials

Eight mulberry clones, four white (*Morus alba*) clones: MA1, MA2, MA3, and MA4, and four black (*Morus nigra*) clones: MN1, MN2, MN3, and MN4, were collected in Orihuela (latitude 38°04′08″N × longitude 0°58′58″W, 27 m above sea level) Alicante (South-Eastern Spain) in 2013. Fully mature fruits were manually harvested and immediately transported to the lab. Five trees were selected for each clone and sixty fruits per clone (12 fruits per tree) were randomly picked at the commercially ripe stage for analytical determinations. Three subsamples by clone (each one composed by 20 fruits) were randomly prepared, and then fruits from each subsample were freeze-dried and ground to obtain the samples as a fine powder.

2.3. Extraction of phenolic compounds

The phenolic compounds in mulberries were extracted according to previous reports (González-Barrio, Borges, Mullen, & Crozier, 2010; Pérez-Gregorio, Regueiro, Alonso-González, Pastrana-Castro, & Simal-Gándara, 2011). The mixture of 200 mg of freeze-dried powder and 1 mL of 80% aqueous methanol acidified with formic acid (1%) was sonicated for 25 min. The mixture was centrifuged at 10,480 *g* for 5 min (model EBA 21, Hettich Zentrifugen, Tuttlingen, Germany) at room temperature. Two additional extractions were performed for each sample with additional 0.5 mL of the same solvent as described earlier, after which they were centrifuged. The three supernatants were pooled and filtered through a 0.45 μm polyvinylidene fluoride (PVDF) filter (Millex HV13, Millipore, Bedford, MA, USA) previous to HPLC analyses.

2.4. Total phenolic content by Folin–Ciocalteau's reagent

Total phenolic content (TPC) was determined by the Folin–Ciocalteu's reagent method adapted to microscale (Mena, Martí, Saura, Valero, & García-Viguera, 2013). TPC was evaluated by measuring the variation in absorbance at 765 nm after 1 h of reaction. Assays were measured by using 96-well microplates (Nunc, Roskilde, Denmark) and Infinite® M200 micro plate reader (Tecan, Grödig, Austria). Results were expressed as mg of gallic acid equivalents (GAE) per g of dry material.

2.5. HPLC analysis, identification and quantification of phenolic compounds

Chromatographic analyses were carried out on a Luna C18 column (250 × 4.6 mm, 5 mm particle size; Phenomenex, Macclesfield, UK) with a security guard C18-ODS (4.0 × 3.0 mm) cartridge system (Phenomenex). Water/formic acid (95:5, v/v) and acetonitrile were used as mobile phases A and B, respectively, with a flow rate of 1 mL/min. The gradient started with 1% of solvent B, keeping isocratic conditions during 5 min, reaching 22% solvent B at 55 min, and 40% at 60 min, which was maintained up to 70 min. The injection volume was 20 μL. Samples were processed on a Merck-Hitachi D-7000 HSM PC

based chromatography data system. Chromatograms were recorded at 280, 320, 360, and 520 nm. Chromatographic comparison with analytical standards, absorbance spectra, and mass spectra, using MS^n (data not shown) experiments, were used to identify compounds previously reported (Juan et al., 2012; Memon, Memon, Luthria, Bhanger, & Pitafi, 2010; Wang et al., 2013), and were quantified by the absorbance of their corresponding peaks. Benzoic acid derivatives were quantified as gallic acid (detected at 280 nm), cinnamic acid derivatives as chlorogenic acid (detected at 320 nm), flavonols as rutin (at 360 nm), and anthocyanins as cyanidin 3-glucoside (at 520 nm).

2.6. *Antioxidant capacity by DPPH• scavenging activity and ABTS•+ assay methods*

The free radical scavenging activity was determined using $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assays according to Mena et al. (2011). Assays were measured by using 96-well microplates (Nunc, Roskilde, Denmark) and Infinite® M200 micro plate reader (Tecan, Grödig, Austria). Results were expressed as mg trolox equivalents per g of dry material.

2.7. *Determination of mineral elements*

The analysis of mineral elements was carried out after HNO_3—$HClO_4$ (2:1, v/v) acid digestion of the lyophilized material by ICP-spectrometry (OES Thermo ICAP 6000 SERIES®; ThermoElectron Corp., Franklin, MA, USA), diluting the extract aliquot with $LaCl_3$ + CsCl, as reported elsewhere (Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010). The total C and N in the samples were determined in a Thermo FlashEA 1112 autoanalyzer (Thermo Fisher Scientific SA, Madrid, Spain).

2.8. *Determination of protein*

Protein content was calculated from the nitrogen content (%N × 6.25) analyzed by Kjeldahl method.

2.9. *Statistical analysis*

Statistical analyses were performed using SPSS 20.0 for Windows (SPSS Science, Chicago, IL, USA). A basic descriptive statistical analysis was followed by an analysis of variance (ANOVA) test for mean comparisons. The method used to discriminate among the means (multiple range test) was Fisher's least significant difference (LSD) procedure at a 95.0% confidence level. Pearson correlation and principal component analyses (PCA) were also performed.

3. Results and discussion

3.1. *Total phenolic content of mulberry fruits*

The phenolic compounds found in fruits and vegetables have attracted much interest due to their beneficial properties (Moo-Huchin et al., 2014). The total phenolic content (TPC) of mulberry clones is presented in Fig. 1. The TPC of selected mulberry

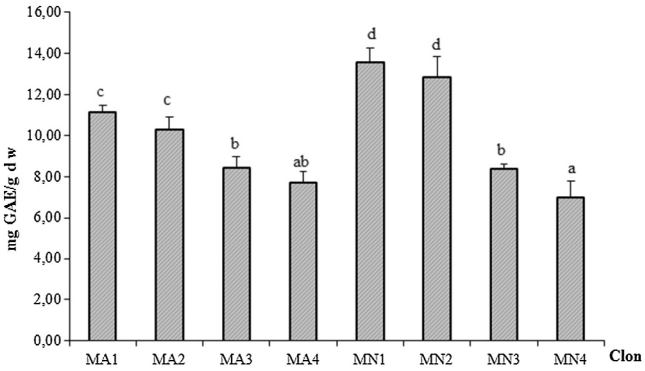

Fig. 1 – Total phenolic compound content in mulberry fruits. Values (means ± SE). Columns marked with different letters are significantly different ($p \leq 0.05$).

clones varied from 6.98 (MN4) to 13.59 (MN1) mg GAE/g dry weight). *M. alba* had the lowest phenolic content (from 7.7 to 11.2 mg GAE/g dw) whereas *M. nigra* clones had the highest levels (from 7.0 to 13.6 mg GAE/g dw). Nonetheless, notable variations among the intra-species clones were recorded, particularly for *M. nigra* clones where the TPC of MN1 was twice the TPC of MN4. Similar values to the ones herein reported have also been found in different genotypes of both *M. alba* and *M. nigra* fruits (Bae & Suh, 2007; Ercisli & Orhan, 2007; Ercisli et al., 2010; Khalid, Fawad, & Ahmed, 2011; Radojković, Zeković, Vidović, Kočar, & Mašković, 2012). However, Lin and Tang (2007) and Imran, Khan, Shah, and Khan (2010) found higher values of total phenolics in mulberry fruit from 1516 to 1650 mg/100 g fw, respectively. The variation of phenolic compounds in berries, as well as in other fruits, depends on many factors, such as degree of maturity at harvest, genetic differences, and environmental conditions during fruit development (Zadernowski, Naczk, & Nesterowicz, 2005). All the same, our results showed that the mulberry fruits of selected clones grown in Spain could be good sources of phenolic constituents and they deserved special attention focused on the study of their phytochemical profile.

3.2. *Phenolic compounds in mulberry fruits*

The phenolic compounds detected in the present work for *Morus alba* and *Morus nigra* clones were similar to those already reported in previous studies (Butkhup, Samappito, & Samappito, 2013; Gundogdu et al., 2011; Lin & Lay, 2013; Pawlowska et al., 2008). In general, the major phenolic groups were benzoic acid derivatives > cinnamic acid derivatives > flavonols > anthocyanins (Table 1). Only MN1 clone revealed a different pattern, the content in anthocyanins being higher than the amount of flavonols.

Benzoic acid derivatives (protocatechuic acid, *p*-hydroxybenzoic acid, and vanillic acid) were the main class of mulberry phenolics (Table 1). The concentration of these compounds ranged from 0.48 (MN4) to 2.55 (MN2) mg/g dw. The predominant hydroxybenzoic acid in mulberry fruits was protocatechuic acid (ranged from 0.41 (MN4) to 2.22 mg/g dw (MN2)), followed by *p*-hydroxybenzoic acid (from 0.01(MN4) to 0.19 mg/g dw (MN2)), and vanillic acid (from 0.06 (MN4) to 0.14 mg/g dw (MN2)).

Table 1 – Phenolic compounds in mulberry fruits (milligrams per g dry weight).

Phenolic compounds	Clones							
	White mulberry (*Morus alba*)				Black mulberry (*Morus nigra*)			
	MA1	MA2	MA3	MA4	MN1	MN2	MN3	MN4
Protocatechuic acid	2.04 ± 0.12 e	1.97 ± 0.04 e	0.91 ± 0.06 c	0.69 ± 0.02 b	1.17 ± 0.03 d	2.22 ± 0.05 f	0.55 ± 0.03 ab	0.41 ± 0.00 a
p-Hydroxybenzoic acid	0.18 ± 0.10 d	0.14 ± 0.02 bcd	0.07 ± 0.03 abc	0.05 ± 0.01 ab	0.17 ± 0.03 cd	0.19 ± 0.02 d	0.02 ± 0.00 a	0.01 ± 0.00 a
Vanillic acid	0.11 ± 0.00 b	0.11 ± 0.01 b	0.10 ± 0.01 b	0.07 ± 0.00 a	0.14 ± 0.01 c	0.14 ± 0.00 c	0.07 ± 0.00 a	0.06 ± 0.00 a
Total benzoic acid derivatives	*2.33 ± 0.03 f*	*2.23 ± 0.05 f*	*1.08 ± 0.09 d*	*0.81 ± 0.02 c*	*1.49 ± 0.02 e*	*2.55 ± 0.07 g*	*0.65 ± 0.03 b*	*0.48 ± 0.00 a*
Neo-chlorogenic acid (3-CQA)	0.30 ± 0.03 e	0.21 ± 0.02 d	0.01 ± 0.00 a	0.03 ± 0.01 ab	0.44 ± 0.03 f	0.20 ± 0.03 d	0.10 ± 0.01 bc	0.12 ± 0.02 c
Chlorogenic acid (5-CQA)	0.97 ± 0.01 e	0.77 ± 0.03 cd	0.15 ± 0.02 a	0.26 ± 0.03 a	3.18 ± 0.21 f	0.81 ± 0.03 cd	0.35 ± 0.05 ab	0.58 ± 0.05 bc
Caffeic acid	0.01 ± 0.00 a	0.03 ± 0.01 c	0.02 ± 0.00 bc	0.01 ± 0.00 a	0.01 ± 0.00 ab	0.03 ± 0.00 c	0.02 ± 0.00 ab	0.02 ± 0.00 ab
p-Coumaric acid	0.01 ± 0.00 b	0.03 ± 0.00 cd	0.0008 ± 0.00 a	0.02 ± 0.00 bc	0.03 ± 0.01 de	0.02 ± 0.00 bc	0.04 ± 0.00 e	0.02 ± 0.00 bc
Ferulic acid	0.002 ± 0.00 a	0.002 ± 0.00 a	0.02 ± 0.00 cd	0.03 ± 0.00 de	0.01 ± 0.00 bc	0.01 ± 0.00 ab	0.03 ± 0.00 e	0.01 ± 0.00 ab
m-Coumaric acid	0.001 ± 0.00 a	0.01 ± 0.00 b	0.05 ± 0.00 c	0.004 ± 0.00 b	0.06 ± 0.00 d	0.002 ± 0.00 a	0.06 ± 0.00 d	0.01 ± 0.00 b
Total cinnamic acid derivatives	*1.29 ± 0.04 d*	*1.05 ± 0.03 d*	*0.25 ± 0.02 a*	*0.36 ± 0.03 ab*	*3.74 ± 0.22 e*	*1.07 ± 0.06 d*	*0.60 ± 0.06 bc*	*0.75 ± 0.08 c*
Quercetin 3-*O*-rutinoside	0.17 ± 0.01 c	0.18 ± 0.01 c	0.02 ± 0.00 a	0.10 ± 0.01 b	0.93 ± 0.06 e	0.28 ± 0.01 d	0.14 ± 0.01 bc	0.13 ± 0.02 bc
Quercetin 3-*O*-glucoside	0.06 ± 0.00 b	0.10 ± 0.01 c	0.003 ± 0.00 a	0.02 ± 0.00 a	0.18 ± 0.02 d	0.12 ± 0.11 c	0.06 ± 0.01 b	0.05 ± 0.02 b
Kaempferol 3-*O*-rutinoside	0.07 ± 0.01 c	0.14 ± 0.01 e	0.02 ± 0.00 ab	0.04 ± 0.01 b	0.00 ± 0.00 a	0.09 ± 0.00 cd	0.11 ± 0.01 d	0.18 ± 0.02 f
Other flavonol derivatives[a]	0.05 ± 0.00 ab	0.08 ± 0.00 c	0.03 ± 0.00 a	0.06 ± 0.01 bc	0.18 ± 0.02 d	0.07 ± 0.00 bc	0.06 ± 0.01 b	0.06 ± 0.01 b
Total flavonols	*0.36 ± 0.02 c*	*0.51 ± 0.03 de*	*0.07 ± 0.01 a*	*0.22 ± 0.02 b*	*1.29 ± 0.09 f*	*0.56 ± 0.01 e*	*0.37 ± 0.03 c*	*0.43 ± 0.06 cd*
Cyanidin 3-*O*-glucoside	–	–	–	–	1.26 ± 0.03 b	0.02 ± 0.00 a	0.004 ± 0.00 a	0.01 ± 0.00 a
Cyanidin 3-*O*-rutinoside	–	–	–	–	0.42 ± 0.03 b	nd	nd	0.004 ± 0.00 a
Pelargonidin 3-*O*-glucoside	–	–	–	–	0.05 ± 0.01 b	nd	nd	nd
Pelargonidin 3-*O*-rutinoside	–	–	–	–	0.08 ± 0.02 b	0.004 ± 0.00 a	0.002 ± 0.00 a	0.004 ± 0.00 a
Total anthocyanins					*1.88 ± 0.09 b*	*0.03 ± 0.01 a*	*0.01 ± 0.00 a*	*0.03 ± 0.00 a*

Values (means ± SE) followed by the same letter, within the same row, are not significantly different according to Fisher's least significant difference (LSD) procedure at 5% significance level.
nd: not detected.

[a] Other flavonol derivatives account for the content in flavonols detected in minor amounts (quercetin, quercetin–acetylglucoside kaempferol, kaempferol hexoside, kaempferol–acetylglucoside, dihydrokaempferol–hexoside, and dihydrokaempferol–hexoside–pentoside).

Protocatechuic acid has already been reported as the main hydroxybenzoic acid derivative in black mulberry fruits (Zadernowski et al., 2005), as well as gallic acid (Butkhup et al., 2013), which was not detected in the present work. Cinnamic acid derivatives were the second major group of mulberry phenolics. The predominant hydroxycinnamate was chlorogenic acid (5-caffeoylquinic acid, between 0.15 and 3.18 mg/g dw for MA3 and MN1, respectively), followed by neo-chlorogenic acid (3-caffeoylquinic acid), while caffeic acid, *p*-coumaric acid, ferulic acid, and *m*-coumaric acid were present at lower amounts (Table 1). The amount in chlorogenic acids of *Morus nigra* fruits was found to be higher than those of *Morus alba*. Butkhup et al. (2013) described that the concentration of chlorogenic acid in white mulberry ranged from 0.01 to 0.06 mg/g dw, while Gundogdu et al. (2011) reported amounts comprised between 0.1 and 3.1 mg/g fw, in agreement with the data herein reported. Arfan, Khan, Rybarczyk, and Amarowicz (2012) also reported that chlorogenic acid was the dominant acid in sugar-free extracts from *Morus nigra* and *Morus alba* fruits. The concentration of chlorogenic acids may be important when fruits are processed as these compounds are considered to be a preferential natural substrate of the catecholase activity of PPO (polyphenol oxidase) (Wojdylo, Oszmianski, & Bielicki, 2013). Therefore, the relative concentration of these compounds could influence the oxidation and color development processes occurring during technological processing. From health promoting point of view, chlorogenic acids have been recognized by their wide biological properties and have been linked with the health-promoting features of coffee (Del Rio et al., 2013). Considering these facts, it is a point worth mentioning that a serving of 100 g of fresh mulberry fruits from some clones like MN1 could provide the same quantity in chlorogenic acids (~120 mg) as an espresso coffee (Crozier, Stalmach, Lean, & Crozier, 2012).

Regarding flavonol derivatives, the main compounds present in mulberry clones were different glycosylated forms of quercetin and kaempferol (Table 1), the content in quercetin glycosides (from 0.02 to 1.11 mg/g dw for MA3 and MN1, respectively) being higher than the amount of kaempferol-*O*-rutinoside (from 0.00 to 0.18 mg/g dw for MN1 and MN4, respectively). Other flavonol derivatives present at minor concentrations were also identified in most of the samples according to their absorption spectra and mass spectra: quercetin aglycone, quercetin-acetylglucoside kaempferol aglycone, kaempferol hexoside, kaempferol–acetylglucoside, dihydrokaempferol–hexoside, and dihydrokaempferol–hexoside–pentoside. These results were in agreement with data reported for both *M. nigra* and *M. alba* by other authors (Butkhup et al., 2013; Pawlowska et al., 2008; Zhang, Han, He, & Duan, 2008). A high content of flavonol derivatives was found in MN1 (1.29 mg/g dw), MN2 (0.56 mg/g dw), and MA2 (0.51 mg/g dw), whereas the lowest content was found for MA3 (0.07 mg/g dw). The highest content in quercetin 3-*O*-rutinoside (rutin), the main flavonol, was found in MN1 (0.93 mg/g dw) followed by MN2 (0.28 mg/g dw). Although flavonol derivatives were not the major phenolic components of mulberry fruits, they should be taken into consideration when studying the phytochemical profile of *Morus* spp. fruits since they may exert critical beneficial features related to human health (Del Rio et al., 2013).

Anthocyanin pigments are of prominent importance in mulberry fruits because of their dual value. First, they constitute an integral part of the sensory attributes since their levels and various forms pertain directly to the coloration of the final product. On the other hand, they have been claimed to possess diverse biological properties and therefore are considered as secondary metabolites with potential nutritional value (Bae & Suh, 2007). For instance, Chen et al. (2006) demonstrated that mulberry anthocyanins could decrease the *in vitro* invasiveness of lung cancer cells. The results for total anthocyanins varied significantly among clones of *M. nigra* (from 0.01 mg/g dw for MN4 to 1.88 mg/g dw for MN1), while *M. alba* lacks of anthocyanins, as expected (Table 1). MN1 clone showed high levels of cyanidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside compared to the other clones. Cyanidin derivatives were the predominant anthocyanins, while pelargonidin derivatives only accounted for limited amounts. Özgen et al. (2009) reported values of total anthocyanins content in black mulberry up to 0.571 mg/g fw. Kim, Bang, Lee, Seuk, and Sung (1999) showed that several cultivated mulberries and a wild variety contained relatively high levels of total anthocyanin (2.45–3.14 mg/g). In contrast, Park, Jung, and Ko (1997) reported that total anthocyanin contents of matured mulberry ranged from 0.19 to 3.29 mg/g. Thus, anthocyanin contents of mulberry fruits can be somewhat variable, depending on cultivar and maturation (Bae & Suh, 2007). All the same, these results reinforce the role of mulberry fruits as potential sources of functional foods due to the several biological and pharmacological effects of anthocyanins.

3.3. *Antioxidant capacity of mulberry fruits*

The antioxidant capacity of foodstuffs is dictated by the different mechanisms of action of their antioxidant constituents; therefore, this capacity could be evaluated by a variety of methods pertaining to the different mechanisms (Pérez-Jiménez et al., 2008). Consequently, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assays were used to properly evaluate the antioxidant activity of mulberry fruits (Fig. 2).

Significant variation ($p < 0.05$) was found in the antioxidant activity of the studied clones. Mean values varied significantly from 3.84 to 20.73 mg trolox g dw ($ABTS^{\bullet+}$ assay) and from 3.62 to 12.91 mg trolox g dw ($DPPH^{\bullet}$ assay). The

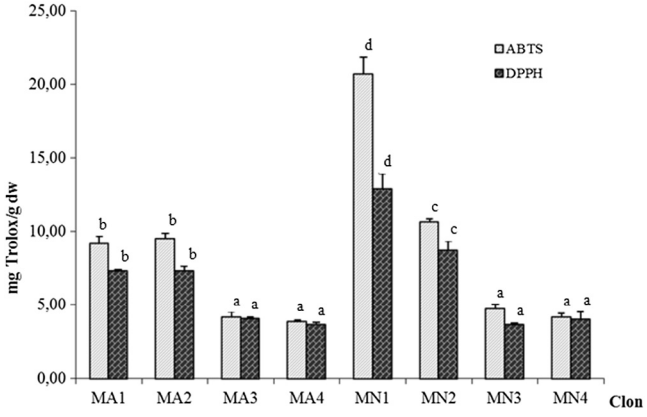

Fig. 2 – Antioxidant activity in mulberry fruits. Values (means ± SE). Columns marked with different letters are significantly different ($p \leq 0.05$).

antioxidant activity of the fruits evaluated was significantly higher in the fruits of *M. nigra* than in those of *M. alba*, for both methods. The clone from black mulberry MN1 showed a significantly ($p < 0.05$) higher antioxidant potential than the rest of clones, even fivefold higher than MA3, MA4, MN3, and MN4. The differences in the antioxidant activity of these mulberry clones could be preliminarily attributed to their different contents in polyphenols. In fact, positive correlations were observed among the TPC and the antioxidant activity measured by the $ABTS^{\bullet+}$ assay ($r = 0.87$, $p \leq 0.05$) and $DPPH^{\bullet}$ assay ($r = 0.92$, $p \leq 0.05$), aspect that emphasizes the great phytochemical value of MN1, in particular in anthocyanins and chlorogenic acids, as it has already been mentioned. Considerable variations in the antioxidant activity of mulberry fruits have been reported by various researchers in previous studies (Ercisli et al., 2010; Gundogdu et al., 2011; Imran et al., 2010; Khalid et al., 2011; Özgen et al., 2009). Factors such as mulberry genotype and sample extraction protocols might certainly account, at least in part, for the observed divergence. In general, mulberry fruits had antioxidant capacities and total phenolic contents comparable with other berries, such as blueberries and cranberries, well known for their high content in phytochemicals with proven health benefits (Wu et al., 2004, 2006). Moreover, some clones (i.e. black mulberries MN1) showed more antioxidant capacity than vegetables like pepper, tomato, and spinach (Halvorsen et al., 2002). On the other hand, Kobus-Cisowska, Gramza-Michalowska, Kmiecik, Flaczyk, and Korczak (2013) demonstrated how the addition of white mulberry fruit to muesli was able not only to increase the antioxidant capacity of the product, but also to reduce the advanced oxidative changes of the product, improving its sensorial characteristics.

3.4. *Mineral composition of mulberry fruits*

The mineral contents of mulberry clones are shown in Table 2 and significant differences among the mulberry clones assayed were observed on the mineral compositions. Among the macroelements (N, P, K, Ca, Na, Mg, and S), the concentration of N ranged between 1.42 (MN3) and 2.13 g/100 g (MA1). The P, K, Ca, Mg, and S values of mulberry species varied from 0.21 (MN3) to 0.31 g/100 g (MA1), 1.48 (MN3) to 2.17 g/100 g (MN1), 0.19 (MA2) to 0.43 g/100 g (MN4), 0.12 (MA2) to 0.19 g/100 g (MA4 and MN1), and 0.07 (MN3) to 0.11 g/100 g (MA1), respectively (Table 2). No differences in the Na content were noted. Ercisli and Orhan (2007) pointed out that common mulberry cultivars grown in Turkey presented concentration of N ranging from 0.75 (*M. alba*) to 0.92% (*M. nigra*), which is by far under the values found in the Spanish mulberries. However, Karlidag, Pehluvan, Turan, and Eyduran (2012) reported higher values of N content, up to 3.19 g/100 g. It should be noted that nitrogen predominated over potassium in white mulberry clones, while potassium prevailed over nitrogen for the black ones. These results agreed with those reported by Karlidag et al. (2012) for *M. alba*, although did not match the values reported by Ercisli and Orhan (2007), who obtained higher values of potassium than nitrogen for *M. alba*. Differences may be related to the different genotypes and growing conditions considered, among other factors.

Among the micro-elements (Fe, Cu, Mn, Zn and Ni), the content of Fe ranged from 23.92 (MN3) to 46.74 mg/kg (MA1). The concentrations of Cu, Mn, Zn, and Ni varied between 2.80 (MN3) and 6.38 mg/kg (MA1), 12.28 (MN3) and 19.38 mg/kg (MA4), 14.89 (MA2) and 22.53 mg/kg (MN1), and 1.22 (MN3) and 2.68 mg/kg (MN1), respectively. Our results were similar with those described by Ercisli and Orhan (2007) and Ercisli et al. (2010), but lower than previously reported by Imran et al. (2010), Karlidag et al. (2012), and Khalid et al. (2011). Again, differences may also be ascribed to different genotypes, fruit maturity, agricultural practice, and ecological conditions, such as climate, altitude and soil factors (Ercisli & Orhan, 2007; Karlidag et al., 2012). The overall decreasing order of mineral elements in the study of *M. alba* fruits was N > K > P > Ca > Mg > S > Na > Fe > Zn > Cu > Ni. On the contrary, the decreasing order of mineral elements in the studied *M. nigra* fruits was K > N > Ca > P > Mg > S > Na > Fe > Zn > Cu > Ni.

Data obtained from white and black mulberries showed that mulberry fruits could match the daily requirements of K, P, and Mg for an adult due to their high content in K, P, and Mg (Ercisli et al., 2010). In addition, the low content in Na of the Spanish mulberry fruits was worth mentioning and could be of interest for low sodium diets.

3.5. *Protein content*

The protein content of fruits of *M. nigra* and *M. alba* was shown in Table 2. They ranged between 8.90% dw (MN3) and 13.33% dw (MN1). The *M. alba* clones (average 12.10% dw) had a higher percentage of protein than the *M. nigra* ones (average 10.06% dw). In the current study, the total protein content in mulberry fruits was higher than those reported by Imran et al. (2010), who reported average values of 0.96 g/100 g dw *M. nigra* and 1.55 g/100 g dw *M. alba*. All the same, these results indicated that the mulberry fruits grown in Spain are a good source of protein, considering that normal adult requires ≈0.8 g of protein per kg of lean body mass per day to maintain normal functions (Ercisli et al., 2010). Most plant diets contain incomplete proteins but combining properly different plant foods (nutrient supplementation) this situation might revert (Ercisli et al., 2010). Therefore, the use of white or black mulberry fruits in an unbalanced plant-based diets might contribute to filling this protein gap.

3.6. *Principal components analysis*

To achieve a better understanding of the trends and relationships among the many variables studied (24) for the different mulberry samples (8 clones), principal component analysis (PCA) was applied. Four principal components were able to explain more than the 95% of the total variation (Table 3). The first component, PC1, representing 62.67% of total variance was positively linked to TPC, antioxidant capacity ($ABTS^{\bullet+}$ and $DPPH^{\bullet}$), total anthocyanins, total cinnamic acid derivatives and total flavonols (Table 3 and Fig. 3). PC2 accounted for 20.78% of the total variance and was positively correlated with total benzoic acid derivatives (Table 3 and Fig. 3). PC3 accounted for 7.03% of the total variance and was positively correlated with kaempferol 3-*O*-rutinoside and *p*-coumaric acid. PC4 accounted for 4.81% of the total variance and was positively correlated with caffeic acid and *m*-coumaric acid.

MN1 clone showed the highest positive value for PC1 (Fig. 3) and was located far from the rest of the clones. This fact pointed

Table 2 – Mineral and protein contents in mulberry fruits (on dry weight basis).

Mineral content	Clones							
	White mulberry (*Morus alba*)				Black mulberry (*Morus nigra*)			
	MA1	MA2	MA3	MA4	MN1	MN2	MN3	MN4
Nitrogen (g/100 g)	2.13 ± 0.08 e	1.62 ± 0.04 bc	1.94 ± 0.02 d	2.05 ± 0.01 de	1.74 ± 0.06 c	1.67 ± 0.04 bc	1.42 ± 0.02 a	1.61 ± 0.03 b
Phosphorus (g/100 g)	0.31 ± 0.01 d	0.24 ± 0.00 b	0.28 ± 0.01 cd	0.28 ± 0.01 c	0.28 ± 0.01 cd	0.28 ± 0.01 c	0.21 ± 0.01 a	0.28 ± 0.01 c
Potassium (g/100 g)	2.13 ± 0.04 d	1.62 ± 0.01 ab	1.72 ± 0.07 bc	1.86 ± 0.06 c	2.17 ± 0.10 d	1.86 ± 0.07 c	1.48 ± 0.05 a	1.50 ± 0.01 a
Calcium (g/100 g)	0.26 ± 0.02 c	0.19 ± 0.00 a	0.24 ± 0.01 bc	0.37 ± 0.00 e	0.30 ± 0.01 d	0.25 ± 0.01 bc	0.21 ± 0.02 ab	0.43 ± 0.01 f
Sodium (g/100 g)	0.01 ± 0.00 a	0.01 ± 0.00 a	0.01 ± 0.00 a	0.01 ± 0.00 a	0.01 ± 0.00 a	0.01 ± 0.00 a	0.01 ± 0.00 a	0.01 ± 0.00 a
Magnesium (g/100 g)	0.18 ± 0.01 c	0.12 ± 0.00 a	0.14 ± 0.01 b	0.19 ± 0.01 c	0.19 ± 0.01 c	0.14 ± 0.01 ab	0.13 ± 0.01 ab	0.17 ± 0.00 c
Sulfur (g/100 g)	0.11 ± 0.00 d	0.08 ± 0.00 b	0.09 ± 0.00 c	0.10 ± 0.00 d	0.09 ± 0.01 bc	0.08 ± 0.00 b	0.07 ± 0.00 a	0.08 ± 0.00 b
Iron (mg/kg)	46.74 ± 2.44 e	28.20 ± 0.68 ab	30.01 ± 2.60 bc	40.53 ± 2.53 d	35.54 ± 0.91 cd	30.55 ± 1.90 bc	23.92 ± 1.20 a	37.09 ± 2.46 d
Zinc (mg/kg)	19.58 ± 0.94 c	14.89 ± 0.13 a	17.35 ± 0.69 abc	17.95 ± 0.39 bc	22.53 ± 1.38 d	16.02 ± 1.14 ab	15.69 ± 0.92 ab	19.87 ± 0.78 c
Manganese (mg/kg)	17.36 ± 0.66 c	12.33 ± 0.22 a	14.35 ± 0.49 b	19.38 ± 0.41 d	17.39 ± 0.78 c	12.48 ± 0.60 a	12.28 ± 0.73 a	18.08 ± 0.32 cd
Boron (mg/kg)	15.51 ± 0.43 cd	14.38 ± 0.14 bc	13.78 ± 0.42 b	19.48 ± 0.66 e	16.75 ± 0.46 d	13.86 ± 0.60 b	12.03 ± 0.57 a	13.19 ± 0.19 ab
Copper (mg/kg)	6.38 ± 0.19 f	4.22 ± 0.05 b	5.65 ± 0.21 e	4.52 ± 0.07 bc	5.21 ± 0.21 de	4.92 ± 0.23 cd	2.80 ± 0.04 a	4.53 ± 0.11 bc
Molybdenum (mg/kg)	0.82 ± 0.02 d	0.11 ± 0.01 a	0.31 ± 0.04 b	0.57 ± 0.02 c	0.10 ± 0.00 a	0.11 ± 0.01 a	0.10 ± 0.00 a	1.07 ± 0.03 e
Nickel (mg/kg)	2.62 ± 0.08 e	1.40 ± 0.11 ab	1.98 ± 0.17 cd	2.35 ± 0.08 dc	2.68 ± 0.24 e	1.76 ± 0.16 bc	1.22 ± 0.13 a	2.67 ± 0.05 e
Lead (mg/kg)	0.29 ± 0.02 a	0.26 ± 0.05 a	0.25 ± 0.01 a	0.29 ± 0.04 a	0.24 ± 0.03 a	0.31 ± 0.06 a	0.34 ± 0.03 a	0.26 ± 0.03 a
Aluminum (mg/kg)	48.31 ± 2.75 d	12.09 ± 0.21 a	21.86 ± 1.12 b	42.08 ± 4.46 cd	17.95 ± 1.67 ab	23.78 ± 1.68 b	22.74 ± 2.34 b	38.66 ± 2.82 c
Carbon (g/100 g)	44.85 ± 1.26 c	43.01 ± 0.06 ab	43.26 ± 0.22 ab	44.87 ± 0.15 c	44.04 ± 0.21 bc	42.45 ± 0.04 a	43.10 ± 0.08 ab	44.13 ± 0.02 bc
Lithium (mg/kg)	0.75 ± 0.05 e	0.19 ± 0.02 abc	0.49 ± 0.09 d	0.22 ± 0.02 bc	0.11 ± 0.01 ab	0.10 ± 0.00 a	0.24 ± 0.03 a	0.44 ± 0.02 d
Strontium (mg/kg)	46.65 ± 3.51 c	29.95 ± 0.08 a	38.19 ± 1.17 b	62.91 ± 0.85 d	45.69 ± 2.26 c	35.80 ± 2.44 ab	31.86 ± 2.15 a	69.15 ± 2.12 e
Titanium (mg/kg)	1.74 ± 0.11 b	0.60 ± 0.09 a	0.70 ± 0.10 a	1.39 ± 0.10 b	0.67 ± 0.01 a	0.84 ± 0.08 a	0.73 ± 0.02 a	1.52 ± 0.28 b
Protein (%)	13.33 ± 0.47 e	10.15 ± 0.23 bc	12.13 ± 0.13 d	12.81 ± 0.04 de	10.85 ± 0.41 c	10.44 ± 0.27 bc	8.90 ± 0.14 a	10.04 ± 0.17 b

Values (means ± SE) followed by the same letter, within the same row, are not significantly different according to Fisher's least significant difference (LSD) procedure at 5% significance level.

Table 3 – Eigenvalues, proportion of variation and eigenvectors associated with each principal component.

Principal components (axes)	1	2	3	4
Eigenvalues	15.04	4.99	1.69	1.15
Cumulated proportion of variation (%)	62.67	83.46	90.48	95.29
Characters (abbreviation for Fig. 3)	Eigenvectors			
ABTS	0.256	0.051	–0.004	0.004
DPPH	0.248	0.119	–0.013	–0.006
TPC	0.219	0.201	–0.121	0.100
Cyanidin 3-O-glucoside (Cy-glc)	0.238	–0.160	–0.049	–0.052
Cyanidin 3-O-rutinoside (Cy-rut)	0.237	–0.164	–0.049	–0.052
Pelargonidin 3-O-glucoside (Pg-glc)	0.245	–0.117	–0.062	0.002
Pelargonidin 3-O-rutinoside (Pg-rut)	0.238	–0.161	–0.028	–0.043
Total anthocyanins (Tot_Anth)	0.238	–0.161	–0.048	–0.052
Other flavonol derivatives (Oth_flavonol)	0.239	–0.111	0.148	0.038
Total flavonols (Tot_Flavonol)	0.247	–0.050	0.191	0.027
Protocatechuic acid (PCA)	0.085	0.412	–0.071	0.052
p-Hydroxybenzoic acid (*p*-OHbenzA)	0.174	0.311	–0.154	–0.065
Vanillic acid (VA)	0.187	0.242	–0.271	0.202
Total benzoic acid derivatives (Tot_benz)	0.098	0.403	–0.088	0.048
Neo-chlorogenic acid (3-CQA)	0.233	0.095	0.132	–0.197
Chlorogenic acid (5-CQA)	0.253	–0.054	0.041	–0.122
Caffeic acid (CA)	–0.003	0.232	0.087	0.676
p-Coumaric acid (*p*-CoumA)	0.110	–0.173	0.409	0.300
Ferulic acid (FA)	–0.075	–0.326	–0.294	0.320
m-Coumaric acid (*m*-CoumA)	0.083	–0.309	–0.191	0.416
Total cinnamic acid derivatives (Tot_ cinn)	0.253	–0.046	0.051	–0.109
Quercetin 3-O-rutinoside (Q-rut)	0.253	–0.081	0.031	–0.017
Quercetin 3-O-glucoside (Q-glc)	0.234	0.069	0.239	0.194
Kaempferol 3-O-rutinoside (K-rut)	–0.111	0.105	0.654	0.042

out that MN1 clone was characterized by a high content of phytochemicals (as anthocyanins, flavonols, and chlorogenic acids) and high antioxidant activity, notably superior to the rest of the clones assayed. MA1, MA2, and MN2 clones exhibited similar PC1 and PC2 values. They were mainly defined by their high PC2 value, corresponding to high amounts of benzoic acid derivatives. Finally, MA3, MA4, MN3 and MN4, clones formed a cluster with negative values for both PC1 and PC2, characterized mainly by their low content in phenolic compounds. Hence, these clones would be those mulberry fruits less interesting from a nutritional point of view.

It should be noted that *M. alba* and *M. nigra* clones were not gathered according to their taxonomic classification, as initially expected. Contrary to the phenotypic identification, PCA of the phytochemical profile produced a different pattern of sample clustering with three well-defined, different sample groups. Phytochemical differences may not account for the differences stressed by taxonomy tools. In this sense, intra-species or clone variability was revealed to be higher than inter-species variability when considering their phenolic contents. Nevertheless, further genotypic studies could confirm this hypothesis on the existing differences between classic taxonomy and phytochemical profiling. On the whole, this novel classification based on the phenolic content and antioxidant activity of the mulberry fruits studied represents a valuable information for future nutritional studies.

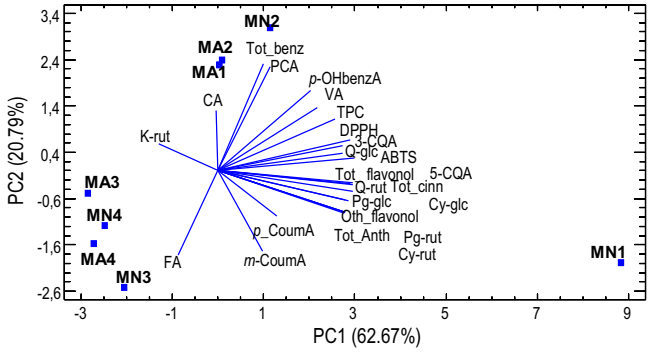

Fig. 3 – Principal component analysis (PC1 and PC2) of mulberry fruits: loading plot and distribution of samples in the consensus space. Abbreviations at Table 3.

4. Conclusions

Many studies have revealed that the consumption of mulberries may have positive effects on human health. The results reported herein showed that white and black mulberry fruits grown in Spain display significant differences in phenolic content, antioxidant activities and mineral composition. In general, the black mulberry clones showed higher antioxidant activities and amounts of phenolic compounds than the white mulberry clones, although a wide intra-species variability was noted. According to these results, some clones of the two species of mulberry could be used for their industrial transformation into phenolic-rich foodstuffs. Similarly, their high content of bioactive phenolic compounds together with their balanced content in proteins and mineral turn them into suitable food matrices for nutritional interventions focused on the health-promoting features of mulberry fruits.

Further studies on mulberry composition should consider non extractable polyphenols since they may increase and enrich the phenolic value of mulberry fruits. Moreover, although seeds only represent a small fraction of mulberry fruits and their recovery and industrialization could be difficult due to their very small size and the fact that mulberries are mainly consumed as fresh fruits, it would be interesting to shed light on mulberry seed composition.

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8.3. PUBLICACIÓN 3

(Poly)phenolic compounds and antioxidant activity of white (*Morus alba*) and black (*Morus nigra*) mulberry leaves: Their potential for new products rich in phytochemicals

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(Poly)phenolic compounds and antioxidant activity of white (*Morus alba*) and black (*Morus nigra*) mulberry leaves: Their potential for new products rich in phytochemicals

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ABSTRACT

The (poly)phenolic fingerprint and antioxidant activities of the leaves of white and black mulberry clones grown in Spain, one of the main European producers, were evaluated for the first time in order to examine their phytochemical potential. Data showed that mulberry leaves are rich in caffeoylquinic acids (6.8–8.5 mg/g dw) and flavonols (3.7–9.8 mg/g dw). It was also evidenced that a wide intra-species variability existed according to principal component analysis. These results can be useful for value-added utilisation of this underused vegetal matrix by the food/pharma industries. In addition, owing to their (poly)phenolic composition with proven biological activities, these data can serve as starting point for further nutritional studies with mulberry leaf-derived products.

1. Introduction

Mulberry tree is present in almost all continents and has been cultivated traditionally for its leaves [food for silkworms (*Bombyx mori* L.) and animal fodder] and as ornaments (Vijayan, 2010). Nowadays, due to its nutritive value, mulberry fruit is consumed in both fresh and processed forms (Gundogdu, Muradoglu, Gazioglu, & Yilmaz, 2011). Several studies have shown that mulberries may have positive effects on human health, especially in people with diabetes (Wang, Xiang, Wang, Tang, & He, 2013); these effects being mainly linked to their phenolic composition (Gundogdu et al., 2011; Tutin, 1996). Previous studies have also shown that mulberry fruits have a high content of bioactive

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phenolic compounds and a balanced content of proteins and minerals (Sánchez-Salcedo, Mena, García-Viguera, Martínez, & Hernández, 2015). However, the health-promoting features of mulberry tree are limited not only to their fruits, but also to the leaves that have shown wide biological properties.

Recent reports indicate that mulberry (*Morus alba* L.) leaves are a rich source of (poly)phenolic substances, including phenolic acids and flavonoids such as caffeic acid, caffeoylquinic acids, kaempferol-3-*O*-(6-malonyl)–glucoside, quercetin-3-*O*-(6-malonyl)–glucoside, and quercetin-3-*O*-glucoside (Memon, Memon, Luthria, Bhanger, & Pitafi, 2010; Thabti, Elfalleh, Hannachi, Ferchichi, & Campos, 2012). Mulberry leaves or leaf-derived extracts exhibit significant hypoglycaemic, hypolipidaemic, and anti-atherogenic effects on humans and on certain animal models (Chung, Kim, Kim, & Kwon, 2013; Enkhmaa et al., 2005; Hunyadi et al., 2013; Jeszka-Skowron et al., 2014; Naowaboot, Pannangpetch, Kukongviriyapan, Kongyingyoes, & Kukongviriyapan, 2009). For instance, Wu et al. (2013) reported that mulberry leaf phenolic extract is able to reduce hepatic lipid accumulation through activation of the AMP-activating protein kinase signalling pathway. Park, Lee, Lee, and Kim (2013) also indicated that mulberry leaf extract could be used to prevent diseases characterised by chronic inflammation through inhibition of NF-kB. Thus, mulberry leaves have been considered as a valuable low-cost material that can be used in the design of new strategies for prevention and treatment of type 2 diabetes and some cardiovascular diseases (Jeszka-Skowron et al., 2014). On the other hand, mulberry leaves are common foodstuffs in some Asian countries. Mulberry leaf powder is used with wheat flour to make *paratha*, the most common food item in breakfast and dinner in the Indian diet (Srivastava, Kapoor, Thathola, & Srivastava, 2003). Consumption of mulberry leaves as infusion and powdered juice is widespread in Korea and Japan and demand has been increasing for them in the last few years (Desmukh, Pathak, & Takalikar, 1993; Katsube, Tsurunaga, Sugiyama, Furuno, & Yamasaki, 2009; Thabti et al., 2012). Therefore, considering the growing number of mulberry-derived foodstuffs and the increasing literature on their biological properties, the phytochemical characterisation of mulberry leaves should be carried out in order to conduct further nutritional studies.

The objective of this study was to determine and quantify, for the first time, as far as we are aware, the main polyphenolic compounds and antioxidant activity of mulberry leaves from different clones grown in Spain, one of the main European producers, in order to evaluate their phytochemical potential.

2. Materials and methods

2.1. Chemicals

The compounds 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulphonic acid) diammonium salt (ABTS), Folin–Ciocalteu's reagent, chlorogenic acid, and rutin (quercetin 3-β-rutinoside) were purchased from Sigma-Aldrich (Steinheim, Germany); formic acid, methanol, acetonitrile, and anhydrous sodium carbonate, all of analytical grade, were from Panreac Química S.A (Barcelona, Spain). The compounds 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and gallic acid were from Fluka Chemika (Neu-Ulm, Switzerland). Milli-Q water was produced using an Elix®3 Millipore water purification system coupled to a Milli-Q module (model Adventage10) (Molsheim, France).

2.2. Plant materials

Fresh leaves samples of eight mulberry clones, four white (*M. alba*) clones: MA1, MA2, MA3, and MA4, and four black (*Morus nigra*) clones: MN1, MN2, MN3, and MN4, were manually collected in Orihuela (latitude 38°04′08″N × longitude 0°58′58″W, 27 m above sea level, Alicante, SE Spain) in June 2013. After collection, the samples were washed with distilled water and lyophilised. The dried materials were ground into a fine powder with a domestic coffee grinder (Moulinex, Écully, France) (particle size < 0.4 mm) and stored in airtight container at 20 °C for 2 months before analysis.

2.3. Extraction of phenolic compounds

Phenolic compounds were extracted according to previous reports (González-Barrio, Borges, Mullen, & Crozier, 2010; Pérez-Gregorio, Regueiro, Alonso-González, Pastrana-Castro, & Simal-Gándara, 2011). The mixture of 200 mg of freeze-dried powder and 1 mL of 80% aqueous methanol acidified with formic acid (1%) was sonicated for 25 min. The mixture was centrifuged at 10,480 *g* for 5 min (model EBA 21, Hettich Zentrifugen, Tuttlingen, Germany) at room temperature. Two additional extractions were performed for each sample with additional 0.5 mL of the same solvent, as described above. The last extraction was kept at 4 °C overnight and sonicated again for 25 min, after which it was centrifuged. The three supernatants were pooled and filtered through a 0.45 μm polyvinylidene fluoride filter (Millex HV13, Millipore, Bedford, MA, USA) before HPLC analysis.

2.4. Total phenolic content by Folin–Ciocalteu's reagent

Total phenolic content (TPC) was determined by the Folin–Ciocalteu's reagent method adapted to microscale (Mena, Martí, Saura, Valero, & García-Viguera, 2013). TPC was evaluated by measuring the variation in absorbance at 765 nm after 1 h of reaction. Assays were measured by using 96-well microplates (Nunc, Roskilde, Denmark) and Infinite® M200 microplate reader (Tecan, Grödig, Austria). Results were expressed as mg of gallic acid equivalents (GAE) per g of dry material.

2.5. HPLC analysis, identification and quantification of phenolic compounds

Chromatographic analyses were carried out on a Luna C18 column (250 × 4.6 mm, 5 mm particle size; Phenomenex, Macclesfield, UK) with a security guard C18-ODS (4.0 × 3.0 mm) cartridge system (Phenomenex). Water/formic acid (95:5, v/v) and acetonitrile were used as mobile phases A and B, respectively, with a flow rate of 1 mL/min. The gradient started with 8% of solvent B, reaching 15% solvent B at 25 min, 22% at 55 min, and 40% at 60 min, which was maintained up to 70 min. The

injection volume was 20 μL. Samples were processed on a Merck-Hitachi D-7000 HSM PC based chromatography data system. Chromatograms were recorded at 280, 320, and 360. Chromatographic comparison with analytical standards, absorbance spectra, and mass spectra, using MS^n (data not shown) experiments, were used to identify compounds previously reported (Dugo et al., 2009; Memon et al., 2010; Wang et al., 2013). Compounds were quantified by the absorbance of their corresponding peaks: cinnamic acid derivatives as chlorogenic acid (detected at 320 nm) and flavonols as rutin (at 360 nm).

2.6. Antioxidant capacity by DPPH• scavenging activity and ABTS•+ assay methods

The free radical scavenging activity was determined using the free radical DPPH• as well as the ABTS•+ method in aqueous media according to Mena et al. (2011). Assays were measured by using 96-well microplates (Nunc) and Infinite® M200 microplate reader (Tecan). Results were expressed as mg Trolox equivalents per g of dry material.

2.7. Statistical analysis

Statistical analyses were performed using SPSS 20.0 for Windows (SPSS Science, Chicago, IL, USA). A basic descriptive statistical analysis was followed by an analysis of variance (ANOVA) test for mean comparisons. The method used to discriminate among the means (Multiple Range Test) was Fisher's Least Significant Difference (LSD) procedure at a 95.0% confidence level. Pearson correlation and principal component analyses (PCA) were also performed.

3. Results and discussion

3.1. Total phenolic content in mulberry leaves

Phenolic compounds are effective antioxidant constituents of plant foods that have been pointed out as the main phytochemical compounds of mulberry leaves with proven biological properties (Chung et al., 2013; Enkhmaa et al., 2005; Hunyadi et al., 2013; Jeszka-Skowron et al., 2014; Naowaboot et al., 2009). The total phenolic content (TPC) in the mulberry leaves from eight Spanish clones ranged from 12.81 to 16.13 mg GAE/g dry weight (dw), for MA2 and MN3, respectively (Table 1). Among the studied clones, *M. alba* had the lowest phenolic content (from 12.81 to 15.50 mg GAE/g dw) whereas the *M. nigra* clones displayed levels slightly higher (from 13.48 to 16.13 mg GAE/g dw). These results were in agreement with a previous report on mulberry leaves from Pakistan (Iqbal et al., 2012), which also found higher TPC values for *M. nigra* than for *M. alba*. Radojković, Zeković, Vidović, Kočar, and Mašković (2012) reported higher values of total phenolics in mulberry leaves, from 66.76 (*M. alba*) to 115.23 (*M. nigra*) mg GAE/g dw. Nevertheless, Thabti et al. (2012) registered lower values of total phenols in mulberry leaves (from 3.45 to 6.31 mg GAE/g dw). An explanation behind the huge variability among the phenolic content in mulberry leaves could be found in the different extraction procedures and analytical methods used in each work. In addition, it has been suggested that phenolic substances in leaves vary according to conditions such as drought, temperature changes, pollution, UV light, and pathogen attacks, among others (Paliyath, Pinhero, Rao, Murr, & Fletcher, 1997). All the same, these results showed that the leaves of selected mulberry clones grown in Spain could be a good source of phenolic constituents. Actually, a previous study in mulberry fruits from Spanish clones showed lower TPC than these found for mulberry leaves (Sánchez-Salcedo et al., 2015). This agrees with a recent study showing that the leaves of different berries (chokeberry, cranberry, blackcurrant, and bilberry) contain significantly more polyphenols than the fruits (Teleszco & Wojdyło, 2015). Accordingly, the value of mulberry leaf as a matrix rich in phenolic substances prompts us to assess the individual (poly)phenolics making up its composition.

3.2. (Poly)phenolic compounds in mulberry leaves

The phytochemical fingerprint of Spanish mulberry leaves was determined on the basis of the previous reports focused on the phenolic composition of mulberry leaves (Dugo et al., 2009; Memon et al., 2010). Fourteen phenolic compounds were identified in the eight mulberry clones analysed: four caffeoylquinic acids (CQAs) and ten flavonols. The content of CQAs was slightly higher than the content of flavonol derivatives, for all the samples (Table 2). In mulberry fruits seventeen phenolic compounds were identified: three benzoic acid derivatives, six cinnamic acid derivatives, four flavonols and four anthocyanins. In this case, the major phenolic groups were benzoic acid derivatives > cinnamic acid derivatives > flavonols > anthocyanins (Sánchez-Salcedo et al., 2015).

The total content of CQAs of mulberry leaves ranged from 6.78 to 8.48 mg/g dw (MA3 and MA4, respectively) for the *M. alba* clones and from 6.43 to 10.05 mg/g dw (MN4 and MN3, respectively) for the *M. nigra* clones, although no significant differences were recorded among clones (Table 2). Chlorogenic

Table 1 – Total phenolic content (mg GAE/g dw) and antioxidant activity (mg Trolox/g dw) in mulberry leaves.

Variable	Clones							
	White mulberry (*Morus alba*)				Black mulberry (*Morus nigra*)			
	MA1	MA2	MA3	MA4	MN1	MN2	MN3	MN4
Total phenols	13.46 ± 0.27 a	12.81 ± 0.14 a	13.16 ± 0.41 a	15.50 ± 0.56 bc	13.48 ± 0.46 a	13.92 ± 0.72 ab	16.13 ± 0.55 c	14.01 ± 0.90 ab
ABTS•+	11.63 ± 0.23 bc	11.34 ± 0.19 abc	10.82 ± 0.32 ab	12.00 ± 0.24 c	10.60 ± 0.26 a	10.86 ± 0.45 ab	13.15 ± 0.24 d	11.41 ± 0.48 abc
DPPH•	12.64 ± 0.22 e	12.34 ± 0.30 de	11.89 ± 0.21 cd	11.17 ± 0.01 ab	10.62 ± 0.26 a	12.15 ± 0.08 de	11.41 ± 0.41 bc	11.77 ± 0.08 ab

Values (means ± SEM) followed by the same letter, within the same row, are not significantly different according to Fisher's least significant difference (LSD) procedure at 5% significance level.

Table 2 – (Poly)phenolic compounds in mulberry leaves (mg/g dw).

(Poly)phenolic compounds	Clones							
	White mulberry (*Morus alba*)				Black mulberry (*Morus nigra*)			
	MA1	MA2	MA3	MA4	MN1	MN2	MN3	MN4
Neo-chlorogenic acid (3-CQA)	0.71 ± 0.02 ab	1.12 ± 0.12 c	0.94 ± 0.09 bc	0.58 ± 0.14 a	1.09 ± 0.05 c	0.97 ± 0.06 bc	0.81 ± 0.20 abc	0.54 ± 0.02 a
Chlorogenic acid (5-CQA)	6.32 ± 0.46 a	6.19 ± 0.94 a	5.29 ± 0.24 a	7.18 ± 1.83 a	7.98 ± 0.14 a	8.30 ± 0.62 a	8.39 ± 1.94 a	5.44 ± 0.15 a
Crypto-Chlorogenic acid (4-CQA)	0.27 ± 0.06 ab	0.17 ± 0.01 a	0.33 ± 0.04 ab	0.42 ± 0.12 bc	0.45 ± 0.07 bc	0.38 ± 0.04 abc	0.56 ± 0.12 c	0.42 ± 0.01 bc
Chlorogenic acid isomer (1-CQA)	0.33 ± 0.01 c	0.13 ± 0.03 ab	0.22 ± 0.03 bc	0.29 ± 0.09 bc	0.26 ± 0.10 bc	0.33 ± 0.06 c	0.29 ± 0.07 bc	0.03 ± 0.01 a
Total Caffeoylquinic acids	7.64 ± 0.41 a	7.61 ± 1.03 a	6.78 ± 0.30 a	8.48 ± 2.17 a	9.78 ± 0.24 a	9.98 ± 0.71 a	10.05 ± 2.33 a	6.43 ± 0.19 a
Quercetin-3,7-O-β-glucopyranoside	0.14 ± 0.02 b	0.07 ± 0.01 ab	0.02 ± 0.00 a	0.07 ± 0.03 ab	0.13 ± 0.02 b	0.03 ± 0.00 ab	0.40 ± 0.10 c	0.05 ± 0.01 ab
Quercetin-3-O-β-glucopyranosyl-(1→6)-β-glucopyranoside	0.24 ± 0.03 abc	0.18 ± 0.02 ab	0.29 ± 0.03 bc	0.34 ± 0.09 c	0.37 ± 0.01 c	0.80 ± 0.04 d	0.33 ± 0.07 c	0.11 ± 0.01 a
Quercetin-rutinoside isomer	0.23 ± 0.03 bcd	0.14 ± 0.01 ab	0.26 ± 0.02 cd	0.10 ± 0.03 a	0.16 ± 0.02 abc	0.53 ± 0.02 e	0.33 ± 0.08 d	0.14 ± 0.00 ab
Kaempferol-3,7-glucopyranoside	0.11 ± 0.01 cd	0.04 ± 0.01 a	0.09 ± 0.01 bc	0.14 ± 0.04 cde	0.16 ± 0.01 e	0.18 ± 0.01 e	0.15 ± 0.01 de	0.05 ± 0.01 ab
Quercetin-rutinoside (rutin)	1.14 ± 0.10 bc	0.69 ± 0.07 ab	0.59 ± 0.04 a	1.21 ± 0.31 c	1.80 ± 0.15 d	0.94 ± 0.05 abc	0.95 ± 0.22 abc	0.58 ± 0.04 a
Quercetin-3-O-glucoside (isoquercitrin)	1.34 ± 0.15 a	1.04 ± 0.08 a	0.92 ± 0.10 a	1.53 ± 0.36 a	1.67 ± 0.23 a	1.51 ± 0.06 a	3.73 ± 0.92 b	1.28 ± 0.09 a
Quercetin-3-O-(6-malonyl)-β-glucopyranoside	1.07 ± 0.08 bc	1.06 ± 0.14 bc	0.82 ± 0.05 ab	1.39 ± 0.36 bcd	0.35 ± 0.01 a	1.03 ± 0.07 bc	1.84 ± 0.41 d	1.54 ± 0.05 cd
Quercetin-3-O-6″-O-acetyl-β-glucopyranoside	0.34 ± 0.01 c	0.14 ± 0.06 a	0.18 ± 0.04 ab	0.34 ± 0.10 bc	0.60 ± 0.05 d	0.15 ± 0.02 a	0.27 ± 0.06 abc	0.24 ± 0.02 abc
Kaempferol-3-O-6″-O-acetyl-β-glucopyranoside	0.34 ± 0.07 a	0.11 ± 0.04 a	0.11 ± 0.05 a	0.36 ± 0.12 a	0.32 ± 0.07 a	0.08 ± 0.01 a	0.95 ± 0.23 b	0.18 ± 0.01 a
Kaempferol-3-O-(6-malonyl)glucoside	0.58 ± 0.07 bcd	0.33 ± 0.08 ab	0.36 ± 0.02 abc	0.63 ± 0.16 cd	0.11 ± 0.02 a	0.33 ± 0.03 ab	0.80 ± 0.18 d	0.65 ± 0.03 d
Total Flavonols	5.53 ± 0.57 a	3.79 ± 0.50 a	3.66 ± 0.27 a	6.11 ± 1.57 a	5.69 ± 0.55 a	5.59 ± 0.33 a	9.75 ± 2.28 b	4.81 ± 0.20 a

Values (means ± SEM) followed by the same letter, within the same row, are not significantly different according to Fisher's least significant differences (LSD) procedure at 5% significance level.

acid (5-CQA) was by far the predominant CQA in mulberry leaves, varying between 5.29 (MA3) and 8.39 mg/g dw (MN3), followed by neo-chlorogenic acid (3-CQA), crypto-chlorogenic acid (4-CQA), and chlorogenic acid isomer (1-CQA) (Table 2). This pattern regarding the abundance in CQAs of mulberry leaves matched the pattern reported for *M. alba* leaves by Dugo et al. (2009). The amount of CQAs in *M. nigra* leaves was slightly higher than that of *M. alba*, according to Radojković et al. (2012). However, the variability between species reported for Serbian cultivars (2.6 and 19.6 mg/g dw for *M. nigra* and *M. alba*, respectively) (Radojković et al., 2012) was notably higher than the differences herein reported. Additionally, Sánchez-Salcedo et al. (2015) revealed a lower content of CQAs in mulberry fruits (between 0.16 and 3.62 mg/g dw) than in mulberry leaves.

Total content of flavonol derivatives in mulberry leaves varied between 3.66 and 9.75 mg/g dw (MA3 and MN3, respectively), without significant statistical differences among clones. The flavonols present in mulberry leaves were a mixture of different glycosylated quercetins and kaempferols, prevailing quercetin derivatives over kaempferol ones (Table 2). The predominant flavonols in mulberry leaves of *M. alba* and *M. nigra* species were quercetin-3-*O*-glucoside (isoquercitrin) > quercetin-3-*O*-(6-malonyl)-β-glucopyranoside = quercetin-rutinoside (rutin) > kaempferol-3-*O*-(6-malonyl) glucoside. In particular, they ranged from 0.92 to 3.73 mg/g dw (MA3 and MN3, respectively) for quercetin-3-glucoside, from 0.35 to 1.84 mg/g dw (MN1 and MN3, respectively) for quercetin-3-*O*-(6-malonyl)-β-glucopyranoside, between 0.58 to 1.80 mg/g dw (MN4 and MN1, respectively) for rutin, and from 0.11 to 0.80 mg/g dw (MN1 and MN3, respectively) for kaempferol-3-*O*-(6-malonyl)glucoside (Table 2). A similar trend has been evidenced by other authors (Dugo et al., 2009; Katsube et al., 2006). However, Thabti et al. (2012) reported values between 7- and 120-fold higher than the results herein obtained for the content of malonyl-glucosides of kaempferol and quercetin (13.33 and 12.59 mg/g dw, respectively). Other flavonols (quercetin-3,7-*O*-β-glucopyranoside, quercetin-3-*O*-β-glucopyranosyl-(1→6)-β-glucopyranoside, quercetin-rutinoside isomer, kaempferol-3,7-glucopyranoside, quercetin-3-*O*-6″-*O*-acetyl-β-glucopyranoside, kaempferol-3-*O*-6″-*O*-acetyl-β-glucopyranoside) were also detected for all the samples at concentrations not exceeding 0.95 mg/g dw, in accordance with Dugo et al. (2009). The content of flavonols was almost 10-fold higher in mulberry leaves than in mulberry fruits (Sánchez-Salcedo et al., 2015).

Significant differences among clones were found in the (poly)phenolic profile of mulberry leaves (Table 2), which could be useful for authentication purposes. MN1 clone showed significant higher levels of rutin and quercetin-3-*O*-6″-*O*-acetyl-β-glucopyranoside. Similarly, when compared with other clones, MN2 had higher levels of quercetin-3-*O*-β-glucopyranosyl-(1→6)-β-glucopyranoside and quercetin-rutinoside isomer. MN3 clone displayed levels of quercetin-3,7-*O*-β-glucopyranoside and quercetin-3-glucoside (isoquercitrin) significantly higher than other clones.

The mean content of CQAs and flavonol derivatives of Spanish mulberry leaves was 8.3 and 5.6 mg/g dw, respectively. Mulberry leaves can hence be considered promising sources of bioactives with proven biological activities (Del Rio et al., 2013; Rodríguez-Mateos et al., 2014). In this sense, a cup of mulberry leaves tea could provide the same quantity of CQAs

as an espresso coffee (Ludwig et al., 2014), with the benefit for some consumers of a caffeine-free beverage. It would also provide similar quantities of CQAs and flavonols than a cup of black tea (3.8 and 10.2 mg/g dw, respectively) (Del Rio et al., 2004). On the other hand, the high content of (poly)phenolic compounds in mulberry leaves may support the key role of these phytochemicals in the health-promoting features related to the supplementation of mulberry leaf (Chung et al., 2013; Enkhmaa et al., 2005; Hunyadi et al., 2013; Jeszka-Skowron et al., 2014; Naowaboot et al., 2009). These results also shed light in the composition of Spanish mulberry leaves, a relevant topic for the revalorisation of this underused vegetal matrix by the food/pharmaceutical industry.

3.3. *Antioxidant capacity of mulberry leaves*

The antioxidant capacity of foodstuffs is dictated by the different mechanisms of action of their antioxidant constituents; therefore, this capacity should be evaluated by a variety of methods dealing with different mechanisms (Pérez-Jiménez et al., 2008). Two assays widely spread, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$, were used to properly evaluate the antioxidant activity of mulberry leaves (Table 1). Significant variations ($p < 0.05$) were found in the antioxidant activity of the studied clones.

Mean values varied from 10.60 (MN1) to 13.15 (MN3) mg Trolox/g dw for $ABTS^{\bullet+}$ and 10.62 (MN1) to 12.64 (MA1) mg Trolox/g dw for $DPPH^{\bullet}$. The antioxidant capacity of the leaves evaluated by the $ABTS^{\bullet+}$ method was similar for both *M. nigra* and *M. alba*. Nevertheless, when considering the $DPPH^{\bullet}$ method, the antioxidant capacity of *M. alba* clones was slightly higher than *M. nigra* ones. Lower levels for *M. alba* leaves were reported by Thabti et al. (2014), ranging for the $ABTS^{\bullet+}$ method from 2.1 to 3.5 mg Trolox/g dw. Considerable variations in the antioxidant activity of mulberry leaves have been reported in previous studies (Iqbal et al., 2012; Thabti et al., 2014; Zou et al., 2012). Factors such as mulberry genotype, growing environment, and sample extraction protocols may account, at least in part, for the observed divergence.

When compared to a previous study in mulberry fruits carried out with the same clones (Sánchez-Salcedo et al., 2015), mulberry leaves showed higher mean values of antioxidant capacity for both the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ methods. Similar results were found by Teleszco and Wojdyło (2015) for berry leaves (cranberry, blackcurrant, and bilberry). This fact may account for the potential use of mulberry leaves in the development of food items with high antioxidant capacity.

A high positive correlation ($r = 0.81$, $p \leq 0.05$) between the TPC and the antioxidant capacity measured by the $ABTS^{\bullet+}$ method was observed. On the contrary, TPC only had a weakly negative correlation with $DPPH^{\bullet}$ ($r = -0.22$, $p \leq 0.05$). Differences in the contribution of each (poly)phenolic compound to the antioxidant response in each antioxidant capacity assay can be behind this fact (Mena et al., 2011).

3.4. *Principal components analysis*

To achieve a better understanding of the trends and relationships among the many studied variables (19) for the different mulberry samples (8 clones), principal component analysis (PCA) was applied. The first five principal components (PCs) explained >97.20% of the total variation (Table 3). Nearly 70.23% of the observed variability was explained by the first two PCs. The first component (PC1), representing 43.93% of total variance, was positively linked to the content of chlorogenic acid isomers (except to neo-chlorogenic acid, 3-CQA), total flavonols, antioxidant capacity by $ABTS^{\bullet+}$ method, and TPC (Table 3, Fig. 1). PC2 accounted for the 26.30% of the total variance. It was positively correlated with two chlorogenic acid isomers

Table 3 – Eigenvalues, proportion of variation and eigenvectors associated with each principal component.

Principal components (axes)	1	2	3	4	5
Eigenvalues	**8.35**	**5.0**	**2.77**	**1.34**	**1.01**
Cumulated proportion of variation	**43.93**	**70.23**	**84.81**	**91.87**	**97.20**
Characters	Eigenvectors				
Neo-chlorogenic acid (3-CQA)	–0.076	0.301	0.079	0.442	0.439
Chlorogenic acid (5-CQA)	0.270	0.245	0.051	0.007	0.100
Crypto-Chlorogenic acid (4-CQA)	0.283	–0.013	–0.157	–0.346	0.238
Chlorogenic acid isomer (1-CQA)	0.185	0.249	0.163	0.079	–0.535
Total Chlorogenic acids	0.258	0.274	0.055	0.051	0.132
Quercetin-3,7-O-β-glucopyranoside	0.295	–0.081	–0.024	0.390	0.113
Quercetin-3-O-β-glucopyranosyl-(1→6)-β-glucopyranoside	0.099	0.322	0.307	–0.313	0.026
Quercetin-rutinoside isomer	0.102	0.189	0.461	–0.157	0.153
Kaempferol-3,7-glucopyranoside	0.230	0.301	0.032	–0.214	–0.172
Quercetin-rutinoside (rutin)	0.131	0.289	–0.348	0.082	–0.272
Quercetin-3-O-glucoside (isoquercitrin)	0.326	–0.061	0.030	0.163	0.222
Quercetin-3-O-(6-malonyl)-β-glucopyranoside	0.171	–0.351	0.181	–0.118	0.034
Quercetin-3-O-6″-O-acetyl-β-glucopyranoside	0.101	0.175	–0.498	0.071	–0.200
Kaempferol-3-O-6″-O-acetyl-β-glucopyranoside	0.312	–0.115	–0.070	0.259	0.015
Kaempferol-3-O-(6-malonyl)glucoside	0.180	–0.357	0.096	–0.093	–0.224
Total Flavonols	0.342	–0.045	0.012	0.072	0.030
TPC	0.306	–0.140	–0.005	–0.213	–0.059
$ABTS^{\bullet+}$	0.257	–0.268	0.073	0.186	–0.101
$DPPH^{\bullet}$	–0.103	0.016	0.453	0.377	–0.377

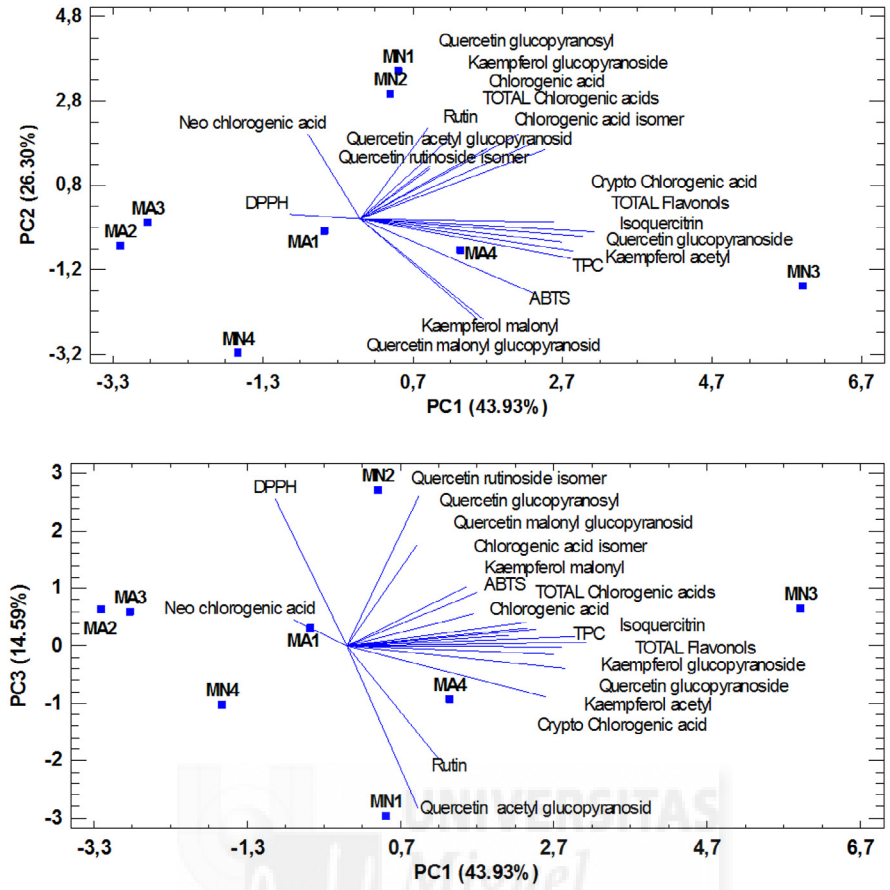

Fig. 1 – Principal component analysis (PC1 *vs* PC2 and PC1 *vs* PC3) of mulberry leaves: loading plot and distribution of samples in the consensus space.

(1-CQA and 5-CQA), quercetin-3-*O*-β-glucopyranosyl-(1→6)-β-glucopyranoside, kaempferol-3,7-glucopyranoside, and rutin, and negatively correlated to quercetin-3-*O*-(6-malonyl)-β-glucopyranoside, kaempferol-3-*O*-(6-malonyl)glucoside, and antioxidant capacity by the $ABTS^{\bullet+}$ method (Table 3, Fig. 1). PC3 accounted for the 14.58% of observed variability and was positively correlated to quercetin-rutinoside isomer and antioxidant capacity by $DPPH^{\bullet}$ method.

PC4 accounted for 7.06% of the total variance and was positively correlated to neo-chlorogenic acid (3-CQA) and quercetin-3,7-*O*-β-glucopyranoside and negatively to crypto-chlorogenic acid (4-CQA). PC5 only accounted for 5.33% of the total variance and was negatively associated with chlorogenic acid isomer (1-CQA) (Table 3).

The MN3 clone showed the highest positive value for PC1 (Fig. 1), being far from the rest of the clones. This indicated that MN3 clone is characterised by a high content of total flavonols, total chlorogenic acids, TPC and antioxidant capacity by $ABTS^{\bullet+}$ method. MN1 and MN2 shared similar values for PC1 and PC2 but they differed in their values for PC3. They displayed PC1 scores around 0 and were the mulberry clones with the highest PC2 scores, pointing out their high amounts in some flavonols and their high $ABTS^{\bullet+}$ values. However, whereas MN2 had a positive PC3 accounting for its high content of the quercetin-rutinoside isomer and $DPPH^{\bullet}$ values, MN1 showed negative scores, which evidenced a phytochemical profile with reduced amounts in quercetin-rutinoside isomer.

MA2 and MA3 clones showed similar values for PC1, PC2, and PC3. These clones presented negative values for PC1 and values close to 0 for PC2 and PC3 (Fig. 1). According to these results, this group of clones is characterised by a low content of (poly)phenolic compounds, although a moderate content of neo-chlorogenic acid (3-CQA), and high values of antioxidant capacity by the $DPPH^{\bullet}$ method. Regarding MA1, MA4, and MN4 clones, they were defined by mean scores for PC1 and PC3 and negative scores for PC2 and they can thus be considered as clones with intermediate characteristics with respect to the rest of the clones aforementioned. Concerning these results, selection of interesting clones with specific quality characteristics for agro-food/pharmaceutical industry could be carried out.

4. Conclusions

Some studies have pointed out that mulberry leaves may constitute a good source of (poly)phenolic compounds able to positively impact human health. The results reported herein showed that the leaves of white and black mulberry clones grown in Spain are rich in CQAs and flavonols and have a wide

intra-species variability in their (poly)phenolic fingerprint. If compared to mulberry fruits, leaves exhibit a higher content in phenolic compounds and antioxidant activity. As a consequence, mulberry leaves could be used for the elaboration of mulberry products by the functional food industry whereas fruits could be earmarked for fresh or juice consumption. This fact may pave the way to the revalorisation of this underused vegetal material in order to develop mulberry leaf-derived products rich in phenolics. Actually, attending to their phenolic composition, mulberry leaves could be used for the development of teas and other beverages. Their extracts could also be used as additives to create or fortify foods such as muesli bars, yoghurts, etc. Moreover, owing to their particular phenolic composition, they can be considered promising sources of phytochemical compounds with proven biological activities. Nevertheless, whether the consumption of a mulberry leaves tea can be beneficial to human health is a question that deserves further works on their bioavailability and biological properties.

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8.4. PUBLICACIÓN 4

Fatty acids composition of Spanish black (*Morus nigra* L.) and white (*Morus alba* L.) mulberries

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ABSTRACT

This research has determined qualitatively and quantitatively the fatty acids composition of white (*Morus alba*) and black (*Morus nigra*) fruits grown in Spain, in 2013 and 2014. Four clones of each species were studied. Fourteen fatty acids were identified and quantified in mulberry fruits. The most abundant fatty acids were linoleic (C18:2), palmitic (C16:0), oleic (C18:1), and stearic (C18:0) acids in both species. The main fatty acid in all clones was linoleic (C18:2), that ranged from 69.66% (MN2) to 78.02% (MA1) of the total fatty acid content; consequently Spanish mulberry fruits were found to be rich in linoleic acid, which is an essential fatty acid. The fatty acid composition of mulberries highlights the nutritional and health benefits of their consumption.

1. Introduction

Morus alba L. and *Morus nigra* L. (both known as mulberry) are deciduous trees belonging to the family Moraceae (Zelová et al., 2014). Mulberry plants have edible and tasty fruits, while various plant parts have been used in traditional Chinese medicine for centuries. However, in most European countries, mulberries are grown for fruit production rather than foliage. Ethno-botanical usage of mulberry fruits include preparation of jams, marmalades, ice-creams, vinegars, juices, wine and other food and cosmetic products (Natic et al., 2014). Studies related to variability in health promoting compound contents in different colored fruits in *M. alba*, *M. rubra* and *M. nigra* have also been reported (Calín-Sánchez et al., 2013; Ercisli & Orhan, 2007; Ercisli et al., 2010).

The mulberry seed oil, very rich in linoleic acid, may be a healthy valuable and dietary fat. However, mulberry seed oil is rather poor in linolenic acid. Low levels of linolenic acid are desired in edible oils, because high levels of this fatty acid can cause unfavourable odor and taste. Additionally and because linolenic acid is easily oxidized due its three double bonds on the hydrocarbon chain, the stability and/or shelf-life of an oil rich in linolenic acid would be too short (Baydar & Akkurt, 2001). It has been established that a diet rich in saturated fatty acid (SFA) increases the risk of hypercholesterolaemia, diabetes and atherosclerosis, whereas unsaturated fatty acid (UFAs) such as linolenic and linoleic acids, which are present in significant amounts in plant seeds, have several beneficial health-related effects (Simopoulos, 1999).

Fatty acids, especially unsaturated fatty acids (USFAs), are important as nutritional substances and metabolites in living organisms. Many kinds of fatty acids play an important role in the regulation of a variety of physiological and biological functions. Fatty acids are organic compounds formed by a hydrocarbonated chain and a carboxylic group which are normally bounded with glycerol-forming acylglycerides (mono-, di- or triglycerides) (James & Martin, 1952). The use of the oil in industry is determined by the composition of fatty acids, and this is highly dependent on its natural origin. The fatty acid composition of oils from vegetable sources varies depending on plant origin, genetic factors, ripening grade of fruits and specific climatic conditions (Davis & Poneleit, 1974; Velasco, Rojas-Barros, & Fernández-Martínez, 2005), and are involved also in plant response to diverse environmental stresses, including pathogen attack (Feussner & Wasternack, 2002; Norman, Krizek, & Mirecki, 2008; Palma, Marangoni, & Stanley, 1995).

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However, there are few published studies on fatty acid composition of mulberry fruits. The object of the present study was to determine qualitatively and quantitatively the fatty acid composition of 8 clones of mulberry fruits (four of *M. alba* and 4 *M. nigra*), all grown in Spain.

2. Materials and methods

2.1. Chemicals and reagents

All reagents used in the lab procedure were GC grade: hexane, methanol, and boron trifluoride (BF_3) were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany); methylene chloride from Labscan, Ltd (Dublin, Ireland); sodium hydroxide and anhydrous sodium sulfate were from Panreac (Castellar del Vallès, Barcelona, Spain). Three fatty acid methyl ester (FAME) mixes, from Supelco, GLC-50, GLC-80, GLC-100, were used together with individual standards of linoleic (C18:2), linolenic (C18:3), arachidonic (C20:4), eicosapentaenoic (C20:5), docosahexaenoic (C22:6), and heptadecanoic (C17:0) acids. All these mixes and standards were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

2.2. Plant materials

Eight mulberry clones, four white (*M. alba*) clones: MA1, MA2, MA3, and MA4, and four black (*M. nigra*) clones: MN1, MN2, MN3, and MN4, were collected in Orihuela (latitude 38°04′08″N × longitude 0°58′58″W, 27 m above sea level in the province of Alicante, Spain) in the 2013 and 2014. Fully mature fruits were manually harvested and immediately transported to the UMH (Universidad Miguel Hernández de Elche) laboratories. Five trees were selected for each clone and 60 fruits per clone (12 fruits per tree) were randomly picked for analytical determinations at the commercially ripe stage, based on total soluble solids (TSS), titratable acidity (TA), and maturity index (MI); details on the values of these three parameters at harvest time are shown in Fig. 1. Three subsamples by clone (each one of 20 fruits) were randomly prepared, and then fruits from each subsample were dried in a hot air oven at 50 °C until reaching constant weight and then milled.

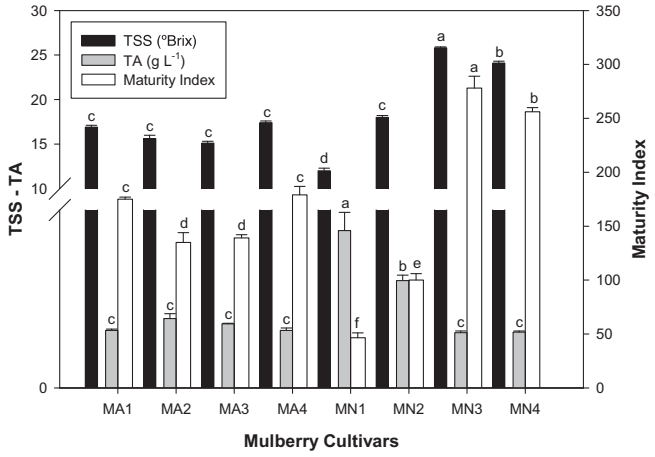

Fig. 1. Main chemical parameters (TSS = total soluble solids, TA = titratable acidity, and MI = maturity index) at harvest time Bars (means ± SE) with the same letter, for each quality parameter, were not significantly different according to Fisheŕs least significant difference (LSD) procedure at 5% significance level ($n = 6$).

2.3. Chemical determinations

Total soluble solids (TSS) and titratable acidity (TA) contents were measured as previously reported by Ercisli and Orhan (2007); measurements were conducted in six replications. The maturity index (MI) was calculated as the ratio between TSS/TA. Results were expressed as mean ± SE (standard error).

2.4. Fatty acid extraction

2.4.1. Methylation procedure

Fatty acids were *in situ* methylated according to Trigueros and Sendra (2015) with some modifications. Briefly, 0.50 g dried mulberry fruit were transferred into a test tube. The internal standard used was C17:0; the standard was freshly prepared as 20 mg/mL solution in HPLC grade *n*-hexane. 30 μL of C17:0 in *n*-hexane solution were added to each tube containing the dried sample. Then, 100 μL of methylene chloride and 1 mL of 0.5 N NaOH in methanol were added and the tubes were heated in a water bath at 90 °C for 10 min. 1 mL of BF_3 in methanol was added and the mixture, and samples were left at room temperature (25 °C) for 30 min to prevent intraisomerization of long chain fatty acids isomers (Werner, Luedecke, & Shultz, 1992). 1 mL of distilled water and 600 μL hexane were added, and then FAMEs were extracted by vigorous shaking for about 1 min. Following centrifugation, the aliquots were dried with anhydrous sodium sulfate and the top layer was transferred into a vial, which was flushed with nitrogen, and vials were stored at −20 °C until analyzed by gas chromatography.

2.4.2. Gas chromatography (GC) analysis

The fatty acid composition of FAMEs was analyzed on an Agilent gas chromatography unit (model 6890, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and a DB-23 capillary column (30 m length, 0.25 μm film, 0.25 mm internal diameter; J&W Scientific, Agilent Technologies). The flow rate of the carrier gas (helium) was 5 mL/min and 30 mL/min at the make-up point; the injector and detector temperatures were 240 and 245 °C, respectively. The injection volume was 0.5 μL (splitless). The temperature program was as follows: initial temperature 60 °C, rate of 4 °C/min from 60 to 220 °C and hold for 10 min, and rate of 1 °C/min from 220 to 225 °C. Oven temperature program was set in order to obtain a good peak resolution. Identification of the FAME peaks was performed by comparing the retention times of the FAME standards; a model chromatogram is shown in Fig. 2. Agilent technologies software (G2072AA Rev.A.05.02 Chemstation) was used for integration of peaks. Fatty acid concentrations in mulberry fruits were expressed as percentage of total fatty acids and also were quantified unitarily in mg/100 g dw. Analyses were conducted in triplicate.

2.5. Statistical analysis

The fatty acid composition of fruits mulberry was investigated in two consecutive years, 2013 and 2014; however, results did not vary significantly ($p > 0.05$) between the two years. Therefore, results are presented as the average of the two years. Each value is a mean of 6 replications, three of each year ($n = 6$). Statistical analyses were performed using SPSS 22.0 for Windows (SPSS Science, Chicago, IL, USA). A basic descriptive statistical analysis was followed by an analysis of variance (ANOVA) test for mean comparisons. The method used to discriminate among the means (Multiple Range Test) was Fisher's Least Significant Difference (LSD) procedure at a 95.0% confidence level. Principal component analyses (PCA) was also performed using SPSS.

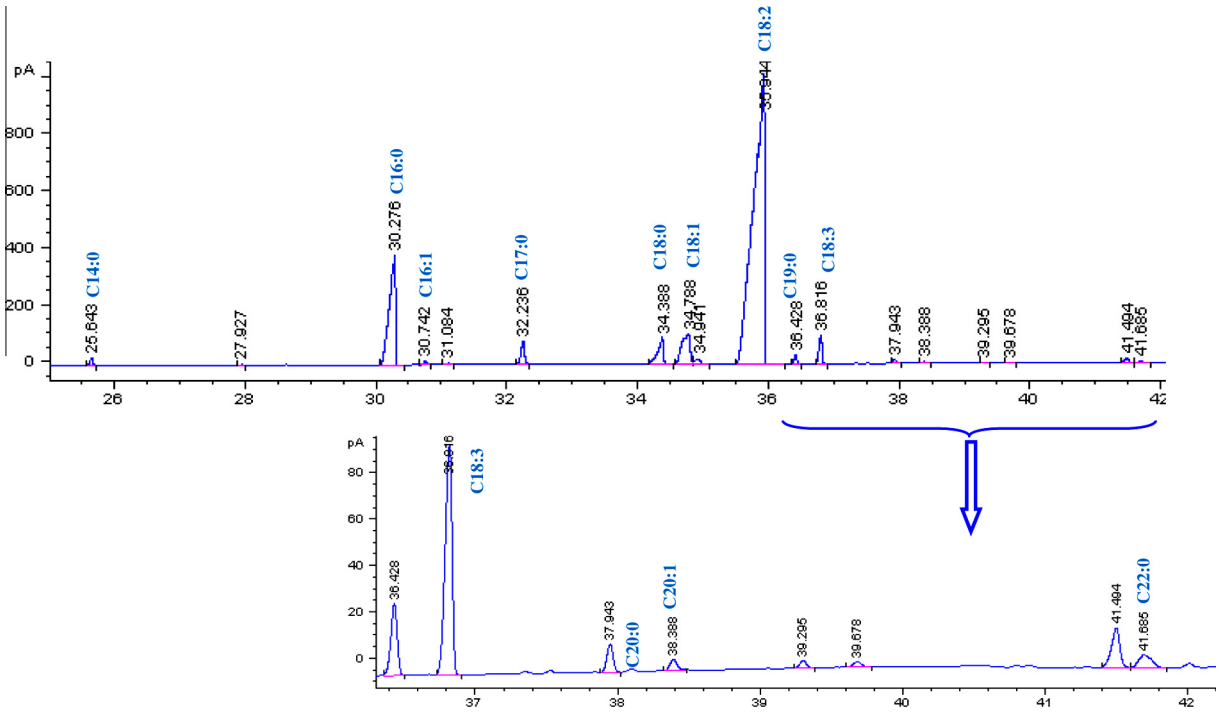

Fig. 2. Chromatogram of fatty acid profile of black mulberry fruits (e.g. clone MN1).

3. Results and discussion

3.1. Fatty acid composition of mulberry fruits

The fatty acid composition expressed as percentage of the total fatty acid content of the eight mulberry clones is shown in Table 1. Fourteen fatty acids were identified and quantified in mulberry fruits. The most abundant fatty acids were linoleic (C18:2), palmitic (C16:0), oleic (C18:1) and stearic (C18:0) acids, in the fruits of both species *M. alba* and *M. nigra*, which together comprised approximately 96.77% of total identified fatty acids (Fig. 2). This agrees with the results previously obtained by Ercisli and Orhan (2007), Yang, Yang, and Zheng (2010), and Gecgel, Velioglu, and Velioglu (2011).

The most abundant fatty acid found in all clones was linoleic acid (C18:2) and this fatty acid ranged from 69.66% (MN2) to 78.02% (MA1) of total fatty acids content. These results agree with those previously found by Gecgel et al. (2011), who reported an average content of 73.70% (C18:2) in black mulberry (*M. nigra* L.), and also with Yang et al. (2010) who observed an average of 79.37% (C18:2) in *M. alba* L. Among the studied clones, *M. nigra* had the lowest C18:2 levels (average 72.97%), whereas *M. alba* clones had the highest levels of this fatty acid (average 74.90%). However, Ercisli and Orhan (2007) in Turkish mulberry fruits found that *M. nigra* had higher C18:2 levels (average 61.85%) than *M. alba* clones (average 57.26%).

Linoleic acid was followed by palmitic acid (C16:0), with its content ranging from 10.72% (MA1) to 17.97% (MN2), oleic acid (C18:1) from 5.32% (MN2) to 7.26% (MN4), and stearic acid (C18:0) from 3.03% (MN2) to 3.79% (MN4). Similar results were reported by Gecgel et al. (2011) and Yang et al. (2010), but these authors observed lower C16:0 values. Nevertheless, Ercisli and Orhan (2007) reported higher values of these fatty acids, except C16:0, in *M. nigra*. The most representative fatty acids reported for other berries (bilberry, cranberry, rose hip, strawberry, elder, and black currant) are also linoleic, linolenic and oleic acids (Helbig, Böhm, Wagner, Schubert, & Jahreis, 2008). The linolenic acid (C18:3) ranged from 1.42% (MN4) to 2.40% (MA3). Gecgel et al. (2011) found lower levels of linolenic (0.45%) in *M. nigra* than those found in the current study, whereas Ercisli and Orhan (2007) did not detect it in *M. nigra* and *M. alba*.

The mulberry clones had high percentages of essential fatty acids (C18:2, and C18:3); for example, *M. alba* contained about 76.67% essential fatty acids (74.90% of C18:2, and 1.78% of C18:3), and *M. nigra* contain about 74.70% essential fatty acids (72.97% of C18:2, and 1.73% of C18:3).

Other fatty acids, such as nonadecanoic (19:0), myristic (C14:0), behenic (C22:0), palmitoleic acid (C16:1) and eicosenoic (C20:1) were present in minor quantities. Gecgel et al. (2011) also detected the behenic (C22:0) and palmitoleic (C16:1) acids in black mulberry. However, Ercisli and Orhan (2007) detected behenic acid (C22:0), and palmitoleic acid (C16:1) at concentrations of 0.26% and 0.67%, respectively, but only in *M. alba*.

Other fatty acids, such as cetoleic (C22:1), arachidic (C20:0), heneicosanoic (C21:0), and arachidonic (20:4) acids were present only in some clones and in small amounts. For example, cetoleic acid was only detected in clones MA1, MA2, and MA4; arachidic acid only in MA4, MN1, and MN3; heneicosanoic acid in MN3 and MN4; and, finally arachidonic acid only in the clone MA3.

These results suggest that the fatty acid profile could be a good parameter to separate different species/clones, but the observed values are easily affected by several factors. There are several factors that can affect fatty acid composition, including plant origin, genetic factors, ripening of fruits, environmental conditions, and temperature during the time elapsed between flowering and ripening (Davis & Poneleit, 1974; Gecgel et al., 2011; Lajara, Díaz, & Díaz, 1990; Velasco et al., 2005).

After grouping of mulberry fatty acids, the abundance order was PUFA > SFA > MUFA. Polyunsaturated fatty acids (PUFA) were the major fraction of the fatty acids, representing at least 76.68% (mean of all four clones) in *M. alba* and 74.74% in *M. nigra*. Monounsaturated fatty acids (MUFA) also represent a considerable part of the fatty acids, with percentages of 16.77% in *M. alba* and 18.75% in *M. nigra*. Saturated fatty acids (SFA) were present only

Table 1
Fatty acid composition of mulberry fruits as % of total fatty acid profile.[a]

Fatty acids (%)	Clones							
	White mulberry (*Morus alba*)				Black mulberry (*Morus nigra*)			
	MA1	MA2	MA3	MA4	MN1	MN2	MN3	MN4
Myristic acid C14:0	0.21 ± 0.01 ab	0.38 ± 0.05 bc	0.24 ± 0.07 ab	0.21 ± 0.04 ab	0.35 ± 0.07 bc	0.50 ± 0.06 c	0.53 ± 0.14 c	0.09 ± 0.01 a
Palmitic acid C16:0	10.72 ± 0.63 a	14.55 ± 1.20 bc	12.78 ± 1.00 ab	11.05 ± 0.74 a	11.93 ± 1.30 ab	17.97 ± 1.94 c	14.04 ± 1.31 ab	12.21 ± 1.04 ab
Palmitoleic acid C16:1	0.12 ± 0.00 a	0.16 ± 0.02 a	0.12 ± 0.01 a	0.13 ± 0.02 a	0.15 ± 0.03 a	0.24 ± 0.04 b	0.10 ± 0.01 a	0.10 ± 0.00 a
Stearic acid C18:0	3.17 ± 0.18 ab	3.76 ± 0.24 b	3.52 ± 0.16 ab	3.17 ± 0.45 ab	3.50 ± 0.08 ab	3.03 ± 0.07 a	3.66 ± 0.06 b	3.79 ± 0.14 b
Oleic acid C18:1	5.55 ± 0.42 a	5.63 ± 0.45 ab	7.12 ± 0.42 c	6.51 ± 0.30 bc	6.00 ± 0.14 ab	5.32 ± 0.16 a	6.49 ± 0.19 bc	7.26 ± 0.36 c
Linoleic acid C18:2	78.02 ± 1.06 c	72.55 ± 1.82 ab	72.81 ± 1.75 ab	76.21 ± 0.62 bc	75.85 ± 1.82 bc	69.66 ± 2.18 a	72.06 ± 1.93 ab	74.30 ± 1.19 abc
Linolenic acid C18:3	1.50 ± 0.13 a	1.56 ± 0.12 a	2.40 ± 0.19 c	1.64 ± 0.15 ab	1.51 ± 0.27 a	1.91 ± 0.10 abc	2.07 ± 0.21 bc	1.42 ± 0.20 a
Nonadecanoic acid C19:0	0.29 ± 0.07 ab	0.70 ± 0.06 c	0.42 ± 0.10 ab	0.44 ± 0.05 abc	0.35 ± 0.09 ab	1.0 ± 0.18 d	0.55 ± 0.09 bc	0.13 ± 0.01 a
Arachidic acid C20:0	nd	nd	nd	0.07 ± 0.04 b	0.02 ± 0.01 a	nd	0.01 ± 0.01 a	nd
Eicosenoic acid C20:1	0.08 ± 0.00 b	0.04 ± 0.02 a	0.11 ± 0.01 b	0.09 ± 0.01 b	0.08 ± 0.01 b	0.10 ± 0.00 b	0.10 ± 0.01 b	0.09 ± 0.01 b
Arachidonic acid C20:4	nd	nd	0.02 ± 0.01 b	nd	nd	nd	nd	nd
Heneicosanoic acid C21:0	nd	nd	nd	nd	nd	nd	0.08 ± 0.02 c	0.04 ± 0.01 b
Behenic acid C22:0	0.17 ± 0.05 a	0.43 ± 0.14 abc	0.46 ± 0.16 bc	0.33 ± 0.11 abc	0.20 ± 0.01 ab	0.22 ± 0.02 abc	0.27 ± 0.05 abc	0.53 ± 0.11 c
Cetoleic acid C22:1	0.17 ± 0.06 b	0.24 ± 0.08 b	nd	0.16 ± 0.07 b	nd	nd	nd	nd
Unidentified fatty acid	nd	nd	nd	nd	0.06 ± 0.03 c	0.05 ± 0.02 bc	0.04 ± 0.02 bc	0.04 ± 0.02 b
Total MUFA	5.92	6.07	7.35	6.89	6.23	5.66	6.69	7.45
Total PUFA	79.52	74.11	75.23	77.85	77.42	71.62	74.17	75.76
Total SFA	14.56	19.82	17.42	15.27	16.35	22.72	19.14	16.79

[a] Values (means ± SE) followed by the same letter, within the same row, are not significantly different according to Fisheŕs least significant difference (LSD) procedure at 5% significance level ($n = 6$). PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids. nd, not detected.

Table 2
Fatty acid content of mulberry fruits (dry weight).[a]

Fatty acids (mg 100 g^{-1} dw)	Clones							
	White mulberry (*Morus alba*)				Black mulberry (*Morus nigra*)			
	MA1	MA2	MA3	MA4	MN1	MN2	MN3	MN4
Myristic acid C14:0	27.8 ± 3.0 bc	31.9 ± 3.8 c	18.0 ± 4.5 ab	24.15 ± 4.17 bc	26.4 ± 1.5 bc	29.4 ± 2.6 c	34.1 ± 6.5 c	8.65 ± 0.65 a
Palmitic acid C16:0	1417 ± 142 c	1208 ± 85 c	992 ± 85 a	1278 ± 72 bc	972 ± 59 a	1062 ± 80 ab	1000 ± 70 a	1236 ± 145 abc
Palmitoleic acid C16:1	16.0 ± 2.1 d	13.4 ± 1.9 bcd	9.33 ± 1.05 ab	15.4 ± 1.9 cd	11.1 ± 0.8 ab	13.6 ± 1.6 bcd	7.15 ± 0.48 a	10.6 ± 1.6 abc
Stearic acid C18:0	429 ± 60 c	313 ± 19 abc	277 ± 23 ab	3863 ± 69 bc	316 ± 56 abc	189 ± 24 a	2751 ± 37 ab	390 ± 58 bc
Oleic acid C18:1	773 ± 139 d	470 ± 40 a	558 ± 49 bcd	782 ± 93 d	524 ± 73 abc	325 ± 28 a	490 ± 73 abc	754 ± 127 cd
Linoleic acid C18:2	10531 ± 1295 d	6138 ± 469 ab	5872 ± 767 ab	9015 ± 791 cd	6878 ± 1228 abc	4365 ± 596 a	5502 ± 870 ab	7789 ± 1515 bcd
Linolenic acid C18:3	193 ± 10 b	129 ± 5 a	187 ± 17 b	188 ± 12 b	120 ± 19 a	115 ± 8 a	146 ± 8 a	156 ± 47 ab
Nonadecanoic acid C19:0	35.4 ± 5.2 bc	58.6 ± 4.4 d	30.4 ± 3.4 ab	49.0 ± 3.2 cd	25.2 ± 4.1 ab	61.2 ± 13.2 d	37.4 ± 1.9 bc	12.9 ± 2.3 a
Arachidic acid C20:0	nd	nd	nd	8.11 ± 4.29 b	2.80 ± 1.44 a	nd	1.22 ± 0.55 a	nd
Eicosenoic acid C20:1	9.99 ± 0.62 d	4.04 ± 1.36 a	8.16 ± 0.72 bcd	10.1 ± 1.1d	6.16 ± 1.02 ab	6.44 ± 0.88 abc	7.36 ± 0.55 bcd	9.80 ± 2.85 cd
Arachidonic acid C20:4	nd	nd	1.40 ± 0.68 b	nd	nd	nd	nd	nd
Heneicosanoic acid C21:0	nd	nd	nd	nd	nd	nd	5.61 ± 0.82 c	3.98 ± 0.47 b
Behenic acid C22:0	20.3 ± 3.7 ab	35.5 ± 11.8 bc	36.7 ± 12.4 bc	37.3 ± 13.2 bc	16.9 ± 2.1 ab	12.8 ± 0.9 a	17.5 ± 1.9 ab	51.2 ± 0.1 c
Cetoleic acid C22:1	19.0 ± 5.0 b	19.4 ± 6.5 b	nd	19.4 ± 7.8 b	nd	nd	nd	nd
Unidentified fatty acid	nd	nd	nd	nd	7.16 ± 0.90 c	4.59 ± 0.73 b	4.83 ± 0.79 b	4.71 ± 0.75 b

[a] Values (means ± SE) followed by the same letter, within the same row, are not significantly different according to Fisheŕs least significant difference (LSD) procedure at 5% significance level ($n = 6$). nd, not detected.

in low percentages with 6.56% in *M. alba* and 6.51% in *M. nigra*. These results agree quite well with those previously reported by Gecgel et al. (2011); these authors reported values of 74.43% of PUFA, but they detected higher amounts of MUFA (10.12%). PUFAs are known to improve the nutritional value and protect consumers against many diseases (Umano & Shibamoto, 1988).

3.2. Fatty acid content of mulberry fruits

The fatty acid compositions expressed in mg/100 g of dried mulberry fruits are shown in Table 2. The main fatty acids were:

- linoleic acid, with concentration ranging from 4365 in MN2 to 10,531 mg/100 g dw in MA1,
- palmitic acid, ranging from 972 in MN1 to 1416.54 mg/100 g dw in MA1,
- oleic acid, ranging from 325 in MN2 to 782 mg/100 g dw in MA4,
- stearic acid, ranging from 189 in MN2 to 429 mg/100 g dw in MA1, and
- linolenic acid, ranging from 115 in MN2 to 193 mg/100 g dw in MA1.

Linoleic acid is an essential fatty acid from which the whole ω-6 fatty acid family is derived. These fatty acids are important components of the cell membranes and are precursors of other substances involved in many physiological responses (Lai et al., 2015). By contrast, palmitic acid is considered as an atherogenic compound, when consumed in high amount (Lai et al., 2015). The results indicate that the Spanish mulberries were found to be rich in the essential linoleic acid. Taking into account these facts, it is worth mentioning that 100 g of fresh mulberries, from clones such as MA1, contain as much as 2.82 g of linoleic acid (about 28.20% of RDI, Recommended Daily Intake) (European Food Safety Authority, 2009).

Other fatty acids were found in intermediate concentrations, including nonadecanoic acid (ranging from 12.89 to 61.24 mg/100 g dw in MN4 and MN2, respectively), myristic acid (from 8.65 to 34.10 mg/100 g dw in MN4 and MN3, respectively), behenic acid (from 12.83 to 51.22 mg/100 g dw in MN2 and

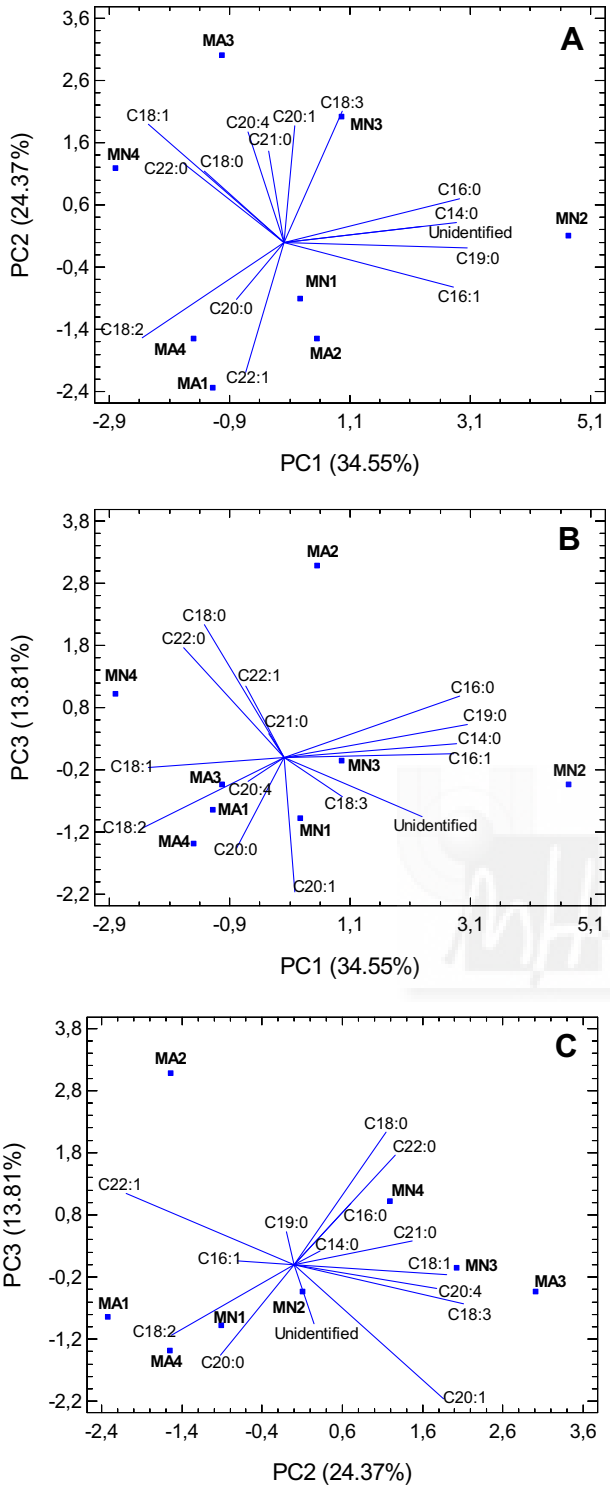

Fig. 3. Principal component analysis (PC1, PC2, and PC3) of mulberry fruits.

MN4, respectively), palmitoleic acid (from 7.15 to 16.04 mg/100 g dw in MN3and MA1, respectively), and eicosenoic acid (from 4.04 to 10.06 mg/100 g dw in MA2 and MA4, respectively). Cetoleic, arachidic, heneicosanoic, and arachidonic acids were only found in some clones and only in low quantities.

The present study showed that the clone MA1 presented the highest total content of fatty acids followed by MA4, MN4, MN1, MA2, MA3, MN3, and MN2. In general, among the studied clones, *M. alba* (from 7991 mg/100 g dw in MA3 to 13,470 mg/100 g dw in MA1) had higher total contents of fatty acids than *M. nigra* (from 6184 mg/100 g dw in MN2 to 10,426 mg/100 g dw in MN4).

The clones MA1, MA4, MN4, and MN1 of Spanish mulberries could be good source of essential fatty acids, especially linoleic acid.

4. Principal components analysis

To achieve a better and easier understanding of the trends and relationships among the many studied variables (15) for the different mulberry samples (8 clones), principal component analysis (PCA) was applied to experimental data generated (Fig. 3).

The first four principal components explained 85.28% of the total variation (Table 3). A 58.92% of the observed variability of the studied data was explained by the first two components (Table 3). The first component, PC1 representing 34.55% of total variance was positively linked to the contents of the following fatty acids: myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), and nonadecanoic (19:0), and negatively linked to linoleic acid (C18:2) (Table 3 and Fig. 3A and B). PC2 accounted for 24.37% of the total variance, and was positively correlated with the content of oleic (C18:1), linolenic (C18:3), and arachidonic (20:4) acids, but negatively with cetoleic (C22:1) acid (Table 3 and Fig. 3A and C). PC3 accounted for 13.81% of the total variance and was positively correlated with arachidic (C20:0) and eicosenoic (C20:1) acids and negatively with stearic (C18:0) and behenic (C22:0) acids. The principal component PC1 (Table 3 and Fig. 3A and B) allowed separation of the clones MA1, MA4, MN4, and MA3, which are located in the negative PC1 axis, and MN1, MA3, MN3, and MA2 in the positive axis and clearly differentiated the MN2 clone with a high positive value.

This indicates that the MA1, MA4 and MN4 clones are characterized by a high content of linoleic acid (C18:2), and low contents of myristic (C14:0), palmitic (C16:0), nonadecanoic (19:0), and palmitoleic (C16:1) acids. These data suggest that MA1, MA4, and MN4 clones are good sources of fatty acids, because they have a high content of polyunsaturated fatty acids (PUFA) and low content of saturated fatty acids (SFA). The MN2 clone that presented the opposite behavior was characterized by a low content of linoleic acid (C18:2) and high contents of myristic (C14:0), palmitic (C16:0), nonadecanoic (19:0), and palmitoleic (C16:1) acids. The principal component PC2 (Fig. 2A and C) allowed separation in

Table 3
Eigenvalues, proportion of variation and eigenvectors associated with each principal component.

Principal components (axes)	1	2	3	4
Eigenvalues	5.18	3.66	2.07	1.88
Cumulated proportion of variation (%)	34.55	58.92	72.73	85.28
Characters	Eigenvectors			
Myristic acid C14:0	0.381	0.060	−0.051	0.198
Palmitic acid C16:0	0.388	0.132	−0.221	−0.057
Palmitoleic acid C16:1	0.374	−0.134	−0.014	−0.222
Stearic acid C18:0	−0.174	0.214	−0.477	0.247
Oleic acid C18:1	−0.298	0.356	0.037	0.007
Linoleic acid C18:2	−0.312	−0.288	0.254	0.074
Linolenic acid C18:3	0.129	0.395	0.141	−0.267
Nonadecanoic acid C19:0	0.405	−0.018	−0.118	−0.174
Arachidic acid C20:0	−0.104	−0.172	0.325	−0.037
Eicosenoic acid C20:1	0.023	0.350	0.483	−0.030
Arachidonic acid C20:4	−0.080	0.332	0.085	−0.471
Heneicosanoic acid C21:0	−0.033	0.275	−0.086	0.533
Behenic acid C22:0	−0.220	0.237	−0.394	−0.224
Cetoleic acid C22:1	−0.084	−0.392	−0.257	−0.214
Unidentified fatty acid	0.304	0.047	0.214	0.369

the positive axis MN4, MN3, MA3, and MN2 clones. MA3 was clearly separated from the others, and MN2 had a value around 0. The MA3 clone was the only clone that contained arachidonic (20:4) acid and had higher content of linolenic (C18:3), oleic (C18:1) than the majority of the clones studied. The clones MN3 and MA2 had similar positive values in the PC1 axis; however and according to the principal component PC3 (Fig. 2B and C), these two clones can be clearly differentiated. The MA2 clone differs from the others by its low amount of eicosenoic (C20:1) acid and high of cetoleic (C22:1) acid. Finally, PC2 and PC3 were not able to differentiate MA4 and MN1 clone; however, PC1 (Fig. 2A and B) allowed the establishment of small differences between MA4 and MN1 clones.

5. Conclusions

Results of this study illustrate the nutritional potential of the fatty acid profile of white and black Spanish mulberry fruits. The most abundant fatty acids were linoleic, palmitic, and oleic. Linoleic acid, an essential polyunsaturated fatty acid, dominated the fatty acid profile (73.9% of total fatty acids) in all the clones. In addition, GC-FID analysis revealed that the clones MA1, MA4, MN4, and MN1 contained higher contents of fatty acids, especially of linoleic acid, than the other clones. These results showed that black and white mulberries could be considered as a natural source of "essential" fatty acids, particularly linoleic acid, and support their high potential for their use in the food, cosmetic, and pharmaceutical industries.

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8.5. PUBLICACIÓN 5

(Poly)phenolic fingerprint and chemometric analysis of white (*Morus alba* L.) and black (*Morus nigra* L.) mulberry leaves by using a non-targeted UHPLC-MS approach

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(Poly)phenolic fingerprint and chemometric analysis of white (*Morus alba* L.) and black (*Morus nigra* L.) mulberry leaves by using a non-targeted UHPLC–MS approach

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ABSTRACT

This study reports the (poly)phenolic fingerprinting and chemometric discrimination of leaves of eight mulberry clones from *Morus alba* and *Morus nigra* cultivated in Spain. UHPLC–MS^n (Ultra High Performance Liquid Chromatography–Mass Spectrometry) high-throughput analysis allowed the tentative identification of a total of 31 compounds. The phenolic profile of mulberry leaf was characterized by the presence of a high number of flavonol derivatives, mainly glycosylated forms of quercetin and kaempferol. Caffeoylquinic acids, simple phenolic acids, and some organic acids were also detected. Seven compounds were identified for the first time in mulberry leaves. The chemometric analysis (cluster analysis and principal component analysis) of the chromatographic data allowed the characterization of the different mulberry clones and served to explain the great intraspecific variability in mulberry secondary metabolism. This screening of the complete phenolic profile of mulberry leaves can assist the increasing interest for purposes related to quality control, germplasm screening, and bioactivity evaluation.

1. Introduction

Mulberry plant (genus *Morus,* family Moraceae) is a monoecious or dioecious plant, growing up to 10–12 m high, widely distributed in India, China, Japan, North Africa, South Europe, etc (Thabti et al., 2014). There are 24 species of *Morus* and one subspecies, with at least 100 known varieties (Ercisli & Orhan, 2007). The most commonly known species in the *Morus* genus are white mulberry (*Morus alba* L.), black mulberry (*Morus nigra* L.), and red mulberry (*Morus rubra* L.) (Gundogdu, Muradoglu, Sensoy, & Yilmaz, 2011).

Owing to its nutritive value, mulberry fruit is consumed in both fresh and processed forms, such as juice, jams, syrups, beverages, natural dyes, or dehydrated fruits (Ercisli & Orhan, 2007; Gundogdu et al., 2011). Mulberry leaves are probably best known by their role in the silk production, as food for silkworms (*Bombyx mori* L.). However, they have moved under the spotlight of nutritional research over the last years since some studies have pointed out that mulberry leaves may constitute a good source of (poly)phenolic compounds able to positively impact on human health (Sánchez-Salcedo, Mena, García-Viguera, Hernández, & Martínez, 2015a). In fact, they exhibit significant hypoglycaemic, hypolipidaemic, anti-inflammatory, and anti-atherogenic effects in humans and in animal models (Chung, Kim, Kim, & Kwon, 2013; Enkhmaa et al., 2005; Harauma et al., 2007; Hunyadi et al., 2013; Jeszka-Skowron et al., 2014). The phenolics associated with these biological effects are mainly phenolic acids and flavonol derivatives (Dugo et al., 2009; Enkhmaa et al., 2005; Hunyadi et al., 2013; Sánchez-Salcedo et al., 2015a; Thabti, Elfalleh, Hannachi, Ferchichi, & Campos, 2012). All these facts are paving the way to the revalorisation of this underused vegetal material in order to develop mulberry leaf-derived products rich in phenolics such as teas, fortified beverages, muesli bars, yogurts, etc (Nam, Jang, & Shibamoto, 2012). Some efforts aiming at the identification of phenolic compounds in mulberry leaves have been done (Dugo et al., 2009; Thabti et al., 2012). Nevertheless, the comprehensive characterization of this phenolic-rich matrix is still lacking. Compositional data, along with genetic, morphological, and

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agronomical parameters, may also lead to a full characterization of plant material, with the objective of its retrieval and biodiversity valorization. Moreover, the screening of the complete phenolic profile of mulberry leaves could be used to study environmental adaptations (Calani et al., 2013).

The aim of this study was therefore to investigate the fingerprint and chemometric discrimination of (poly)phenolic compounds in leaves of eight mulberry clones with industrial interest from *Morus alba* and *Morus nigra* grown in Spain, one of the main European producers. The assessment of the (poly)phenolic profile of mulberry leaves was carried out by using ultra-HPLC (UHPLC) separation and MS^n linear ion trap mass spectrometric characterization. The phytochemical discrimination of the clones was obtained by chemometric analysis, which assisted in the unravelling of the great intra- and inter-specific variability in mulberry secondary metabolites. This combined recourse to UHPLC–MS^n high-throughput qualitative profiling and multivariate statistical analysis overcame the limitations of traditional approaches for evaluating the phytochemical profile of plant matrices based on a list of target analytes. It also represented a relevant combination to comprehensively assess large batches of plant products.

2. Materials and methods

2.1. Chemicals

Quinic, caffeic, protocatechuic, neochlorogenic, and chlorogenic acids, as well as rutin (quercetin 3-β-rutinoside), were purchased from Sigma-Aldrich (Steinheim, Germany). The solvents were supplied by Carlo Erba Reagents (Milan, Italy).

2.2. Plant materials

Fresh leaves samples of eight mulberry clones, four white (*M. alba*) clones: MA1, MA2, MA3, and MA4, and four black (*M. nigra*) clones: MN1, MN2, MN3, and MN4, were manually collected in Orihuela (latitude 38°04′08″N × longitude 0°58′58″W, 27 m above sea level, Alicante, SE Spain) in June 2013. After collection, the samples were washed with distilled water and freeze-dried. The freeze-dried materials were grounded into fine powders (particle size <0.4 mm) with a domestic grinder (Moulinex, Écully, France) and stored in airtight container at 20 °C until use.

2.3. Extraction of phenolic compounds

The (poly)phenolic compounds in mulberry leaves were extracted according to a previous report (Sánchez-Salcedo et al., 2015a). The mixture of 150 mg of freeze-dried powder and 1 mL of 80% aqueous methanol acidified with formic acid (1%) was sonicated at 25 ± 5 °C for 60 min. The mixture was centrifuged at 10,480*g* for 5 min at room temperature and the supernatant was collected. Two additional extractions were performed for each sample with additional 0.5 mL of the same solvent, as described above. The last extraction was kept at 4 °C overnight and sonicated again for 25 min, after which it was centrifuged. The three supernatants were pooled before UHPLC–MS analysis. Each sample was extracted in triplicate.

2.4. Characterization by ultra-high performance liquid chromatography–mass spectrometry (UHPLC–MS)

Methanolic extracts of mulberry leaf powder were analyzed using an Accela UHPLC 1250 equipped with a linear ion trap-mass spectrometer (MS) (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray ionization probe (H-ESI-II; Thermo Fisher Scientific Inc., San Jose, CA, USA). Separations were performed using a BlueOrchid C18 column (50 × 2 mm), 1.8 μm particle size (Knauer, Berlin, Germany). Volume injected was 5 μL and column oven was set to 30 °C. Each sample was analyzed in duplicate.

Two MS experiments in negative mode were performed (Mena et al., 2012). An experiment optimized for flavonoid analysis after infusion with epicatechin (Experimental Conditions 1) was carried out using the following conditions: the MS worked with a capillary temperature equal to 275 °C, while the source heater temperature was set to 200 °C. The sheath gas flow was 40 units, while both auxiliary and sweep gas were set to 5 units. The source voltage was 4 kV. The capillary and tube lens voltages were −42 and −118 V, respectively. The mobile phase was 0.1% (v/v) aqueous formic acid (phase A) and 0.1% (v/v) formic acid in acetonitrile (phase B). Elution was performed at a flow rate of 0.2 mL/min. The gradient started with 100% A, held until 2 min, 20% B at 5 min, 40% B at 13 min, 80% B at 14 min, 80% B held until 17 min followed by 4 min to re-equilibrate the column. Analyses were carried out using full scan mode, data-dependent MS^3 scanning from *m/z* 100–2000, with collision induced dissociation (CID) equal to 30 (arbitrary units). Pure helium gas was used for CID.

In a second experimental framework, the MS worked with conditions optimized for hydrolysable tannin analysis, after infusion with punicalagin standard (mixture of two isomers) (Experimental Conditions 2). The capillary temperature was set to 275 °C, while the source heater temperature was 250 °C. The sheath gas flow was 60 units, while auxiliary and sweep gas were set to 15 and 4 units, respectively. The source voltage was 4 kV. The capillary and tube lens voltages were −49 and −153 V, respectively. Analyses were carried out using full scan mode, data-dependent MS^3 scanning from *m/z* 100–2000, with CID equal to 30 (arbitrary units). The chromatographic conditions were identical to those used for Experimental Conditions 1.

Data processing was performed using Xcalibur 2.2 SP1.48 software from Thermo Scientific. Quantification was performed in selected ion monitoring mode by selecting the relative base peak at the corresponding mass to charge ratio (*m*/*z*) under Experimental Conditions 1.

2.5. Statistical analysis

Peak area values of each (poly)phenolic compound were used for the different multivariate analyses applied. Each peak area used for the analyses was the mean of six replicates (two analytical duplicates for each one of the extraction triplicates). Statistical analyses were performed using STATGRAPHICS Plus 5.0 software (Manugistics, Inc., Rockville, MD). Cluster analysis was applied to obtain hierarchical associations using the Euclidean distance and Ward's method as dissimilarity measure and amalgamation rule, respectively. Principal component analysis was carried out using the analytical data as variables.

3. Results and discussion

3.1. Identification of phytochemicals compounds in mulberry leaves

In this study, the leaves of four clones of *M. alba* and four of *M. nigra* were compared. The phytochemical composition of these plant matrixes, focused on the phenolic fraction, was investigated by a UHPLC-ESI-MS^n fingerprint method paired with multivariate analysis to facilitate a rapid, comprehensive screening of their potential (Calani et al., 2013). The phytochemical profile of mulberry leaves was determined by using a non-targeted procedure for

Table 1
Mass spectral characteristics of (poly)phenolic compounds detected in mulberry leaves.

Id.	Compounds	RT (min)	$[M-H]^-$ (*m/z*)	MS^2 ion fragments (*m/z*)[c]	MS^3 ion fragments (*m/z*)[c]
1	L-Malic acid	0.84	**133**	**115**[d](100), 87(5)	71(100)
2	Quinic acid[a]	0.86	**191**	**127(100)**, 173(85), 85(70), 93(40)	85(100), 99(55), 109(40)
3	Citric acid	1.11	**191**	**111(100)**, 173(32)	111(100), 67(50)
4	Dihydroxybenzoic acid (protocatechuic acid)[a,b]	3.43	**153**	**109(100)**	
5	Caffeoylquinic acid glucoside	5.96	**515**	**179(100)**, 353(60), 341(50), 191(10)	135(100)
6	3-Caffeoylquinic acid (neochlorogenic acid)[a]	6.14	**353**	**191(100)**, 179(40), 135(9), 173 (5)	173(100), 127(90), 111(60), 85(35)
7	Caffeic acid-hexoside	6.25	**341**	**179(100)**	135(100)
8	5-Caffeoylquinic acid (chlorogenic acid)[a]	6.72	**353**	**191(100)**, 173(25), 179(18)	127(100), 173(90), 85(55), 93(55)
9	Quercetin-hexoside-hexoside	6.74	**625**	**301(100),** 463(48)	179(100), 151(45)
10	Caffeic acid[a]	6.81	**179**	**135(100)**	135(100)
11	Kaempferol-hexoside-hexoside	6.87	**609**	**447(100)**, 285(10)	284(100), 327(30), 255(10)
12	4-Caffeoylquinic acid (cryptochlorogenic acid)	6.91	**353**	**191(100)**, 173(10), 179(5)	127(100), 173(90), 85(70), 93(50)
13	Quercetin malonyl-dihexoside[b]	6.91	**711**	**667(100)**	505(100), 463(40), 301(20), 547(10), 625(10)
14	Kaempferol rutinoside hexoside[b]	6.96	**755**	**593(100)**	285(100)
15	Caffeoylquinic acid isomer	7.01	**353**	**191(100)**, 179(10)	127(100), 173(88), 85(74), 93(56)
16	Kaempferol-malonyl-dihexoside[b]	7.15	**695**	**651(100)**, 489(18), 531(5)	489(100), 285(54), 447(20), 531(18)
17	Quercetin-dihexoside	7.33	**625**	300(100), **301(85)**, 445(79), 463(45), 505(25)	179(100), 151(58)
18	Quercetin-rhamnose-hexose-rhamnose[b]	7.35	**755**	**301(100)**, 591(60), 489(40), 609(30), 271(18)	271(100), 255(50), 179(10), 151(8)
19	Quercetin-rutinoside isomer	7.59	**609**	**301(100)**, 445(20), 489(15)	271(100), 255(65), 179(5), 151(3)
20	Quercetin-rutinoside (rutin)[a]	7.77	**609**	**301(100)**	179(100), 151(45), 257(20), 273(15)
21	Kaempferol-hexoside-rhamnoside	7.88	**593**	**284(100)**, 429(80), 447(20), 473(10)	255(100), 227(5)
22	Quercetin-malonyl-rutinoside[b]	7.88	**695**	**651(100)**	300(100), 609(80), 591(30)
23	Quercetinhexoside (isoquercitrin)	7.94	**463**	**301(100)**	179(100), 151(65), 273(15), 257(12)
24	Quercetin-acetylhexoside	8.19	**505**	**301(100)**, 463(20)	179(100), 151(65), 273(15), 257(15)
25	Kaempferolrutinoside	8.19	**593**	**285(100)**	257(100), 267(51), 229(47), 241(41), 213(21)
26	Quercetin-malonyl-hexoside	8.21	**549**	**505(100)**	301(100), 179(6), 151(3)
27	Kaempferol-malonyl-rutinoside[b]	8.30	**679**	**635(100)**	284(100), 471(40), 255(25), 593(15), 489(10), 429(5)
28	Quercetin-acetylhexoside	8.38	**505**	**301(100)**, 445(45), 463(25)	179(100), 151(75), 273(15), 257(10)
29	Kaempferol hexoside (astragalin)	8.42	**447**	**285(100)**, 327(25)	257(100), 267(49), 229(45), 241(40), 213(22)
30	Quercetin-acetylhexoside	8.60	**505**	**301(100)**, 463(20), 445(10)	179(100), 151(55), 273(20), 257(15)
31	Kaempferol-acetylhexoside	8.83	**489**	**285(100)**	257(100), 229(50), 267(40), 241(35), 213(20)

[a] Compounds identified by comparing retention times and MS data with those of reference compounds.
[b] Compounds (tentatively) identified for the first time in mulberry leaf.
[c] The relative ionic abundance for each ion is reported in parentheses.
[d] MS^2 ions in bold were those subjected to MS^3 fragmentation.

screening of (poly)phenolic compounds with diverse ionization attitudes and consisting of two complementary MS conditions (Mena et al., 2012). This approach allowed the full characterisation of the phenolic fraction of mulberry leaves and the tentative identification of a total of 31 compounds (Table 1), the widest phenolic characterisation of this material to date. This set of compounds was present in all the clones tested, regardless of the species considered, *M. alba* or *M. nigra*. More than 120 mass spectrum outputs were analysed for each analytical replicate and experimental condition. Considering the number of (poly)phenolic compounds identified in mulberry leaf, flavonols were the most relevant. Caffeoylquinic acids and some simple phenolic acids were also detected. Moreover, other phytochemicals such as organic acids were found.

Six compounds were identified by comparison with reference standards, while the remaining 25 compounds were tentatively identified based on the interpretation of their fragmentation patterns obtained from mass spectra (MS^2 and MS^3 experiments) and by comparison with the literature. The interpretation of the MS fragmentation patterns present in the literature was not discussed unless of special interest. The retention times and mass spectrum data along with peak assignments for the identified phytochemicals are described in Table 1.

A total of 20 flavonols were identified in mulberry leaf, which pointed out flavonols as the main class of phenolics by number of compounds. They were glycosylated forms of quercetin and kaempferol. Both flavonol aglycones showed a wide number of derivatives with different sugar moieties and were variously acylated. Among the 12 quercetin derivatives detected, 9 (compounds **9**, **17**, **19**, **20**, **23**, **24**, **26**, **28**, and **30**) had been previously identified in leaves of *M. alba* (Dugo et al., 2009; Thabti et al., 2012), while, as far as we know, compounds **13**, **18**, and **22** have been described in *Morus* leaves for the first time (Dall'Asta et al., 2012; Kazuma, Noda, & Suzuki, 2003; Mikulic-Petkovsek, Slatnar, Stampar, & Veberic, 2012). Regarding kaempferol derivatives, compounds **11**, **21**, **25**, **29**, and **31** had already been described in mulberry leaf (Dugo et al., 2009; Thabti et al., 2012). Three kaempferol glycosides (compounds **14**, **16**, and **27**) have been described for the first time in this plant material, to our knowledge, in this study. Compound **14** was identified by comparison with previously reported data (Bresciani et al., 2015). Compounds **16** and **27** exhibited molecular ions at *m/z* 695 and 679, respectively, and were tentatively identified as kaempferol-malonyl-rutinoside and kaempferol-malonyl-dihexoside, according to the characteristic fragmentation pattern of flavonols esterified with malonic acid (Dugo et al., 2009; KajdŽanoska, Gjamovski, & Stefova, 2010; Kazuma et al., 2003). Flavonols have been reported to be the main group of flavonoids in mulberry leaves, with concentrations ranging from 3.7 to 9.8 mg/g dw in Spanish clones (Sánchez-Salcedo et al., 2015a), while reaching up to 85.4 mg/g for Tunisian *M. rubra* (Thabti et al., 2012). Quercetin derivatives prevail over kaempferol

ones, and isoquercitrin (compound **23**) and quercetin-malonyl-glucoside (**26**) have been reported among the main individual flavonoids of mulberry leaves (Dugo et al., 2009; Sánchez-Salcedo et al., 2015; Thabti et al., 2012).

A total of five caffeoylquinic acid derivatives were found in this study. In particular, four caffeoylquinic acid isomers (compounds **6**, **8**, **12**, and **15**) and a caffeoylquinic acid glucoside (**5**) were identified (Dugo et al., 2009; Jaiswal, Müller, Müller, Karar, & Kuhnert, 2014). Caffeoylquinic acids are the phenolic compounds present in larger quantities in mulberry leaves, being chlorogenic acid (5-caffeoylquinic acid) the predominant one. Total caffeoylquinic acid concentrations varies between 6.8 and 8.5 mg/g dw for Spanish *M. alba* and *M. nigra* clones, without significant inter-species differences. Nevertheless, great intra- and interspecific variations in caffeoylquinic acid content have been evidenced in other studies (Dugo et al., 2009; Radojković, Zeković, Vidović, Kočar, & Mašković, 2012; Thabti et al., 2012).

Other phenolic compounds, such as protocatechuic acid (**4**), caffeic acid (**10**), and caffeic acid-hexoside (**7**), were identified by comparison with their commercial standard or, in the case of caffeic acid-hexoside, by its fragmentation pattern (Dall'Asta et al., 2012). Caffeic acid and its glycosylated form have been described at very low concentrations (<1 mg/g dw) in *M. alba* leaf (Skupień, Kostrzewa-Nowak, Oszmiański, & Tarasiuk, 2008). However, to our knowledge, this is the first time that protocatechuic acid is reported in *Morus* species. Some organic acids of mulberry fruits (Mikulic-Petkovsek, Schmitzer, Slatnar, Stampar, & Veberic, 2012; Sánchez et al., 2014), such as L-malic acid (**1**), quinic acid (**2**), and citric acid (**3**) were also detected in the leaves of *M. alba* and *M. nigra* clones.

This high-throughput phytochemical screening evidenced the broad array of (poly)phenolic structures and other chemicals present in mulberry leaves and, although the phenolic profile of mulberry leaf had been extensively studied (Dugo et al., 2009; Enkhmaa et al., 2005; Hunyadi et al., 2013; Skupień et al., 2008; Sánchez-Salcedo et al., 2015a; Thabti et al., 2012), this work allowed the tentative identification of 7 compounds not previously reported in this plant.

The lack of authentic standards for the tentatively identified compounds hinders accurate calibration and absolute quantification of phytochemicals when mass spectrometry detection is used. Slight structural differences may entail significant differences in electro-spray ionization ability and, hence, quantitation of a class of compounds on the basis of a reference standard can be cumbersome and lead to abnormal quantification (Calani et al., 2013). As an alternative strategy, non-targeted screening and chemometric analysis of the chromatographic data allow phytochemical discrimination, overcoming the limitations of traditional methods. Actually, based on multivariate statistics, this approach may produce relevant information for the assessment of the functional, nutritional, and technological potential of mulberry leaves from different clones and species.

3.2. Chemometric classification

Two unsupervised pattern recognition methods, cluster analysis (CA) and principal component analysis (PCA), were applied. The peak area values of the 31 compounds identified were used as variables.

CA was carried out to obtain hierarchical associations among clones as a dendrogram (Fig. 1). Three major groups were clustered based on the existing similarities with respect to the variables analyzed. The first group consisted of four clones divided in two subclusters (MA1 and MA3, and MA2 and MN2), the second included one clone (MN1), and the third one comprised three clones (MA4, MN3, and MN4). These groups were highly dissimilar,

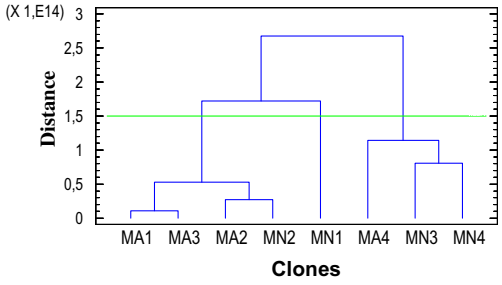

Fig. 1. Dendrogram of the hierarchical cluster analysis among eight mulberry fruit clones using Ward's method based on squared Euclidean distance.

indicating clear differences among clones (intra-species variability) irrespectively of the species considered (inter-species variability). This point has already been emphasized when dealing with the phytochemistry of Spanish mulberries (Sánchez-Salcedo, Mena, García-Viguera, Martínez, & Hernández, 2015b; Sánchez-Salcedo et al., 2015a).

To achieve a better understanding of the characteristics of the different groups, PCA was applied. The first six uncorrelated principal components (PCs) explained 97.9% of the total variation. The variable percentage contributions to the main PCs are described in Table 2. Loading and score plots are reported in Fig. 2 and Supplemental Fig. S1, respectively.

Main PCs were correlated attending to the kind of glycoside attached to the flavonol moiety. Concerning this, the first component (PC1) was positively linked to flavonols containing *O*-hexoside units, like quercetin-hexoside-hexoside (**9**), kaempferol-hexoside-hexoside (**11**), quercetinmalonyl-dihexoside (**13**), kaempferol-malonyl-dihexoside (**16**), quercetinhexoside (isoquercitrin) (**23**), quercetin-acetylhexosides (**24**, **28**, and **30**), quercetin-malonyl-hexoside (**26**), and kaempferol-acetylhexoside (**31**), and negatively correlated to caffeic acid (**10**) (Table 2, Fig. 2). PC2, associated mainly with caffeoylquinic acids and flavonols containing *O*-rutinoside units, had positive loadings from caffeic acid-hexoside (**7**), 5-caffeoylquinic acid (chlorogenic acid) (**8**), and kaempferol-malonyl-rutinoside (**27**), while negative loads from 4-caffeoylquinic acid (cryptochlorogenic acid) (**12**), kaempferolrutinosidehexoside (**14**), caffeoylquinic acid (**15**), quercetin-rutinoside (rutin) (**20**), and kaempferolrutinoside (**25**) (Table 2, Fig. 2). PC3 was negatively correlated to quinic acid (**2**) and mainly flavonols containing *O*-rhamnoside units such as quer cetin-rhamnose-hexose-rhamnose (**18**) and kaempferol-hexoside-rhamnoside (**21**), but also quercetin-malonyl-rutinoside (**22**) (Table 2, Fig. 2). PC4 and PC5 had positive loadings from protocatechuic acid (**4**) and from quercetin-dihexoside (**17**), the isomer of quercetin-rutinoside (**19**) and kaempferolhexoside (astragalin) (**29**), respectively (Table 2). PC6 correlated negatively to L-malic acid (**1**), citric acid (**3**), caffeoylquinic acid glucoside (**5**), and 3-caffeoylquinic acid (neochlorogenic acid) (**6**) (Table 2).

The clusterization obtained by PCA was very similar to that aforementioned for the hierarchical CA. Again, three groups of mulberry clones were differenced. The first group, comprising MN3, MN4, and MA4 and having positive PC1 scores, was characterized by a high content in flavonols containing *O*-hexoside units and a low content in caffeic acid. They showed a variable content in flavonols containing rutinosides (PC2), while MN3 and MN4 (positive PC3) showed a lower content in compounds **18**, **21**, and **22** than MA4 (negative PC3).

Clones MA1, MA2, MA3, and MN2 were grouped attending to their negative scores for PC1 and positives ones for PC2 (Fig. 2). They displayed a low content in flavonols containing *O*-hexoside units and compounds **12**, **14**, **15**, **20**, and **25**, whereas a high amount of **7**, **8**, and **27**. Their content in rutinoside-containing

Table 2
Eigenvalues, proportion of variation and eigenvectors associated with each principal component.

Principal components (axes)		1	2	3	4	5	6
Eigenvalues		10.72	6.34	4.44	3.46	3.15	2.24
Cumulated proportion of variation (%)		34.59	55.03	69.35	80.51	90.68	97.90
Code	**Compound**	**Eigenvectors**					
1	L-Malic acid	−0.129	−0.123	−0.034	−0.161	0.034	−0.529
2	Quinic acid	−0.031	−0.154	−0.398	−0.017	−0.186	0.102
3	Citric acid	0.117	0.043	−0.198	0.268	−0.035	−0.430
4	Dihydroxybenzoic acid (protocatechuic acid)	0.030	0.096	−0.101	0.461	0.005	0.002
5	Caffeoylquinic acid glucoside	0.020	0.014	−0.298	−0.261	0.100	−0.363
6	3-Caffeoylquinic acid (neochlorogenic acid)	−0.200	0.009	0.153	−0.224	0.005	−0.317
7	Caffeic acid-hexoside	−0.163	0.200	0.180	−0.143	0.201	−0.184
8	5-Caffeoylquinic acid (chlorogenic acid)	−0.092	0.225	−0.147	0.284	0.064	−0.299
9	Quercetin-hexoside-hexoside	0.261	−0.167	−0.017	−0.148	0.013	−0.037
10	Caffeic acid	−0.251	−0.119	−0.065	−0.149	−0.124	0.050
11	Kaempferol-hexoside-hexoside	0.240	−0.192	0.015	−0.019	0.187	−0.126
12	4-Caffeoylquinic acid (cryptochlorogenic acid)	0.049	−0.358	−0.120	−0.146	0.069	−0.023
13	Quercetin malonyl-dihexoside	0.270	0.048	−0.118	−0.093	−0.186	0.031
14	Kaempferol rutinoside hexoside	−0.017	−0.346	−0.001	0.250	−0.027	−0.089
15	Caffeoylquinic acid isomer	0.070	−0.307	−0.270	−0.017	−0.019	0.064
16	Kaempferol-malonyl-dihexoside	0.279	0.063	−0.036	0.020	−0.192	−0.024
17	Quercetin-dihexoside	0.106	−0.109	0.167	0.114	0.440	0.068
18	Quercetin-rhamnose-hexose-rhamnose	−0.054	−0.170	−0.387	−0.166	0.030	0.044
19	Quercetin-rutinoside isomer	0.000	0.079	−0.229	−0.090	0.444	0.124
20	Quercetin-rutinoside (rutin)	−0.020	−0.331	0.022	0.249	−0.117	−0.124
21	Kaempferol-hexoside-rhamnoside	−0.066	0.157	−0.265	0.262	0.280	0.010
22	Quercetin-malonyl-rutinoside	0.106	0.204	−0.291	−0.179	0.040	0.233
23	Quercetinhexoside (isoquercitrin)	0.197	−0.164	0.150	−0.046	0.310	0.039
24	Quercetin-acetylhexoside	0.294	0.064	0.022	−0.089	−0.017	−0.082
25	Kaempferolrutinoside	−0.133	−0.285	0.012	0.246	−0.013	0.080
26	Quercetin-malonyl-hexoside	0.295	0.013	0.008	−0.086	−0.055	−0.118
27	Kaempferol-malonyl-rutinoside	0.158	0.249	−0.248	0.109	0.079	−0.005
28	Quercetin-acetylhexoside	0.281	0.061	0.124	−0.102	−0.069	−0.046
29	Kaempferolhexoside (astragalin)	0.202	−0.165	0.121	0.066	0.309	0.012
30	Quercetin-acetylhexoside	0.231	0.048	0.132	0.100	−0.300	−0.032
31	Kaempferol-acetylhexoside	0.296	0.035	0.003	0.079	0.003	−0.113

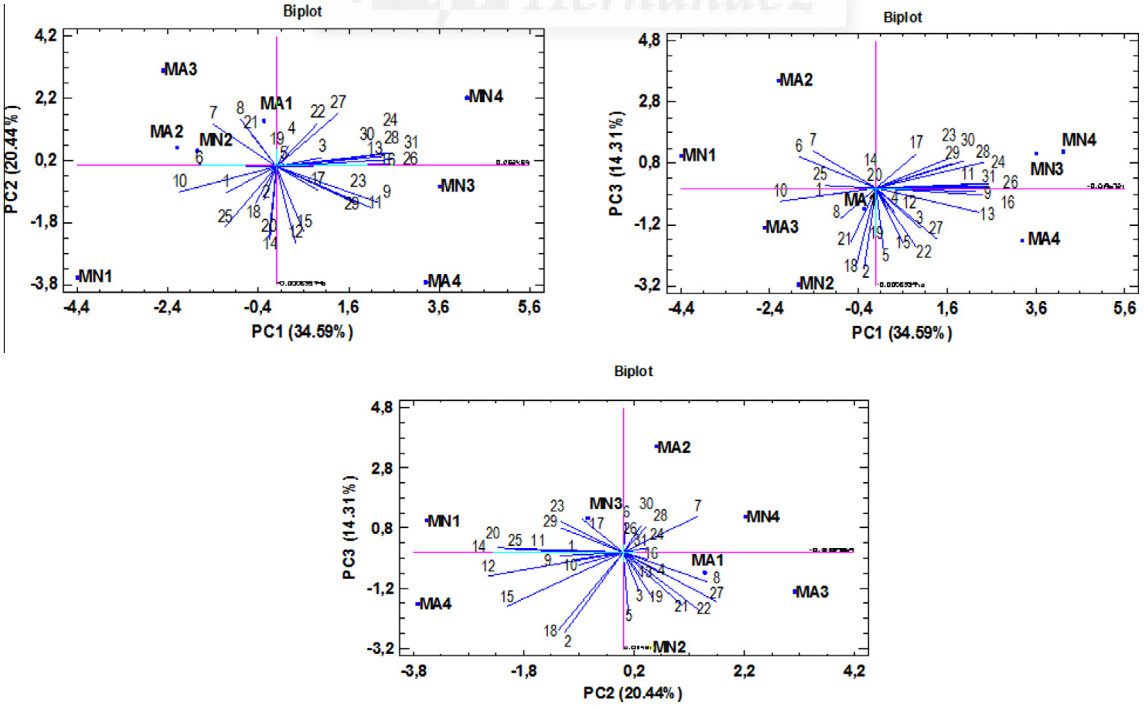

Fig. 2. Main biplots (loadings and scores) obtained from the PCA of the mulberry leaves.

flavanols depended on each clone, as they showed different values for PC3. It should be noted that MA1 showed the most balanced phytochemical profile among all the clones studied, exhibiting medium contents for all the phytochemicals identified.

The MN1 clone showed the lowest value for PC1 and PC2, while medium scores for PC3 (Fig. 2). It was clearly separated from the rest of mulberry clones and could be regarded as the clone having the lowest content in hexoside-containing flavonols and caffeic

acid-hexoside, but with a higher content in caffeic acid and cryptochlorogenic acid.

PCA results partially reflected the information obtained for these same clones when multivariate analysis was applied to their main (poly)phenolic compounds, total phenol content, and different antioxidant capacity assays (Sánchez-Salcedo et al., 2015a). Taking into account the different parameters studied, this chemometric discrimination, far from the taxonomical one, was expected. Nevertheless, the wide intra-species variability in the (poly)phenolic fingerprint of each clone regardless the species, *M. alba* or *M. nigra*, was newly evidenced.

4. Conclusions

These results comprehensively summarize the specific phytochemical profile of leaves belonging to different mulberry clones. They may allow the selection of the most appropriate plant genetic resource of interest for agro-food/pharmaceutical industries. Moreover, this detailed high-throughput phytochemical screening can be used for quality control and authentication/adulteration purposes. On the other hand, if mulberry leaves are used for the elaboration of products rich in bioactive compounds (tea beverages, for instance), most of these clones could have promising prospects. Even if their content in single compounds varied notably, they all contained high and similar amounts of caffeoylquinic acids and flavonols (Sánchez-Salcedo et al., 2015a), which have been described as potentially beneficial toward several pathophysiological processes in humans (Del Rio et al., 2013; Dellafiora, Mena, Del Rio, & Cozzini, 2014; Rodriguez-Mateos et al., 2014). Thus, considering the complex metabolism of these substances, where severe deconjugation steps occur in the intestinal lumen before absorption, breeding programs or germplasm selection should be focused on the total content in caffeoylquinic, quercetin, and kaempferol moieties of the leaf, rather than on its content in individual compounds. Finally, this non-targeted approach investigating the phenolic fingerprinting and chemometric discrimination of eight clones allowed the tentative identification of 7 phenolic structures not previously reported in this plant. This fact extends the array of substances contributing to the definition and bioactivity of this plant material, and represents a remarkable milestone in the adequate assessment of their biological and nutritional properties.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.05.121.

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8.6. PUBLICACIÓN 6

Phytochemical evaluation of eight white (*Morus alba* L.) and black (*Morus nigra* L.) mulberry clones grown in Spain based on UHPLC-ESI-MS^n metabolomics profiles

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Phytochemical evaluation of eight white (*Morus alba* L.) and black (*Morus nigra* L.) mulberry clones grown in Spain based on UHPLC-ESI-MS^n metabolomic profiles

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ABSTRACT

Mulberry (*Morus spp.*) fruits have moved under the spotlight of nutrition research owing to their phytochemical composition. The aim of this study was to investigate the (poly)phenolic profile and chemometric discrimination of fruits from eight Spanish clones, four belonging to *Morus alba* and four to *Morus nigra* species. The assessment of the phytochemical fingerprint of mulberry, carried out by UHPLC-ESI-MS^n high-throughput analysis, allowed the tentative identification of up to 64 compounds, including 20 flavonols, 6 flavanones, 2 flavan-3-ols, 1 flavone, 1 flavanonol, 1 dihydrochalcone, 4 anthocyanins (only in *M. nigra*), 13 hydroxycinnamic derivatives, 4 hydroxybenzoic acids, 3 other low-molecular-weight phenolics, 6 lignans, and 3 organic acids. Among the compounds detected, 21 have been reported for the first time in mulberry fruit. Multivariate analysis served to quickly characterize the different samples of mulberry fruits according mainly to their (poly)phenolic fingerprint. This approach may produce relevant information for the evaluation of the functional, nutritional, and technological prospects of mulberry fruits.

1. Introduction

Phytochemical compounds are secondary metabolites found naturally in plant foods, including fruits, vegetables and grains. Among the different kinds of phytochemicals, (poly)phenolic compounds are those studied to a greater extent. They contribute to some physiological functions of plants and have also been linked to the benefits of an increased dietary consumption of fruits and vegetables (Del Rio et al., 2013; Rodriguez-Mateos et al., 2014), including reduction of cardiovascular risk, type 2 diabetes, neurodegenerative disorders, and of the loss of functionality associated with aging, as well as reduced incidence of some kinds of cancer (Boffetta et al., 2010; Leenders et al., 2013; Mursu, Virtanen, Tuomainen, Nurmi, & Voutilainen, 2014; Tresserra-Rimbau et al., 2014; Valls-Pedret et al., 2012).

Berry fruits have been recognized to be rich sources of different phytochemicals and, in particular, (poly)phenolic compounds with biological activity (Del Rio, Borges, & Crozier, 2010). Among them, mulberries (*Morus spp.*) have moved under the spotlight of nutrition research owing to their phytochemical composition, which might, as said, positively impact on human health (Sánchez-Salcedo, Mena, García-Viguera, Martínez, & Hernández, 2015). Several works have reported that mulberry fruits contain high amounts of polyphenolic substances (Juan, Jianquan, Junni, Zijian, & Ji, 2012; Natić et al., 2015; Pawlowska, Oleszek, & Braca, 2008; Sánchez-Salcedo, Mena, García-Viguera, Hernández & Martínez, 2015; Sánchez-Salcedo, Mena, García-Viguera, Martínez et al., 2015), organic acids, and sugars (Ercisli & Orhan, 2007; Sánchez et al., 2014). Furthermore, mulberry fruits have been found to be rich in linoleic acid, an essential fatty acid, constituting 73.9% of their total fatty acid content (Sánchez-Salcedo, Sendra, Carbonell-Barrachina, Martínez, & Hernández, 2016). According to this phytochemical profile, mulberries, eaten fresh or used in the production of wine, fruit juice, and jam (Ning, Lu, & Zhang, 2005), might represent a useful ingredient in the development of functional foods able to

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improve health (Liang et al., 2012). Mulberry fruits are also used in traditional Chinese medicine, where they have been claimed to protect against liver and kidney damage, strengthen the joints, improve eyesight, and have *anti*-aging effects (Li, 1982). In addition, mulberry fruits have been used effectively in natural medicine for the treatment of sore throat, fever, hypertension, and anaemia (Gong & Zhu, 2008; Ma, 2002). Nevertheless, despite these promising health-promoting features, little is known about their phenolic composition, a key point to fully establish their biological effects. Similarly, the contribution of mulberry inter/intra-species variability to phenolic composition has been only scarcely assessed (Butkhup, Samappito, & Samappito, 2013; Memon, Memon, Luthria, Bhanger, & Pitafi, 2010; Natić et al., 2015; Sánchez-Salcedo, Mena, García-Viguera, Hernández et al., 2015; Sánchez-Salcedo, Mena, García-Viguera, Martínez et al., 2015). Therefore, a complete characterization of the polyphenolic profile of mulberry fruits considering genotypic differences is lacking, and metabolomics approaches focused on (poly)phenolic compounds are valuable assets to accomplish this task.

The aim of this study was investigating the (poly)phenolic fingerprinting and the further chemometric discrimination of mulberry fruits from eight Spanish clones, four belonging to *Morus alba* species and four to *Morus nigra*. The evaluation of the phenolic profile of mulberry fruits was carried out by ultra-HPLC (UHPLC) separation and MS^n linear ion trap mass spectrometric characterization. Chemometric analysis using multivariate statistics assisted in the phytochemical discrimination of the clones to unravel the intra- and inter-specific variability. This metabolomics approach joining UHPLC—MS^n high-throughput profiling and multivariate analysis overcame the deficiencies of traditional phytochemistry and allowed to study extensively large batches of plant material.

2. Materials and methods

2.1. Chemicals

Quinic acid, protocatechuic acid, caffeic acid, 3′-caffeoylquinic acid (neochlorogenic acid), 5′-caffeoylquinic acid (chlorogenic acid), catechin, epicatechin, *m*-hydroxybenzoic acid, and quercetin-3-β-rutinoside (rutin) were purchased from Sigma-Aldrich (Steinheim, Germany). All solvents were purchased from Carlo Erba Reagents (Milan, Italy).

2.2. Plant material

Mulberry clones, four white (*M. alba*) clones (MA1, MA2, MA3, and MA4), and four black (*M. nigra*) clones (MN1, MN2, MN3, and MN4), were collected in Orihuela (latitude 38°04′08″N x longitude 0°58′58″ W, 27 m above sea level), Alicante province (SE Spain) in 2013. Fully mature fruits were manually harvested and transported to the lab. Five genetically identical trees were selected for each clone and sixty fruits per clone (12 fruits per tree) were randomly picked at the commercially ripe stage. Three subsamples by clone (each one composed by 20 fruits) were randomly prepared to reduce intra-clone differences due to environmental factors and better present the characteristic of the clone, freeze-dried, and ground as a powder.

2.3. Extraction of (poly)phenolic compounds

The phenolic compounds in mulberry fruits were extracted according to a previous report (Sánchez-Salcedo, Mena, García-Viguera, Hernández et al., 2015; Sánchez-Salcedo, Mena, García-Viguera, Martínez et al., 2015). Briefly, the mixture of 200 mg of freeze-dried powder and 1 mL of 80% aqueous methanol acidified with formic acid (1%) was sonicated for 25 min. The mixture was centrifuged at 10,480 *g* for 5 min at room temperature and the supernatant was collected. Two additional extractions were performed for each sample with additional 0.5 mL of the same solvent, as described above, after which they were centrifuged. The three supernatants were pooled before UHPLC-MS analysis. Each sample was extracted in triplicate.

2.4. Characterization by liquid chromatography-mass spectrometry (UHPLC-MS)

Methanolic extracts of mulberry fruit powder were analyzed using an Accela UHPLC 1250 equipped with a linear ion trap-mass spectrometer (MS) (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray ionization probe (H-ESI-II; Thermo Fisher Scientific Inc., San Jose, CA, USA). Separations were performed using a BlueOrchid C18 (50 × 2 mm), 1.8 μm particle size (Knauer, Berlin, Germany). Volume injected was 5 μL and column oven was set to 30 °C. Three MS experiments were performed, two in negative mode and one using positive ionization (anthocyanins) (Mena et al., 2012). Each sample was analyzed in duplicate.

An experiment optimized for epicatechin analysis (Experimental Conditions 1) was carried out using the following conditions. The MS worked with a capillary temperature equal to 275 °C, while the source heater temperature was set to 200 °C. The sheath gas flow was 40 units, while both auxiliary and sweep gas were set to 5 units. The source voltage was 4 kV. The capillary and tube lens voltages were −42 and −118 V, respectively. Elution was performed at a flow rate of 0.2 mL/min. The gradient started with 100% of 0.1% aqueous formic acid, isocratic conditions for 2 min, followed by a 3-min linear gradient of 0% to 20% acetonitrile 0.1% formic acid, and then an 8-min linear gradient of 20% to 40% acetonitrile 0.1% formic acid was applied. From 13 to 14 min the acidified acetonitrile was increased to 80%, followed to 3 min of 80% acetonitrile and then 4 min at the start conditions to re-equilibrate the column. Analyses were carried out using full scan mode, data-dependent MS^3 scanning from *m/z* 100 to 2000, with collision induced dissociation (CID) equal to 30 (arbitrary units). Pure helium gas was used for CID.

In a second experimental framework, the MS worked with conditions optimized for hydrolysable tannin analysis, after infusion with punicalagin standard (mixture of two isomers) (Experimental Conditions 2). The capillary temperature was set to 275 °C, while the source heater temperature was 250 °C. The sheath gas flow was 60 units, while auxiliary and sweep gas were set to 15 and 4 units, respectively. The source voltage was 4 kV. The capillary and tube lens voltages were −49 and −153 V, respectively. Analyses were carried out using full scan mode, data-dependent MS^3 scanning from *m/z* 100 to 2000, with CID equal to 30 (arbitrary units). The chromatographic conditions were identical to those used for Experimental Conditions 1.

In positive mode, the MS worked with a capillary temperature equal to 275 °C, while the source heater temperature was set to 300 °C. The sheath gas flow was 40 units, while auxiliary gas was set to 5 units, without sweep gas. The source voltage was 4.5 kV. The capillary voltage and tube lens were 20 and 95 V, respectively. As for the previous experiments, the chromatographic conditions were identical to those used for Experimental Conditions 1.

Data processing was performed using Xcalibur software from Thermo Scientific. Area calculation was performed in selected ion monitoring mode by selecting the relative base peak at the corresponding mass to charge ratio (*m/z*) under Experimental Conditions 1.

2.5. Statistical analysis

Peak area values of each (poly)phenolic compound were used for multivariate analysis. Each area value is the mean of three analytical replicates. Principal component analysis (PCA) with varimax was carried out using the IBM SPSS Statistics 19 software package (SPSS Inc., Chicago, IL, USA).

Table 1
Mass spectral characteristics of (poly)phenolic compounds detected in mulberry fruits in negative ionization mode.

Id.	Compounds	RT (min)	$[M\text{-}H]^-$ (m/z)	MS^2 ion fragments (m/z) [c]	MS^3 ion fragments (m/z) [c]
1	Quinic acid[a]	0.76	**191**	**127(100)**[d], 173(90),85(40), 93(30)	127(100), 173(84), 85(60)
2	*L*-Malic acid	0.91	**133**	**115(100),** 87(20), 71(15)	71(100)
3	Citric acid	1.09	**191**	**111(100),** 173(60)	67(100), 93(40)
4	Catechol	4.44	**109**	**109(100)**	–
5	Dihydroxybenzoic acid (protocatechuic acid)[a]	4.48	**153**	**109(100)**	–
6	Gentisoyl-hexoside[b]	5.11	**315**	**153(100)**	109(100)
7	Vanillic acid	5.87	**167**	**152(100),** 123(90), 108(17), 167(10)	108(100), 152(5)
8	Caffeoylquinic acid glucoside[b]	5.92	**515**	**179(100),** 353(70), 341(65)	135(100)
9	Caffeic acid[a]	5.94	**179**	**135(100),** 179(15)	79(100)
10	Caffeoylquinic acid diglucoside[b]	5.95	**677**	**515(100),** 353(20), 503(8)	353(100), 191(40), 323(10), 179(7)
11	3-Caffeoylquinic acid (neochlorogenic acid)[a]	6.12	**353**	**191(100),** 179(45), 135(10)	171(100), 173(80)
12	*p*-Coumaric acid	6.30	**163**	**119(100)**	–
13	Caffeoylquinic acid derivative[b]	6.37	**999**	**353(100),** 645(70), 981(65), 819(35), 837(20)	173(100)
14	5-Caffeoylquinic acid (chlorogenic acid) [a]	6.47	**353**	**173(100),** 191(15), 179(10)	171(100)
15	Ferulic acid	6.55	**193**	**149(100),** 178(60), 134(20), 165(5)	134(100)
16	Dicaffeoylquinic acid	6.56	**515**	**353(100),** 341(55), 179(50)	173(100), 191(5)
17	Piceatannol/oxyresveratrol dihexoside	6.57	**567**	**405(100),** 243(75)	243(100)
18	Quercetin-hexoside-hexoside	6.60	**625**	**301(100),** 463(48)	179(100), 151(45)
19	(+)-Catechin[a]	6.64	**289**	**245(100)**, 205(40), 179(20)	203(100), 227(33), 187(25)
20	4-Caffeoylquinic acid (cryptochlorogenic acid)	6.67	**353**	**191(100),** 173(75), 178(55)	173(100), 127(90), 93(75)
21	Quercetin-acetylhexoside-hexoside[b]	6.82	**667**	**505(100),** 463(30), 301(20), 625(15), 547(4)	300(100), 343(20), 271(15)
22	Kaempferol-rutinoside-hexoside[b]	6.84	**755**	**593(100)**	285(100)
23	Caffeoylquinic acid isomer	6.88	**353**	**173(100),** 191(55), 179(22)	93(100), 111(65), 155(20)
24	Guaiacyl(8-*O*-5)ferulic acid hexoside[b]	7.01	**551**	**389(100),** 193(55), 341(12)	193(100), 341(15), 165(10)
25	Isorhamnetin-hexoside	7.07	**477**	**314(100)**, 315(25), 449(11)	300(100)
26	Coumaroylquinic acid	7.09	**337**	**191(100)**, 163(33)	–
27	Tetrahydroxy-dimethoxyflavone-hexoside	7.15	**507**	**327(100),** 489(25), 345(20), 477(20), 315(12)	312(100), 167(28), 295(20)
28	Quercetin-rhamnose-hexose-rhamnose[b]	7.24	**755**	**301(100),** 591(80), 489(50), 609(30), 737(15)	179(100), 151(25)
29	Quercetin-dihexoside	7.26	**625**	**300(100),** 301(90), 445(60), 463(40), 505(25)	179(100), 151(47)
30	(−)-Epicatechin[a]	7.37	**289**	**245**,205,179,261	203,227,187
31	*m*-Hydroxybenzoic acid[a]	7.40	**137**	93 (100)	93
32	Lariciresinol[b]	7.43	**329**	**178**,299,314,284,193	–
33	Kaempferol-hexoside-hexoside	7.50	**609**	**285(100),** 429(80), 447(15)	257(100), 241(45), 267(25), 229(15), 213(8)
34	Quercetin-rutinoside-hexoside[b]	7.51	**771**	**301(100),** 609(30), 591(10)	179(100), 151(90), 273(40)
35	Quercetin-rutinoside (rutin) [a]	7.63	**609**	**301(100)**	179(100), 151(81), 273(20)
36	*m*-Coumaric acid	7.71	**163**	**119(100)**	–
37	Quercetin-hexoside (isoquercitrin)	7.82	**463**	**301(100)**	179(100), 151(60), 273(15)
38	Taxifolin	7.82	**303**	**285(100)**, 125(25), 177(18)	–
39	Kaempferol rutinoside	8.03	**593**	**285(100)**	257(100), 267(60), 229(55), 241(35), 213(20)
40	Quercetin-acetylhexoside	8.09	**505**	**301(100),** 463(30)	179(100), 151(55), 273(20)
41	Quercetin-malonyl-hexoside[b]	8.21	**549**	**301(100),** 505	179(100), 151(38)
42	Kaempferol-hexoside (astragalin)	8.27	**447**	**285(100),** 327(30)	257(100), 241(60), 199(40), 267(30)
43	Quercetin-acetylhexoside	8.27	**505**	**301(100),** 445(60), 463(25)	179(100), 151(90), 273(15)
44	4-Hydroxy-3-methoxycinnamaldehyde[b] (coniferyl aldehyde)	8.29	**177**	**162(100),** 133(15)	162(100), 134(55), 106(33)
45	Quercetin-acetylhexoside	8.47	**505**	**301(100),** 463(30), 445(10)	179(100), 151(50), 273(22)
46	Eriodictyol-hexoside	8.60	**449**	**287(100)**	151(100), 135(10), 125(5)
47	Syringaresinol[b]	8.61	**417**	**181(100),** 166(30), 402(25), 371(20)	166(100)
48	Kaempferol-acetylhexoside	8.73	**489**	**285(100)**	257(100), 267(80), 241(50)
49	Pinoresinol[b]	8.82	**357**	**151(100),** 136(35), 311(20)	136(100)
50	Phloretin	8.84	**273**	**167(100)**	123(100), 125(10), 151(4)
51	Guaiacyl(*t*8-*O*-4)syringyl(8–8)guaiacyl hexoside[b]	9.02	**745**	**583(100)**	535(100), 387(35), 195(20)
52	Guaiacyl(8-*O*-4)syringyl(8–8)guaiacyl hexoside[b]	9.09	**745**	**583(100)**	535(100), 387(40), 195(15)
53	Quercetin	10.13	**301**	**179(100),** 151(80), 257(50), 273(15)	–
54	Morin	10.21	**301**	**179(100),** 151(35), 257(25), 271(13)	–
55	Tetrahydroxyflavanone-hexoside-pentoside[b]	10.56	**581**	**449(100),** 563(50), 431(25)	431(100), 287(55), 269(5)
56	Tetrahydroxyflavanone-hexoside-pentoside[b]	10.77	**581**	**449(100),** 563(45), 431(20)	431(100), 287(35), 269(5)
57	Tetrahydroxyflavanone-hexoside[b]	10.94	**449**	**431(100),** 287(50), 269(15)	413(100)
58	Tetrahydroxyflavanone-hexoside[b]	11.18	**449**	**431(100),** 287(20), 269(4)	413(100)
59	Kaempferol	11.85	**285**	**257(100),** 267(35), 241(20), 229(20)	–
60	Tetrahydroxyflavanone[b]	13.11	**287**	**269(100),** 241(20), 251(15)	–

[a] Compounds identified by comparing retention times and MS data with those of reference compounds.
[b] Compounds (tentatively) identified for the first time in mulberry fruit.
[c] The relative ionic abundance for each ion is reported in parentheses.
[d] MS^2 ions in bold were those subjected to MS^3 fragmentation.

3. Results and discussion

3.1. Identification of phytochemicals compounds in mulberry fruits

The fruits of eight clones of *M. alba* and *M. nigra* were assessed. The phytochemical profile of these plant materials, focused on (poly)phenolic compounds, was evaluated by a comprehensive UHPLC-ESI-MS^n method combined with multivariate statistics (Calani et al., 2013). In particular, a non-targeted approach for screening of phenolic compounds with diverse ionization attitudes, and consisting of three complementary MS conditions, in both negative and positive mode, was used to determine the phytochemical composition of the fruits (Mena et al., 2012). About 400 mass spectrum outputs were studied for each analytical replicate. This procedure allowed the tentative identification of up to 64 compounds (Tables 1 and 2), the broadest characterisation of the (poly)phenolic fingerprint of mulberry fruits to date. Among the different classes of (poly)phenolic compounds identified in mulberry fruit, flavonols were the most relevant (Fig. 1). Other flavonoids (flavanones, flavan-3-ols, dihydrochalcones, flavanonols, and anthocyanins), phenolic acids, and lignans were observed, as well as some organic acids.

Nine compounds were identified by comparison with reference standards, while the remaining 55 compounds were identified based on their fragmentation patterns obtained from mass spectra (MS^2 and MS^3 experiments) and by comparing their spectra with the available literature. Twenty one compounds (compounds **6**, **8**, **10**, **13**, **21**, **22**, **24**, **28**, **32**, **34**, **41**, **44**, **47**, **49**, **51**, **52**, **55**, **56**, **57**, **58**, and **60**) were tentatively identified for the first time, as far as we know, in mulberry fruits. The interpretation of the MS fragmentation patterns present in the literature was not discussed unless of special interest. The retention times and mass spectrometric data along with peak assignments for the identified compounds are reported in Table 1.

A total of 20 flavonols were identified in mulberry fruit. They were mainly glycosylated forms of quercetin, kaempferol, and isorhamnetin (**25**) (Table 1). Apart from morin (**54**), which was identified only as aglycone, in agreement with McNab, Ferreira, Hulme, and Quye (2009), flavonols showed a wide number of derivatives with different sugar and acyl moieties. Seven of the 12 quercetin derivatives detected (**18**, **29**, **35**, **37**, **40**, **43**, and **53**) had been previously identified in fruits of *M. alba* (Juan et al., 2012; Natić et al., 2015; Sánchez-Salcedo, Mena, García-Viguera, Hernández et al., 2015; Sánchez-Salcedo, Mena, García-Viguera, Martínez et al., 2015), while other 3 quercetin derivatives detected (**28**, **41**, and **45**) had been described only in mulberry leaves (Sánchez-Salcedo, Mena, García-Viguera, Hernández, et al., 2015). Quercetin-acetylhexoside-hexoside (*m/z* 667) (**21**) and quercetin-rutinoside-hexoside (*m/z* 771) (**34**) were identified according to their characteristic quercetin fragment ions and to the loss of their sugar/acetyl units. In the case of compound **21**, the fragment ions at *m/z* 505 and 463 were formed by the loss of an hexoside and acetylhexoside units, respectively. Compound **34** fragmented to *m/z* 609 (loss of hexoside) and 301 (loss of rutinoside-hexoside). Regarding kaempferol derivatives, compounds **33**, **42**, **48**, and **59** had already been described in mulberry fruits (Juan et al., 2012; Natić et al., 2015; Sánchez-Salcedo, Mena, García-Viguera, Hernández et al., 2015; Sánchez-Salcedo, Mena, García-Viguera, Martínez et al., 2015), while kaempferol-rutinoside-hexoside (**22**) and kaempferol-rutinoside (**39**) have been recently identified in mulberry leaves (Dugo et al., 2009; Sánchez-Salcedo, Tassotti, Del Rio, Hernández, Martínez, & Mena, 2016), but not in fruits. Some flavonols reported for Serbian mulberry fruits (Natić et al., 2015) were not detected in the present study, which could account for geographical and genotypic differences. Quantitatively, flavonols have been described to be one of the main phenolic classes in mulberry fruits, with concentrations ranging between 0.1 and 1.3 mg/g dw in Spanish clones (Sánchez-Salcedo, Mena, García-Viguera, Hernández et al., 2015; Sánchez-Salcedo, Mena, García-Viguera, Martínez et al., 2015), similarly to those reported for other mulberry cultivars worldwide (Butkhup et al., 2013; Pawlowska et al., 2008; Zhang, Han, He, & Duan, 2008).

Twelve different flavonoids belonging to five subclasses of flavonoids other than flavonols (flavanones, flavan-3-ols, flavone, flavanonol, dihydrochalcone, and anthocyanins) were tentatively identified. Among flavanones (Table 1), eriodictyol-hexoside (**46**) had already been reported in *Morus spp.* (Wang, Xiang, Wang, Tang, & He, 2013). On the contrary, compounds **55**, **56**, **57**, **58**, and **60** have never been described in mulberry fruits previously, to our best knowledge. Compound **60** showed MS^2 fragments similar to those of dihydrokaempferol (Wang et al., 2013), but its late retention time excluded this fact and prompted us to tentatively identify this compound as tetrahydroxyflavanone. Glycosylated derivatives of tetrahydroxyflavanone (**55**–**58**) were identified by their corresponding MS^2 fragment ions, characterized for losses of pentose and hexose units (132 and 164, respectively) and their MS^3 fragments, matching those of compound **60**. Two flavan-3-ols (**19** and **30**) were identified by comparison with their corresponding commercial standards, while compounds **27** (flavone), **38** (flavanonol), and **50** (dihydrochalcone) were tentatively identified according to Fischer, Carle, and Kammerer (2011); Zhang et al. (2008), and Mena et al. (2012), respectively. Four anthocyanins, already reported in mulberry fruits (Pawlowska et al., 2008), were also identified (Table 2). They were hexoside and rutinoside derivatives of pelargonidin and cyanidin and were detected solely in black mulberry clones, as it had been previously reported (Sánchez-Salcedo, Mena, García-Viguera, Hernández et al., 2015; Sánchez-Salcedo, Mena, García-Viguera, Martínez et al., 2015).

Several phenolic acids were tentatively identified (Table 1). Among them, up to thirteen hydroxycinnamic derivatives, which had already been reported to be the main phenolic compounds in Spanish mulberry fruits (Sánchez-Salcedo, Mena, García-Viguera, Hernández et al., 2015; Sánchez-Salcedo, Mena, García-Viguera, Martínez et al., 2015), were detected. Simple hydroxycinnamic acids caffeic acid (**9**), *p*-coumaric acid (**12**) and *m*-coumaric acid (**36**), and ferulic acid (**15**) were characterized by comparison with proper standards or based on literature data (Calani et al., 2014; Natić et al., 2015). Similarly, most of the caffeoylquinic acids were identified according to the fragmentation patterns of their corresponding commercial standards (compounds **11** and **14**) or by literature comparison (**8**, **10**, **16**, **20**, **23**, and **26**) (Calani et al., 2014; Dall'Asta et al., 2012; Dugo et al., 2009; Jaiswal, Műller, Műller, Karar, & Kuhnert, 2014; Simirgiotis, Silva, Becerra, & Schmeda-Hirschmann, 2012; Vallverdú-Queralt, Arranz, Medina-Remón, Casals-Ribes, & Lamuela-Raventós, 2011). Compound **13** (molecular ion *m/z* 999) exhibited a major MS^2 fragment ion at *m/z* 353 and a MS^3 fragment ion at *m/z* 173, corresponding to the characteristic fragmentation pattern of chlorogenic acid. It also showed MS^2 ions at *m/z* 645 (loss of

Table 2
Mass spectral characteristics of anthocyanins detected in mulberry fruits in positive ionization mode.

Id.	Compounds	RT (min)	$[M]^+$ (*m/z*)	MS^2 ion fragments (*m/z*)[a]	MS^3 ion fragments (*m/z*)[a]
1	Cyanidin rutinoside	6.74	**595**	**287(100)**[b], 449(30)	–
2	Cyanidin hexoside	6.67	**449**	**287(100)**	–
3	Pelargonidin hexoside	6.86	**433**	**271(100)**	–
4	Pelargonidin rutinoside	6.91	**579**	**443(100),** 271(25)	271(100)

[a] The relative ionic abundance for each ion is reported in parentheses.
[b] MS^2 ions in bold were those subjected to MS^3 fragmentation.

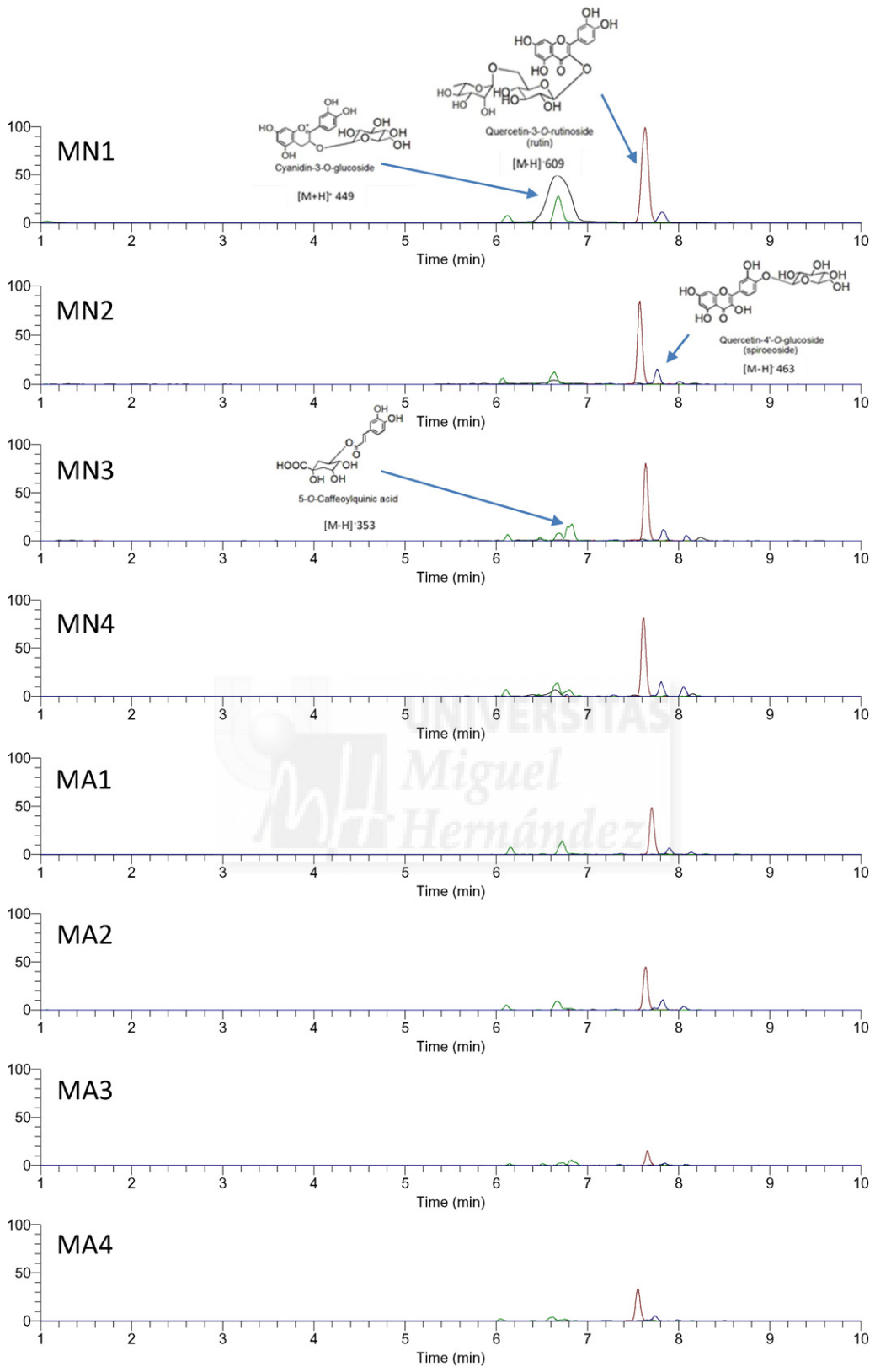

Fig. 1. Chromatograms of some of the most representative compounds for each clone, extracted in selected ion monitoring mode.

caffeoylquinic acid) and 819 (loss of caffeic acid), but the complete structure could not be identified; thus, it was tentatively identified as caffeoylquinic acid derivative. Four hydroxybenzoic acids were identified by comparison with their commercial standards (**5** and **31**) or with literature data (**7** and **6**) (Natić et al., 2015; Sawada et al., 2012). Other phenolic compounds were also identified by literature comparison (**4**, **17,** and **44**) (Barros et al., 2013; Romain et al., 2014; Wang et al., 2013).

Analyses also allowed the tentative identification of 6 lignans (**24**, **32**, **47**, **49**, **51**, **52)** (Table 1)**,** which were characterized according to

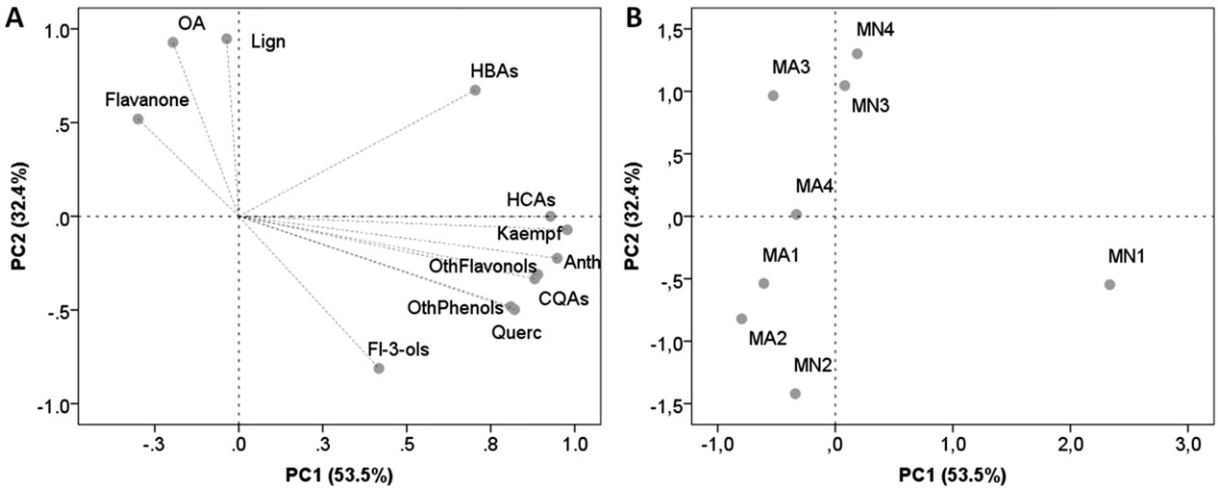

Fig. 2. Principal component analysis of mulberry fruits. A) Loading plot of PC1 versus PC2; B) Distribution of mulberry fruit samples in the consensus space. Quercetin derivatives (Querc) include compounds 18, 21, 28, 29, 34, 35, 37, 40, 41, 43, 45, and 53 (Table 1); Kaempferol derivatives (Kaempf), 22, 33, 39, 42, 48, and 59; Other flavonols (OthFlavonols), 25 and 54; Flavanone (Flavanone), 46, 55, 56, 57, 58, and 60; Flavon-3-ols (Fl-3-ols) 19 and 30; Simple hydroxycinnamic acids (HCAs), compounds 9, 12, 15, and 36 (Table 1); Caffeoylquinic acids (CQAs) 8, 10, 11, 13, 14, 16, 20, 23, and 26; Hydroxybenzoic acids (HBAs) 5, 6, 7, and 31; Lignans (Lign) 24, 32, 47, 49, 51, and 52; Other phenolics (OthPhenols) 4, 17, 27, 38, 44, and 50; Organic acid (OA) 1, 2, and 3; Anthocyanins (Anth), compounds 1, 2, 3, and 4 (Table 2).

their fragmentation patterns and literature data (Eklund, Backman, Kronberg, Smeds, & Sjöholm, 2008; Huis et al., 2012; Mena et al., 2012). Finally, organics acids (**1**, **2**, and **3**) were also detected and identified (Mena et al., 2012).

This high-throughput analysis has highlighted the wide array of phenolic substances and other phytochemicals present in mulberry fruits. Even if some detailed characterizations of mulberry fruits had been performed in the past (Butkhup et al., 2013; Natić et al., 2015; Pawlowska et al., 2008; Wang et al., 2013), this work allowed the tentative identification of 20 compounds not previously reported in these fruits, representing the widest profiling of mulberry fruit phenolics to date.

3.2. Chemometric classification

Due to the lack of authentic standards, quantification of the phenolic compounds identified could be inaccurate. Chemometric analysis helps to overcome this limitation and allows phytochemical discrimination of plant material (Calani et al., 2013). For this reason, a multivariate unsupervised analysis was applied to the samples in order to achieve a better understanding of the impact of genotypic differences on the phytochemical composition of mulberry fruits. Peak area values of the 64 compounds identified were used for the PCA. However, for an easier model, the PCA was carried out using the sum of each class of molecules, without considering individual compounds.

The first two principal components (PCs) explained 85.3% of the total variability (Fig. 2A). Almost 53.5% of the observed variability was explained by the first PC. PC1 was positively linked to kaempferol derivatives, anthocyanins, simple hydroxycinnamic acids, other flavonols, caffeoylquinic acids, other phenolic compounds, quercetin derivatives, and hydroxybenzoic acids. Principal component 2 (PC2) had high negative component loading from flavan-3-ols and was inversely correlated to lignans, organic acids, and flavanones.

Every *M. alba* clone presented a similar negative PC1 value, but they differed in their scores for PC2 (Fig. 2B): MA1 and MA2 formed a subcluster with negative scores for PC2, MA3 had positive values, and MA4 displayed mean values between MA2 and MA3. Attending to these scores, *M. alba* clones can be defined as samples with low content in phenolic compounds, showing variable quantities for specific phytochemicals. On the other hand, *M. nigra* clones displayed very different relationships (Fig. 2B): MN3 and MN4 showed similar positive scores for both PC1 and PC2; MN2 showed negative values for PC1 and PC2, and was close to MA2; while MN1 had an extreme positive value for PC1, which was located quite far from the rest of the clones. These facts pointed out that *M. nigra* clones have a higher content in phytochemicals than *M. alba* ones, being MN1 the one with the highest content in (poly)phenolic compounds.

It should be noted that *M. alba* and *M. nigra* clones were not cluster according to their taxonomic classification, as initially expected. However, these observations match those recorded when quantifying the main phenolic compounds present in these Spanish clones (Sánchez-Salcedo, Mena, García-Viguera, Hernández et al., 2015; Sánchez-Salcedo, Mena, García-Viguera, Martínez et al., 2015). Further genotypic studies could confirm the existing differences between classic taxonomy and phytochemical profiling. On the whole, this novel classification based on the phenolic fingerprint of mulberry fruits represents a valuable information for future nutritional studies.

4. Conclusions

The UHPLC-ESI-MS^n analysis allowed the tentative identification of 64 individual phytochemical compounds in mulberry fruits, including 20 flavonols, 6 flavanones, 2 flavan-3-ols, 1 flavone, 1 flavanonol, 1 dihydrochalcone, 4 anthocyanins (only in *M. nigra*), 13 hydroxycinnamic derivatives, 4 hydroxybenzoic acids, 3 other low-molecular-weight phenolics, 6 lignans, and 3 organic acids. Among the detected compounds, 21 have been reported for the first time in mulberry fruits. Multivariate analysis served to quickly characterize the different samples of mulberry fruits mainly according to their (poly)phenolic fingerprint. This approach may produce relevant information for the assessment of the functional, nutritional, and technological prospects of mulberry fruits. It could be also translated to other plant matrices.

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9. RESUMEN DE RESULTADOS, DISCUSIÓN Y CONCLUSIONES

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9. RESUMEN DE RESULTADOS, DISCUSIÓN Y CONCLUSIONES

9.1. PUBLICACIÓN 1

Physicochemical characterisation of eight Spanish mulberry clones: processing and fresh market aptitudes

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International Journal of Food Science and Technology 2014, 49, 477-483

Factor de impacto: **1,384 Q2** (Categoria: Food Science & Technology)

El **objetivo** de este estudio fue describir y comparar, por primera vez, los principales parámetros físico-químicos de especies de morera blancas y negras cultivadas en España.

9.1.1. RESUMEN DE RESULTADOS Y DISCUSIÓN

En lo que se refiere a los **parámetros morfológicos** y **físicos** en los **frutos,** el peso de los frutos de las especies de morera osciló entre 2,10 g (MA4) y 4,15 g (MN2). Este rango está en consonancia con los obtenidos por Erclisli y Orhan (2007), que obtuvieron un rango de 2,14 a 5,05 g. Entre los clones analizados, se encontró que los frutos más pequeños los presentó el clon MA4 (12,2 mm $ø_{equatorial}$ y 23,2 mm de altura de fruto) y el clon MN4 (12,9 mm $ø_{equatorial}$ y 21,6 mm de altura de fruto) (Tabla 1, publicación 1).

Con respecto al **color,** las variables colorimétricas medidas en el **fruto** fluctuaron entre los valores siguientes: **L***, a partir de 23,9 (MN1) a 62,5 (MA4), **a*** valores de -1,78 (MA3) a 9,50 (MN3), **b*** de -0,88 (MN1) a 27,4 (MA3), **C*** valores de 1,34 (MN1) a 27,4 (MA3) y **h*** entre 22,2 (MN4) a 310 (MN1). Ercisli y Orhan (2007) y Özgen et al. (2009) obtuvieron menores valores de **L*** en clones turcos de *Morus nigra*. El color del zumo fue significativamente diferente al de los frutos frescos.

Respecto a los **sólidos solubles totales (TSS)** se vieron afectados significativamente por el clon ($p<0,05$) y oscilaron entre 12º Brix (MN1) y 25,8º Brix (MN3). Estos resultados están de acuerdo con los obtenidos por Ercisli y Orhan (2007,

2008), a excepción los clones MN3 y MN4, que mostraron valores muy altos de sólidos solubles totales.

En cuanto a la **acidez valorable total,** el clon MN1 tuvo significativamente mayor acidez (>2,6 g de ácido cítrico/L de zumo) que el resto de clones (Tabla 3, publicación 1). Los valores de acidez encontrados en el presente estudio son similares con los mostrados por Elmaci y Altug (2002); Ercisli y Orhan (2007); Imran et al. 2010. Sin embargo, Darias-Martin et al. (2003) obtuvieron valores mayores, 16,2 a 28,1 g de ácido cítrico/ L de zumo.

El **índice de madurez** varió considerablemente de 46,6 del clon MN1 a 278 del clon MN3. Según los resultados obtenidos, los clones estudiados eran lo suficientemente dulces y se recomiendan para el consumo fresco.

Se observaron altos contenidos de **humedad** en los clones MN1, MA2 y MA3, mientras que el valor más bajo se encontró en el clon MN3. Los valores diferían significativamente ($p<0,05$) entre sí, excepto en los clones MA1, MA4 y MN2, que fueron estadísticamente similares. Según Ercisli y Orhan (2007) las especies de morera comunes cultivadas en Turquía presentaron contenidos de humedad del 71,5% al 74,6%, y estos valores estaban dentro del rango en que se encuentran las moras españolas. Sin embargo, Imran et al. (2010) obtuvieron valores mayores de humedad de hasta 82,40%.

El contenido de **fibra cruda** osciló en un estrecho intervalo, entre 1,56 (MN4) y 2,32 (MA4) g/100 g dw. Estos valores son ligeramente superiores a los obtenidos por Imran et al. (2010). Estos resultados mostraron que las moras españolas son también una buena fuente de fibra.

Con referencia a los **azúcares** detectados, el azúcar predominante en el **fruto** fue la fructosa (~ 61%) seguido de la glucosa (~ 39%), mientras que la sacarosa solo se presentó a nivel de trazas. La fructosa y la glucosa fueron significativamente mayores en los frutos de los clones MN3 y MN4 que en otros clones. Los resultados experimentales mostraron que el contenido de azúcar de las especies de *Morus* españolas, eran más altos que los de especies de *Morus* de Pakistán y Turquía (Elmaci y Altug, 2002; Imran et al., 2010; Gundogdu et al., 2011).

El **ácido orgánico** predominante en el **fruto** fue el ácido málico, generalmente seguido del quínico, cítrico, tartárico, fumárico y fórmico. Se han obtenido diferencias

considerables en los contenidos de ácidos orgánicos por distintos autores (Koyuncu, 2004; Özgen et al., 2009; Gundogdu et al., 2011; Mahmood et al., 2012).

El **Análisis** de **conglomerados** ha permitido la agrupación de los clones en tres grupos: (i) MN1 y MN2; (ii) MN3 y MN4; y (iii) MA1, MA2, MA3 y MA4 según sus características físico-químicas.

9.1.2. CONCLUSIONES

Este estudio es probable que represente los primeros datos sobre las propiedades físico-químicas de los frutos de moreras españolas. Las especies de morera blanca y negra muestran diferentes características significativas. Se ha observado variación de los frutos de los ocho clones de morera, que se deben principalmente a las diferencias específicas de genotipo. El análisis de conglomerados ha permitido la clasificación de los ocho clones en tres grupos diferentes: (i) MN1 y MN2, (ii) MN3 y MN4 y (iii) MA1, MA2, MA3 y MA4. Todos los clones de morera estudiados mostraron propiedades físico-químicas muy prometedoras e interesantes, tanto para consumo en fresco como para la industrialización. La clasificación final de los frutos de morera para consumo en fresco o industrialización sería diferente de acuerdo con el parámetro de clasificación. Por ejemplo, si la idoneidad para el consumo en fresco se basa en la "apariencia" (peso de fruto y color atractivo), los mejores clones serían MN1, MN2, MA1 y MA2, sin embargo, si la clasificación se basa en el "sabor" (alta intensidad de dulzor), los mejores clones serían MN3, MN4, MA3 y MA4. Por consiguiente, es fundamental contar con información sobre los requerimientos y necesidades de los consumidores, en esta particular fruta. El presente estudio es el primer paso hacia la identificación de estas frutas como potenciales alimentos saludables que también pueden ser utilizados en la industria alimentaria y farmacéutica. Sin embargo, son necesarios más estudios pomológicos para seleccionar los cultivares o clones de morera que sean los más adecuados para el consumo en fresco o industrialización, estos estudios deberían incluir pruebas hedónicas para vincular las propiedades físico-químicas que los consumidores aceptan.

9.2. PUBLICACIÓN 2

Phytochemical evaluation of white (*Morus alba* L.) and black (*Morus nigra* L.) mulberry fruits, a starting point for the assessment of their beneficial properties

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Journal of Functional Foods 2015, 12, 399-408

Factor de impacto: **3,973 Q1** (Categoria: Food Science & Technology)

El **objetivo** de este estudio fue evaluar por primera vez el contenido fenólico, la actividad antioxidante y la composición mineral, de los frutos de moreras blancas y negras cultivadas en España.

9.2.1. RESUMEN DE RESULTADOS Y DISCUSIÓN

Los **fenoles totales TPC** fueron determinados por el método del reactivo *Folin-Ciocalteau.* Los clones seleccionados variaron de 6,98 a 13,59 mg GAE/g dw. Los clones de *M. nigra* tenían los niveles más altos de fenoles totales (de 7,0 a la 13,6 mg GAE/g dw). No obstante, se registraron variaciones notables entre los clones de la misma especie, en particular para los clones de *M. nigra* donde el TPC del clon MN1 era el doble que el del clon MN4. Los resultados obtenidos son similares a los encontrados en otros estudios en diferentes genotipos de frutos de *M. alba* y *M. nigra* (Bae y Suh, 2007; Ercisli y Orhan, 2007; Ercisli et al., 2010; Khalid et al., 2011; Radojković et al., 2012). Sin embargo, Lin y Tang (2007) e Imran et al. (2010) encontraron valores más altos de fenoles totales en moras 1.516-1.650 mg/100 g fw, respectivamente.

Los **compuestos fenólicos** detectados en los **frutos** de *M. alba* y *M. nigra* en nuestro trabajo son similares a los obtenidos en estudios previos (Butkhup et al., 2013; Gundogdu et al., 2011; Lin y Lay, 2013; Pawlowska et al., 2008). En general, los principales grupos fenólicos fueron, derivados del ácido benzoico>derivados del ácido cinámico>flavonoles>antocianinas (Tabla 1, publicación 2).

La concentración de los derivados del ácido benzoico (ácido protocatéquico, ácido p-hidroxibenzoico y ácido vanílico) oscilaron entre 0,48 a 2,55 mg/g dw. El ácido

hidroxibenzoico predominante en frutos de morera era el ácido protocatéquico, seguido del ácido p-hidroxibenzoico, y el ácido vanílico.

Otros autores observaron que el principal derivado del ácido hidroxibenzoico en moras negras era el ácido protocatéquico (Zadernowski et al., 2005), así como el ácido gálico (Butkhup et al., 2013), que no fue detectado en el presente trabajo. En cuanto a los derivados del ácido cinámico, el hidroxicinamato predominante fue el ácido clorogénico, entre 0,15 y 3,18 mg/g dw, seguido del ácido neo-clorogénico, mientras que el ácido cafeico, ácido p-cumárico, ácido ferúlico, y el ácido m-cumárico estuvieron presentes en cantidades menores (Tabla 1, publicación 2). La cantidad de ácidos clorogénicos en los frutos de *Morus nigra* fue más alta que en los de *Morus alba*. Butkhup et al. (2013) encontraron que la concentración de ácido clorogénico en morera blanca era de 0,01 a 0,06 mg/g dw, mientras que Gundogdu et al. (2011) encontraron cantidades comprendidas entre 0,1 y 3,1 mg/g fw, estos resultados están en consonancia con nuestros resultados. Arfan, et al. (2012) también encontraron que el ácido clorogénico fue el ácido dominante en extractos sin azúcar de frutos de *Morus nigra* y *Morus alba*. Respecto a los derivados de los flavonoles, los principales compuestos que se presentaron en los clones de morera eran diferentes formas glicosiladas de quercetina y kaempferol (Tabla 1, publicación 2); el contenido en glicósidos de quercetina osciló (de 0,02 a 1,11 mg/g dw) siendo mayor que la cantidad de kaempferol-*O*-rutinoside que osciló (de 0 a 0,18 mg/g dw).

Se encontraron un alto contenido de derivados de flavonoles en el clon MN1 (1,29 mg/g dw), MN2 (0,56 mg/g dw), y MA2 (0,51 mg/g dw), mientras que los contenidos más bajos se encontraron en el clon MA3 (0,07 mg/g dw). El flavonol principal fue la rutina (quercetin 3-*O*-rutinóside); el mayor contenido se halló en el clon MN1 (0,93 mg/g dw) seguido del clon MN2 (0,28 mg/g dw). Los resultados de antocianinas totales variaron significativamente entre los clones de *M. nigra* (desde 0,01 mg/g dw del clon MN3 a 1,88 mg/g dw del clon MN1), mientras que *M. alba* carecía de antocianinas como era de esperar (Tabla 1, publicación 2). El clon MN1 mostró niveles más altos de cyanidin 3-*O*-glucóside y cyanidin 3-*O*-rutinóside que el resto de clones. Las antocianinas predominantes fueron los derivados de la cianidina, mientras que los derivados de la pelargonidina representaron cantidades muy pequeñas. Özgen et al. (2009) obtuvieron valores del contenido total de antocianinas en moras negras de hasta 0,571 mg/g de fw. Kim et al. (1999) obtuvieron niveles altos de antocianinas totales

(2,45 a 3,14 mg/g) en moreras de una variedad silvestre. Por el contrario, Park et al. (1997) obtuvieron contenidos de antocianinas totales en las moras maduras que oscilaron entre 0,19 a 3,29 mg/g.

Se utilizaron los ensayos $ABTS^{\bullet+}$ *y* $DPPH^{\bullet}$ para evaluar la **actividad antioxidante** de los **frutos** de morera (Figura 2, publicación 2). Los valores medios variaron significativamente desde 3,84 hasta 20,73 mg de Trolox g dw ($ABTS^{\bullet+}$) y desde 3,62 hasta 12,91 mg de Trolox g dw ($DPPH^{\bullet}$). La actividad antioxidante fue significativamente mayor en los frutos de *M. nigra* que en los de *M. alba*, en ambos métodos. El clon MN1 mostró un mayor potencial antioxidante que el resto de clones, incluso cinco veces más altos que los clones MA3, MA4, MN3 y MN4. De hecho, se observaron correlaciones positivas entre el TPC y la actividad antioxidante medida por el método $ABTS^{\bullet+}$ ($r = 0,87$, $p \leq 0,05$) y el método $DPPH^{\bullet}$ ($r = 0,92$, $p \leq 0,05$), aspecto que destaca el gran valor fitoquímico del clon MN1, en particular en antocianinas y ácidos clorogénicos, como ya se ha mencionado. Estudios previos han puesto de manifiesto las considerables variaciones existentes en cuanto a la actividad antioxidante en los frutos de morera (Ercisli et al., 2010; Gundogdu et al., 2011; Imran et al., 2010; Khalid et al., 2011; Özgen et al., 2009).

En la **composición mineral** de los **frutos**, se observaron diferencias significativas entre los clones de morera ensayados. Entre los macroelementos (N, P, K, Ca, Na, Mg, y S), la concentración de N osciló entre 1,42 y 2,13 g/100 g dw (Tabla 2, publicación 2). Ercisli y Orhan (2007) señalaron que las variedades de morera cultivadas en Turquía presentaron concentraciones de N que fueron desde 0,75% (*M. alba*) a 0,92% (*M. nigra*). Sin embargo, Karlidag, et al. (2012) obtuvieron valores más altos de N, hasta 3,19 g/100 g. Cabe señalar que el nitrógeno predominó sobre el potasio en los clones de morera blanca, mientras que el potasio predominó sobre el nitrógeno para la morera negra. Estos resultados están en la misma línea que los obtenidos por Karlidag et al. (2012) en *M. alba*, aunque Ercisli y Orhan (2007), obtuvieron valores más altos de potasio que de nitrógeno para *M. alba*.

Entre los microelementos (Fe, Cu, Mn, Zn y Ni), el contenido de Fe osciló entre 23,92 a 46,74 mg/kg. Nuestros resultados fueron similares a los obtenidos por Ercisli y Orhan (2007) y Ercisli et al. (2010), pero inferiores a los obtenidos por Imran et al. (2010), Karlidag et al. (2012), y Khalid et al. (2011). En general el orden decreciente de los elementos minerales en el estudio de frutos para *M. alba* fue N>K>P>Ca>Mg>S>

Na>Fe>Zn>Cu>Ni. Por el contrario, el orden decreciente de los elementos minerales en los frutos de *M. nigra* fue K>N>Ca>P>Mg>S>Na>Fe>Zn>Cu>Ni. Además, el bajo contenido en Na de las moras españolas es digno de mencionar ya que podrían ser de interés para dietas bajas en sodio.

El contenido de proteína en los **frutos** osciló entre el 8,90% dw y el 13,33% dw. Los clones de *M. alba* (promedio 12,10% dw) tuvieron un mayor porcentaje de proteína que los de *M. nigra* (promedio de 10,06% dw). En las moreras españolas, el contenido total de proteínas de los frutos fue mayor al obtenido por Imran et al. (2010), en moreras pakistaníes quienes obtuvieron valores medios de 0,96 g/100 g dw en *M. nigra* y 1,55 g/100 g dw en *M. alba.*

El **análisis de componentes principales PCA** permitió separar los clones en tres grupos. El 1º grupo formado por el clon MN1 que se caracteriza por un alto contenido de fitoquímicos (como antocianinas, flavonoles, y ácidos clorogénicos) y alta actividad antioxidante, en particular superior al resto de los clones ensayados. El 2º grupo formado por los clones MA1, MA2, y MN2 se caracterizaron, principalmente por sus altas cantidades de derivados del ácido benzoico. Finalmente, el tercer grupo lo formaron los clones MA3, MA4, MN3 y MN4, caracterizado, principalmente, por su bajo contenido en compuestos fenólicos. Cabe señalar que los clones de *M. alba* y *M. nigra* no se reunieron en función de su clasificación taxonómica, como inicialmente se esperaba.

9.2.2. CONCLUSIONES

Muchos estudios han puesto de manifiesto que el consumo de moras puede tener efectos positivos sobre la salud humana. Los resultados obtenidos en el presente estudio mostraron que los frutos de morera blanca y negra cultivadas en España, mostraron diferencias significativas en el contenido de compuestos fenólicos, actividad antioxidante y composición mineral. En general, los clones de morera negra mostraron un mayor contenido en compuestos fenólicos y una mayor actividad antioxidante que los clones de morera blanca, a pesar de que se observó una amplia variabilidad entre especies. De acuerdo con estos resultados, algunos clones de las dos especies de morera se podrían utilizar para su transformación industrial en productos alimentarios ricos en compuestos fenólicos.

9.3. PUBLICACIÓN 3

(Poly)phenolic compounds and antioxidant activity of white (*Morus alba*) and black (*Morus nigra*) mulberry leaves: Their potential for new products rich in phytochemicals

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Journal of Functional Foods 2015, 18, 1039-1046

Factor de impacto: **3,973 Q1** (Categoria: Food Science & Technology)

El **objetivo** de este estudio fue determinar y cuantificar, los principales compuestos polifenólicos y la actividad antioxidante de las hojas de morera de diferentes clones cultivados en España, con el fin de evaluar su potencial fitoquímico.

9.3.1. RESUMEN DE RESULTADOS Y DISCUSIÓN

El contenido en **fenoles totales (TPC)** en las **hojas** de moreras españolas varió de 12,81 a 16,13 mg GAE/g dw, (Tabla 1, publicación 3). Los clones de *M. alba* presentaron el menor contenido en fenoles totales (de 12,81 a 15,50 mg GAE/g dw), mientras que los clones de *M. nigra* presentaron niveles ligeramente más altos (13,48 a 16,13 mg GAE/g dw). Estos resultados están de acuerdo con los obtenidos por Iqbal et al. (2012) en hojas de moreras Pakistaníes. Radojković et al. 2012 obtuvieron valores en fenoles totales en las hojas de morera que iban desde 66,76 (*M. alba*) a 115,23 (*M. nigra*) mg GAE/g dw superiores a los mostrados por los clones españoles. Sin embargo, Thabti et al. (2012) obtuvieron valores más bajos en fenoles totales en las hojas de morera (3,45-6,31 mg GAE/g dw).

En cuanto a los **compuestos polifenólicos**, la huella digital fitoquímica de las **hojas** de morera españolas fue determinada en base a los estudios anteriores centrados en la composición fenólica de hojas de morera (Dugo et al., 2009; Memon et al., 2010). Se identificaron catorce compuestos fenólicos en los ocho clones de morera analizados: cuatro ácidos cafeilquínicos (CQAs) y diez flavonoles. El contenido de CQAs fue ligeramente mayor que el contenido de los derivados de flavonoles, en todas las muestras (Tabla 2, publicación 3). El contenido medio de CQAs y derivados de flavonoles de las hojas de morera españolas fue del 8,3 y 5,6 mg/g dw, respectivamente.

El contenido total de CQAs de las hojas de morera varió de 6,78 a 8,48 mg/g dw para los clones de *M. alba* y 6,43 a 10,05 mg/g dw para los clones de *M. nigra* (Tabla 2, publicación 3). El ácido clorogénico fue con diferencia el predominante en las hojas de morera, variando entre 5,29 (MA3) y 8,39 mg/g dw (MN3), seguido del ácido neoclorogénico (3-CQA), ácido criptoclorogénico (4-CQA), y el isómero del ácido clorogénico (1-CQA) (Tabla 2, publicación 3). Estos resultados con respecto al contenido en CQAs en las hojas de morera son similares a los obtenidos en *M. alba* por Dugo et al. (2009). El contenido de CQAs en hojas de *M. nigra* fue ligeramente mayor que el de *M. alba*, estos resultados coinciden con los obtenidos por Radojković et al. (2012). Por otra parte, Sánchez-Salcedo et al. (2015) encontraron un menor contenido de CQAs en los **frutos** de morera (0,16 y 3,62 mg/g dw) que en las **hojas** de morera. El contenido total de los derivados de flavonoles en las hojas varió entre 3,66 y 9,75 mg/g dw. En cuanto a los flavonoles predominaron más los derivados de quercetina que los de kaempferol (Tabla 2, publicación 3). Los flavonoles predominantes en las hojas de morera de *M. alba* y *M. nigra* fueron quercetin-3-*O*-glucóside (isoquercitrin)>quercetin-3-*O*-(6-malonyl)-β-glucopyranoside = quercetin-rutinoside (rutina)>kaempferol-3-*O*-(6-malonyl) glucoside. En particular, oscilaron entre 0,92 a 3,73 mg/g dw para el quercetin-3-*O*-glucoside, 0,35 a 1,84 mg/g dw para el quercetin-3-*O*-(6-malonyl)-β-glucopiranoside, entre 0,58 a 1,80 mg/g dw para la rutina, y 0,11 a 0,80 mg/g dw para el kaempferol-3-*O*-(6-malonyl) glucoside (Tabla 2, publicación 3). Similar tendencia ha sido demostrada por otros autores (Dugo et al., 2009; Katsube et al., 2006), sin embargo, Thabti et al. (2012) obtuvieron valores entre 7 y 120 veces más altos que los resultados obtenidos en el presente estudio para el contenido de malonyl-glucoside de quercetina y kaempferol (13,33 y 12,59 mg/g dw, respectivamente). Otros flavonoles también se detectaron en todas las muestras a concentraciones que no excedían de 0,95 mg/g dw, coincidiendo con los estudios Dugo et al. (2009). El contenido de flavonoles fue casi 10 veces mayor en las hojas de morera que en los frutos (Sánchez-Salcedo et al., 2015). Se encontraron diferencias significativas entre los clones en el perfil polifenólico de las hojas de morera (Tabla 2, publicación 3), lo que podría ser útil para propósitos de autentificación.

La actividad antioxidante se determinó por los métodos $ABTS^{\bullet+}$ y $DPPH^{\bullet}$ (Tabla 1, publicación 3). Los valores medios variaron de 10,60 (MN1) a 13,15 (MN3) mg Trolox/g dw por el método $ABTS^{\bullet+}$ y 10,62 (MN1) a 12,64 (MA1) mg Trolox/g dw por

el método *DPPH*$^{\bullet}$. La actividad antioxidante por el método *ABTS*$^{\bullet+}$ fue similar para ambas especies *M. nigra* y *M. alba*. Mientras que, cuando se considera el método *DPPH*$^{\bullet}$, la actividad antioxidante de los clones de *M. alba* fue ligeramente mayor que la de *M. nigra.* Thabti et al. (2014) obtuvieron valores inferiores de actividad antioxidante, por el método *ABTS*$^{\bullet+}$, para las hojas de morera blanca (2,1-3,5 mg de Trolox/g dw). En la actividad antioxidante de las hojas de morera se han registrado variaciones considerables en estudios previos (Iqbal et al., 2012; Thabti et al., 2014; Zou et al., 2012). Las hojas de morera mostraron valores medios más altos de capacidad antioxidante para el método *ABTS*$^{\bullet+}$ y para el método *DPPH*$^{\bullet}$ (Sánchez-Salcedo et al., 2015). Resultados similares fueron encontrados por Teleszco y Wojdyło (2015) para las hojas de bayas (arándano agrio, grosella negra, y el arándano). Estos resultados resaltan el potencial de las hojas de morera en el desarrollo de alimentos con alta capacidad antioxidante. Se observó una alta correlación positiva ($r = 0,81$, $p \leq 0,05$) entre el TPC y la capacidad antioxidante medida por el método *ABTS*$^{\bullet+}$. Por el contrario, TPC sólo presentó una débil correlación negativa con el método *DPPH*$^{\bullet}$ ($r = -0,22$, $p \leq 0,05$).

El **análisis de componentes principales PCA** permitió separar los clones. El clon MN3 se caracterizó por un alto contenido de flavonoles totales, ácidos clorogénicos totales, TPC y una alta actividad antioxidante. Los clones MN1 y MN2 mostraron altas cantidades en algunos flavonoles y altos valores de *ABTS*$^{\bullet+}$. Sin embargo, mientras que el clon MN2 presentó un alto contenido de quercetin-rutinoside y valores de *DPPH*$^{\bullet}$, el clon MN1 mostró un perfil fitoquímico con cantidades muy bajas en quercetin-rutinoside. Respecto a los clones MA2 y MA3 se caracterizaron por un bajo contenido en compuestos polifenólicos, aunque un contenido moderado de ácido neo-clorogénico (3-CQA), y valores altos de actividad antioxidante. Y por último, los clones MA1, MA4, y MN4, pueden ser considerados como clones con características intermedias con respecto al resto de los clones antes mencionados.

9.3.2. CONCLUSIONES

Algunos estudios han señalado que las hojas de morera pueden constituir una buena fuente de compuestos polifenólicos capaces de tener un impacto positivo en la salud humana. Los resultados presentados en este trabajo, muestran que las hojas de los clones de morera blanca y negra cultivadas en España son ricos en CQAs y flavonoles y tienen una amplia variabilidad intraespecífica en sus huellas digitales polifenólicas. Si

comparamos los frutos de morera con las hojas, estas presentan un mayor contenido en compuestos fenólicos y actividad antioxidante. Como consecuencia, las hojas de morera se podrían utilizar para la elaboración de productos de morera por parte de la industria de la alimentación funcional, mientras que las frutas podrían destinarse para su consumo en fresco o zumo. Este hecho puede allanar el camino a la revalorización de este material vegetal infrautilizado, con el fin de desarrollar productos derivados de hoja de morera ricos en compuestos fenólicos. En realidad, atendiendo a su composición fenólica, las hojas de morera se podrían utilizar para el desarrollo de tés y otras bebidas. Sus extractos también se podrían usar como aditivos para crear o enriquecer los alimentos tales como barritas de muesli, yogures, etc. Por otra parte, debido a su composición fenólica en particular, pueden ser considerados fuentes prometedoras de compuestos fitoquímicos con actividades biológicas demostradas. Sin embargo, si el consumo de hojas de morera en té puede ser beneficioso para la salud humana, todavía requiere más estudios de investigación sobre su biodisponibilidad y propiedades biológicas.

9.4. PUBLICACIÓN 4

Fatty acids composition of Spanish black (*Morus nigra* L.) and white (*Morus alba* L.) mulberries

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Food Chemistry 2016, 190, 566-571

Factor de impacto: **4,052 Q1 (2015)** (Categoria: Food Science & Technology)

El **objetivo** de este estudio fue determinar cualitativa y cuantitativamente la composición de ácidos grasos de los frutos de 8 clones de morera (cuatro de *M. alba* y cuatro *M. nigra*) cultivadas en España.

9.4.1. RESUMEN DE RESULTADOS Y DISCUSIÓN

En cuanto a la **composición de ácidos grasos** se identificaron y cuantificaron catorce ácidos grasos en los **frutos** de la morera. Los ácidos grasos más abundantes fueron los ácidos linoleico (C18: 2), palmítico (C16: 0), oleico (C18: 1) y esteárico (C18: 0), en los frutos de ambas especies, los cuales en su conjunto comprendían aproximadamente 96,77% de ácidos grasos totales identificados (Figura 2, publicación 4). Estos resultados son coincidentes con los obtenidos previamente por Ercisli y Orhan (2007), Yang et al. (2010), y Gecgel et al. (2011).

El ácido graso más abundante que se encontró en todos los clones fue el ácido linoleico (C18: 2) oscilando entre el 69,66% (MN2) y el 78,02% (MA1) del contenido total de ácidos grasos. Estos resultados están de acuerdo con los obtenidos por Gecgel et al. (2011), quienes obtuvieron contenidos medios de 73,70% (C18: 2) en morera negra, y con los obtenidos por Yang et al. (2010) que obtuvieron un promedio de 79,37% (C18: 2) en *M. alba*.

El ácido linoleico fue seguido por el ácido palmítico (C16: 0), con contenidos que iban de 10,72% (MA1) a 17,97% (MN2), ácido oleico (C18: 1) a partir de 5,32% (MN2) a 7,26% (MN4), y ácido esteárico (C18: 0) de 3,03% (MN2) al 3,79% (MN4). Resultados similares fueron obtenidos por Gecgel et al. (2011) y Yang et al. (2010), pero estos autores observaron menores valores de C16: 0. Sin embargo, Ercisli y Orhan

(2007) obtuvieron valores más altos de estos ácidos grasos, excepto C16: 0, en *M. nigra*. El ácido linolénico (C18: 3) varió del 1,42% (MN4) al 2,40% (MA3). Gecgel et al. (2011) encontraron niveles más bajos de linolénico (0,45%) en *M. nigra*, mientras que Ercisli y Orhan (2007) no lo detectaron en *M. nigra* y *M. alba*.

Los clones de morera tenían altos porcentajes de ácidos grasos esenciales (C18: 2, y C18: 3); por ejemplo, *M. alba* contenía aproximadamente 76,67% y *M. nigra* el 74,70%.

Otros ácidos grasos, tales como nonadecanoico (C19: 0), mirístico (C14: 0), behénico (C22: 0), palmitoleico (C16: 1) y eicosenoico (C20: 1) estaban presentes en cantidades menores. Gecgel et al. (2011) también detectaron los ácidos behénico (C22: 0) y palmitoleico (C16: 1) en la morera negra. Sin embargo, Ercisli y Orhan (2007) detectaron ácido behénico (C22: 0) y ácido palmitoleico (C16: 1) a concentraciones de 0,26% y 0,67%, respectivamente, pero sólo en *M. alba*.

Otros ácidos grasos, tales como cetoleico (C22: 1), araquídico (C20: 0), heneicosanoico (C21: 0) y araquidónico (20: 4) estaban presentes sólo en algunos clones y en pequeñas cantidades.

Después de agrupar los ácidos grasos de los frutos de la moreras, el orden de abundancia fue: ácidos grasos poliinsaturados PUFA>ácidos grasos saturados SFA>ácidos grasos monoinsaturados MUFA. Los ácidos grasos poliinsaturados (PUFA) eran la fracción más importante de los ácidos grasos, que representaban al menos el 76,68% en *M. alba* y el 74,74% en *M. nigra.* Los ácidos grasos saturados (SFA) también representan una parte considerable de los ácidos grasos, con porcentajes del 16,77% en *M. alba* y del 18,75% en *M. nigra*. Los ácidos grasos monoinsaturados (MUFA) sólo estaban presentes en bajos porcentajes con 6,56% en *M. alba* y el 6,51% en *M. nigra*. Estos resultados están en consonancia con los obtenidos por Gecgel et al. (2011); estos autores obtuvieron valores de 74,43% de PUFA, pero detectaron una mayor cantidad de ácidos grasos monoinsaturados (10,12%).

Respecto al **contenido de ácidos grasos** de los **frutos** de morera expresados en mg/100 g los principales ácidos grasos fueron los siguientes: El ácido linoleico, con una concentración que varía de 4.365 del clon MN2 a 10.531 mg/100 g dw del clon MA1, el ácido palmítico, que va desde 972 del clon MN1 a 1.416,54 mg/100 g dw del clon MA1, el ácido oleico, que van desde 325 del clon MN2 a 782 mg/100 g dw del clon MA4, el ácido esteárico, que va desde 189 del clon MN2 a 429 mg/100 g dw del clon

MA1, y el ácido linolénico, que va desde 115 del clon MN2 a 193 mg/100 g dw del clon MA1.

Otros ácidos grasos se encontraron en concentraciones intermedias (Tabla 2, publicación 4).

El presente estudio demostró que el clon MA1 presentó el mayor contenido total de ácidos grasos seguido por MA4, MN4, MN1, MA2, MA3, MN3, y MN2. En general, entre los clones estudiados, *M. alba* tenían contenidos totales más altos de ácidos grasos que *M. nigra*. Los clones MA1, MA4, MN4, y MN1 de moras españolas podría ser buena fuente de ácidos grasos esenciales, especialmente de ácido linoleico.

El **análisis de componentes principales PCA** indica que los clones MA1, MA4 y MN4 se caracterizan por un alto contenido de ácido linoleico (C18: 2), y bajos contenidos de ácidos mirístico (C14: 0), palmítico (C16: 0), nonadecanoico (19: 0), y palmitoleico (C16: 1). Estos datos sugieren que los clones MA1, MA4, y MN4 son una buena fuente de ácidos grasos, ya que tienen una alto contenido de ácidos grasos poliinsaturados (PUFA) y un bajo contenido de ácidos grasos saturados (SFA). El clon MN2 presentaba el comportamiento opuesto se caracteriza por un bajo contenido de ácido linoleico (C18: 2) y un alto contenido de ácidos mirístico (C14: 0), palmítico (C16: 0), nonadecanoico (19: 0) y palmitoleico (C16: 1). El clon MA3 era el único clon que contenía ácido araquidónico (C20: 4) y tenía un mayor contenido de linolénico (C18: 3), oleico (C18: 1) que la mayoría de los clones estudiados. El clon MA2 se diferencia de los demás clones por su baja cantidad de ácido eicosenoico (C20: 1) y su alto contenido de ácido cetoleico (C22: 1).

9.4.2. CONCLUSIONES

Los resultados de este estudio ponen de manifiesto el potencial nutricional del perfil de ácidos grasos de los frutos de morera blanca y negra españolas. Los ácidos grasos más abundantes fueron linoleico, palmítico y oleico. El ácido linoleico, un ácido graso poliinsaturado esencial, dominó el perfil de ácidos grasos (73,9% de ácidos grasos totales) en todos los clones. Además, el análisis GC-FID reveló que los clones MA1, MA4, MN4, y MN1 contenían altos contenidos de ácidos grasos, especialmente de ácido linoleico, que el resto de clones. Estos resultados mostraron que las moras negras y blancas podrían ser consideradas como una fuente natural de ácidos grasos

"esenciales", en particular el ácido linoleico y esto apoya su alto potencial para su uso en industrias de alimentos, cosmética y farmacéutica.

9.5. PUBLICACIÓN 5

(Poly)phenolic fingerprint and chemometric analysis of white (*Morus alba* L.) and black (*Morus nigra* L.) mulberry leaves by using a non-targeted UHPLC-MS approach

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Food Chemistry 2016, 212, 250-255

Factor de impacto: **4,052 Q1 (2015)** (Categoria: Food Science & Technology)

El **objetivo** de este estudio fue, investigar la huella digital y la discriminación quimiométrica de compuestos polifenólicos en hojas de ocho clones de morera de *Morus alba* y *Morus nigra*.

9.5.1. RESUMEN DE RESULTADOS Y DISCUSIÓN

La **composición fitoquímica** de las **hojas** de los clones de *M. alba* y de *M. nigra*, se centró en la fracción fenólica, y se determinó por el método de huellas digitales UHPLC-ESI-MS^n emparejado con el análisis multivariante para facilitar la detección rápida y completa de su potencial (Calani et al., 2013) y consistente de dos condiciones complementarias MS (Mena et al., 2012). En las **hojas** de morera se identificaron tentativamente un total de 31 compuestos en todos los clones ensayados (Tabla 1, publicación 5), siendo ésta la caracterización fenólica más amplia de este material realizada hasta la fecha.

En el presente estudio, los flavonoles fueron los más relevantes en las hojas de morera. Se detectaron ácidos cafeoilquínicos, algunos ácidos fenólicos simples y también otros fitoquímicos como los ácidos orgánicos.

De los 31 compuestos identificados, seis fueron identificados por comparación con estándares de referencia, mientras que los 25 restantes se identificaron tentativamente basándose en la interpretación de sus patrones de fragmentación obtenidos de los espectros de masas (experimentos MS^2 y MS^3) y por comparación con la literatura (Tabla 1, de la publicación 5).

En las hojas de morera se identificaron un total de 20 flavonoles, se han señalado los flavonoides como la principal clase de compuestos fenólicos (Tabla 1, publicación

5). Estos fueron formas glicosiladas de quercetina y kaempferol. Entre los 12 derivados de quercetina detectados, 9 (compuestos **9, 17, 19, 20, 23, 24, 26, 28,** y **30**) habían sido identificados previamente en hojas de *M. alba* (Dugo et al., 2009; Thabti et al., 2012), mientras que, hasta donde se sabe, los compuestos **13, 18,** y **22** se han descrito por primera vez en hojas de *Morus* (Dall'Asta et al., 2012; Kazuma et al., 2003; Mikulic-Petkovsek et al., 2012). En cuanto a los derivados del kaempferol, los compuestos **11, 21, 25, 29,** y **31** ya habían sido descritos en la hoja de morera (Dugo et al., 2009; Thabti et al., 2012). En este estudio tres glicósidos de kaempferol (compuestos **14, 16,** y **27**) se han descrito por primera vez en esta planta según nuestro conocimiento. Los derivados de quercetina prevalecieron sobre el kaempferol. La isoquercitrina (compuesto **23**) y el quercetin-malonyl-glucoside (**26**) se han indicado entre los principales flavonoides individuales de las hojas de morera (Dugo et al., 2009; Sánchez-Salcedo et al., 2015; Thabti et al., 2012).

En el estudio se identificaron un total de cinco derivados del ácido cafeoilquínico. Cuatro isómeros del ácido cafeoilquínico (compuestos **6, 8, 12,** y **15**) y se identificó un ácido dicafeoilquínico (**5**), todos previamente identificados en hojas de morera (Dugo et al., 2009). Los ácidos cafeoilquínicos son compuestos fenólicos presentes en grandes cantidades en las hojas de morera, siendo el ácido clorogénico el predominante. Las concentraciones de ácido cafeoilquínico total varió entre 6,8 y 8,5 mg/g dw de los clones españoles, y no se encontraron diferencias significativas entre las especies. Sin embargo, grandes variaciones intra e interespecíficas en contenido de ácido cafeoilquínico han sido evidenciadas por otros autores (Dugo et al., 2009; Radojković et al., 2012; Thabti et al., 2012).

Otros compuestos fenólicos, tales como el ácido protocatéquico (**4**), el ácido cafeico (**10**), y el ácido cafeico hexoside (**7**), fueron identificados por comparación con su estándar comercial o, en el caso de ácido cafeico hexoside (**7**), por su patrón de fragmentación (Dall'Asta et al., 2012). El ácido cafeico y su forma glicosilada se han descrito a concentraciones inferiores (<1 mg/g dw) en la hoja de *M. alba* (Skupień et al., 2008).

Es la primera vez que el ácido protocatéquico se identifica en especies de *Morus*, según nuestro conocimiento. Algunos ácidos orgánicos presentes en los frutos de morera (Mikulic-Petkovsek et al., 2012; Sánchez et al., 2014), tales como, el ácido

quínico (**1**) el ácido L-málico (**2**), y el ácido cítrico (**3**) también fueron detectados en las hojas.

Este trabajo permitió la identificación tentativa de 7 compuestos que no se habían indicado anteriormente en esta especie. A pesar de que varios trabajos habían estudiado ampliamente el perfil fenólico de la hoja de morera (Dugo et al., 2009; Enkhmaa et al., 2005; Hunyadi et al., 2013.; Skupień et al., 2008; Sánchez-Salcedo et al., 2015; Thabti et al., 2012).

En el **análisis de Conglomerados (CA)** permitió separar a los clones en tres grupos principales, (i) el primer grupo estaba formado por MA1, MA3, MA2 y MN2, (ii) el segundo incluía el clon MN1, y (iii) el tercero comprendía MA4, MN3 y MN4. Estos grupos fueron muy diferentes, indicando diferencias claras entre los clones (variabilidad intra-especie) con independencia de las especies consideradas (variabilidad entre especies).

El **análisis de componentes principales PCA** separó a los clones en grupos, el (i) primer grupo, estaba formado por los clones MA1, MA2, MA3, y MN2 (Figura 2, publicación 5) mostraron un bajo contenido en flavonoles, unidades de *O*-hexoside y de los compuestos **12, 14, 15, 20** y **25**, mientras que presentaban una alta cantidad de los compuestos **7, 8** y **27**. Su contenido en flavonoles rutinoside dependía de cada clon. Cabe señalar, que el clon MA1 mostró el perfil fitoquímico más equilibrado entre todos los clones estudiados, exhibiendo contenidos medios para todos los fitoquímicos identificados. El (ii) segundo grupo compuesto por el clon MN1 que fue claramente separado del resto de clones de morera y podría ser considerado como el clon que tiene el contenido más bajo en flavonoles y ácido cafeico hexoside, pero con un mayor contenido en ácido cafeico y ácido criptoclorogénico. Y (iii) el tercer grupo comprendía los clones MN3, MN4, y MA4, se caracterizó por un alto contenido en flavonoles que contenían unidades de *O*-hexoside y una bajo contenido en ácido cafeico. Este grupo mostró un contenido variable en flavonoles y contenían rutinosides, mientras que los clones MN3 y MN4 revelaron un menor contenido en compuestos **18, 21,** y **22** que el clon MA4.

9.5.2. CONCLUSIONES

Estos resultados muestran el perfil fitoquímico específico de las hojas de morera. Éstos podrían permitir la selección genética más adecuada como un recurso de interés

para las industrias agroalimentarias/farmacéuticas. Además, este detallado análisis fitoquímico de alto rendimiento puede ser utilizado para el control de calidad y los propósitos de autentificación/adulteración.

Por otra parte, si las hojas de morera se utilizan para la elaboración de productos ricos en compuestos bioactivos (por ejemplo, bebidas de té), la mayoría de estos clones podrían tener buenas perspectivas. Todos los clones estudiados contenían cantidades altas y similares en contenidos de ácidos cafeoilquínicos y flavonoles, incluso si su contenido en compuestos individuales variaba notablemente, (Sánchez-Salcedo et al., 2015a), éstos han sido descritos como potencialmente beneficiosos para varios procesos fisiopatológicos en los seres humanos (Del Río et al., 2013; Dellafiora et al., 2014; Rodríguez-Mateos et al., 2014). Por lo tanto, teniendo en cuenta el complejo metabolismo de estas sustancias, donde la desconjugación severa se produce en el lumen intestinal antes de la absorción, los programas de mejoramiento o selección de germoplasma deben centrarse en el contenido total en la hoja en cafeoilquínico, quercetina, y kaempferol, en lugar de en su contenido en compuestos individuales. Por último, este método no específico en la investigación de la toma de huellas digitales fenólicas, y la discriminación quimiométrica de ocho clones, permitieron la identificación tentativa de 7 estructuras fenólicas que no se identificaron anteriormente en esta especie. Este hecho amplía la gama de sustancias que contribuyen a la definición y bioactividad de este material vegetal, y representa un hito notable en la evaluación adecuada de sus propiedades biológicas y nutricionales.

9.6. PUBLICACIÓN 6

Phytochemical evaluation of eight white (*Morus alba* L.) and black (*Morus nigra* L.) mulberry clones grown in Spain based on UHPLC-ESI-MS^n metabolomics profiles

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Food Research International 2016, 89, 1116-1122.

Factor de impacto: **3,182 Q1 (2015)** (Categoria: Food Science & Technology)

El **objetivo** de este estudio fue, la investigación de las huellas digitales polifenólicas y la discriminación quimiométrica de frutos de ocho clones de morera de las especies *Morus alba* y *Morus nigra*.

9.6.1. RESUMEN DE RESULTADOS Y DISCUSIÓN

Respecto a la **identificación de compuestos fitoquímicos**, se evaluaron los **frutos** de ocho clones de *M. alba* y *M. nigra*. El perfil fitoquímico de estos materiales vegetales, se centró en compuestos polifenólicos, se evaluó mediante un método integral UHPLC-ESI-MS^n combinado con estadística multivariante (Calani et al., 2013). En particular, un enfoque no específico para el cribado de compuestos fenólicos con diversas actitudes de ionización, y que consta de tres condiciones MS complementarias, tanto en modo negativo y positivo, se utilizó para determinar la composición fitoquímica de los frutos (Mena et al., 2012). Se identificaron tentativamente 64 compuestos (Tabla 1 y Tabla 2 publicación 6), la caracterización más amplia de la de huellas digitales polifenólicas de frutos de morera hasta la fecha. Entre las diferentes clases de compuestos polifenólicos identificados en los frutos, los flavonoles fueron los más relevantes (Figura 1 publicación 6). Se observaron otros flavonoides (flavononas, flavan-3-oles, dihidrochalconas, flavanonoles y antocianinas), ácidos fenólicos, y los lignanos, así como algunos ácidos orgánicos.

Nueve compuestos fueron identificados por comparación con estándares de referencia, mientras que 55 se identificaron en base a sus patrones de fragmentación obtenidos a partir de los espectros de masas (experimentos MS^2 y MS^3) y mediante la comparación de sus espectros con la literatura disponible. Se identificaron

tentativamente por primera vez 21 compuestos según nuestro conocimiento (compuestos **6, 8,10, 13, 21, 22, 24, 28, 32, 34, 41, 44, 47, 49, 51, 52, 55, 56, 57, 58,** y **60**), en **frutos** de morera.

En este trabajo se identificaron un total de 20 flavonoles en los **frutos** de la morera. Estos fueron principalmente formas glicosiladas de quercetina, kaempferol, e isorhamnetina (**25**) (Tabla 1 publicación 6). El morin (**54**), se identificó sólo como aglicona, de acuerdo con McNab et al. (2009). Entre los 12 derivados de quercetina detectados, siete (**18, 29, 35, 37, 40, 43,** y **53**) ya habían sido identificados previamente en frutos de *M. alba* (Juan et al, 2012; Natić et al, 2015; Sánchez-Salcedo et al., 2015), mientras que los 3 derivados de quercetina detectados (**28, 41,** y **45**) sólo se habían descrito en hojas de morera (Sánchez-Salcedo et al., 2015). En cuanto a los derivados de kaempferol, los compuestos **33, 42, 48** y **59** ya se había descrito en **frutos** de morera anteriormente (Juan et al., 2012; Natić et al., 2015; Sánchez-Salcedo et al., 2015), mientras que el kaempferol-rutinoside-hexoside (**22**) y kaempferol-rutinoside (**39**) se han identificado recientemente en hojas de morera (Dugo et al., 2009; datos no publicados), pero no en los frutos. En el presente estudio no se detectaron algunos de los flavonoles encontrados en frutos de morera en Serbia (Natić et al., 2015), esto podría ser debido a diferencias geográficas y genotípicas. Cuantitativamente, los flavonoles se han descrito al ser una de las principales clases de fenólicos en **frutos** de morera, con concentraciones que oscilan entre 0,1 y 1,3 mg/g dw en clones españoles (Sánchez-Salcedo et al., 2015), de manera similar a los descritos para otros cultivares de morera de todo el mundo (Butkhup et al., 2013; Pawlowska et al., 2008; Zhang et al., 2008).

Se identificaron tentativamente 12 flavonoides pertenecientes a cinco subclases de flavonoides distintos de los flavonoles (flavanonas, flavan-3-oles, flavona, flavanonol, dihidrochalcona, y antocianinas). Entre las flavanonas (Tabla 1 publicación 6), el eriodictyol-hexoside (**46**) ya había sido identificado en *Morus spp.* (Wang et al., 2013). Por el contrario, los compuestos **55, 56, 57, 58,** y **60** nunca se han descrito en los frutos de morera anteriormente, según nuestro conocimiento. El compuesto **60** se identificó tentativamente como tetrahydroxyflavanone. Los dos flavan-3-oles (**19** y **30**) fueron identificados por comparación con sus correspondientes estándares comerciales, mientras que los compuestos **27** (flavona), **38** (flavanonol), y **50** (dihidrochalcona) fueron identificados tentativamente según Fischer et al. (2011), Zhang et al. (2008), y Mena et al. (2012), respectivamente. Las cuatro antocianinas, ya se habían notificado en

los frutos de morera (Pawlowska et al., 2008), también se identificaron en nuestro estudio (Tabla 2 publicación 6). Estas fueron derivados de hexósido y rutinósido de pelargonidina y cianidina, que fueron detectadas únicamente en los clones de morera negra, tal como se había indicado anteriormente (Sánchez-Salcedo et al., 2015).

Varios ácidos fenólicos se identificaron tentativamente (Tabla 1 publicación 6). Entre ellos fueron detectados, hasta trece derivados hidroxicinámicos, que ya habían sido encontrados como los principales compuestos fenólicos en los frutos de moreras españolas (Sánchez-Salcedo et al., 2015). Los ácidos hidroxicinámicos simples ácido cafeico (**9**), ácido p-cumárico (**12**), ácido m-cumárico (**36**), y ácido ferúlico (**15**) fueron caracterizados por comparación con apropiados estándares o en base a los datos de literatura (Calani et al., 2014; Natić et al., 2015). Del mismo modo, la mayoría de los ácidos cafeoilquínicos eran identificados de acuerdo con sus patrones de fragmentación de sus correspondientes estándares comerciales (compuestos **11** y **14**) o por comparación con la literatura (**8, 10, 16, 20, 23,** y **26**) (Calani et al., 2014; Dall'Asta et al, 2012.; Dugo et al., 2009; Simirgiotis et al., 2012; Vallverdú-Queralt et al., 2011). El compuesto **13**, fue tentativamente identificado como derivado del ácido cafeoilquínico. Los cuatro ácidos hidroxibenzoicos eran identificados por comparación con sus estándares comerciales (**5** y **31**) o con datos de la literatura (**7** y **6**) (Natić et al., 2015; Sawada et al., 2012). También se identificaron otros compuestos fenólicos por comparación con datos de la literatura (**4, 17,** y **44**) (Barros et al., 2013; Romain et al., 2014; Wang et al., 2013).

Los análisis también permitieron la identificación tentativa de 6 lignanos (**24, 32, 47, 49, 51** y **52**) (Tabla 1 publicación 6), que se caracterizaron de acuerdo a sus patrones de fragmentación y los datos de la literatura (Eklund et al., 2008; Huis et al., 2012; Mena et al., 2012). Por último, también fueron detectados e identificados 3 ácidos orgánicos (**1, 2,** y **3**) (Mena et al., 2012).

Nuestro estudio permitió la identificación tentativa de 21 compuestos no identificados previamente en moras, que representa el perfil más amplio de compuestos fenólicos de **frutos** de morera hasta la fecha. Este análisis de alto rendimiento ha puesto de manifiesto la amplia gama de sustancias fenólicas y otros fitoquímicos presentes en los frutos de morera. A pesar de que algunas caracterizaciones detalladas de frutos de morera se habían realizado en el pasado (Butkhup et al., 2013; Natić et al., 2015; Pawlowska et al., 2008; Wang et al., 2013).

Según el **análisis de componentes principales PCA** los clones de *M. alba* se pueden definir como muestras con un contenido bajo en compuestos fenólicos y, con cantidades variables de fitoquímicos específicos. Se observó que los clones de *M. nigra* tenían un mayor contenido en fitoquímicos que los de *M. alba*, siendo MN1 el que tenía el mayor contenido en compuestos polifenólicos. Es importante reseñar, que los clones *M. alba* y *M. nigra* no se agruparon de acuerdo con su clasificación taxonómica, como en un principio se esperaba. Sin embargo, estas observaciones coinciden con las mostradas por Sánchez-Salcedo et al. (2015).

9.6.2. CONCLUSIONES

El análisis UHPLC-ESI-MS^n permitió la identificación tentativa de 64 compuestos fitoquímicos individuales en frutos de morera, incluyendo 20 flavonoles, 6 flavanonas, 2 flavan-3-oles, 1 flavona, 1 flavanonol, 1 dihidrochalcona, 4 antocianinas (sólo en *M. nigra*), 13 derivados del ácido hidroxicinámico, 4 ácidos hidroxibenzoicos, otros 3 fenólicos de bajo peso molecular, 6 lignanos, y 3 ácidos orgánicos. Entre los compuestos detectados 21 han sido identificados por primera vez en los frutos de morera. El análisis multivariante sirve para caracterizar rápidamente las diferentes muestras de frutos de morera de acuerdo principalmente a sus huellas digitales polifenólicas. Este enfoque puede producir información pertinente para la evaluación de las perspectivas funcionales, nutricionales, y tecnológicas de los frutos de morera.

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10. CONCLUSIONES GENERALES

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10. CONCLUSIONES GENERALES

En general, los resultados obtenidos en esta Tesis muestran que:

- Todos los **frutos** de morera estudiados mostraron propiedades físico-químicas muy prometedoras e interesantes, tanto para su consumo en fresco como para la industrialización.
- En general, los **frutos** de morera negra mostraron una mayor actividad antioxidante y un mayor contenido en compuestos fenólicos que los clones de morera blanca. Los **frutos** de morera poseen un alto contenido en compuestos fenólicos bioactivos, junto con un equilibrado contenido en proteínas y minerales.
- Las **hojas** de los clones de morera blanca y negra cultivadas en España son ricas en CQAs y flavonoles. Si comparamos los frutos de morera con las hojas, las hojas presentan un mayor contenido en compuestos fenólicos y una mayor actividad antioxidante. Como consecuencia, las hojas de morera se podrían utilizar para la elaboración de productos de morera por parte de la industria de la alimentación funcional.
- Los ácidos grasos más abundantes en los **frutos** de morera fueron linoleico, palmítico y oleico. El ácido linoleico, un ácido graso poliinsaturado esencial, dominó el perfil de ácidos grasos.
- En las **hojas** de morera se identificaron tentativamente un total de 31 compuestos en todos los clones ensayados, de los cuales 7 compuestos no se habían identificado anteriormente en la morera, siendo esta la caracterización fenólica más amplia de este material realizada hasta la fecha.
- En los **frutos** se identificaron tentativamente 64 compuestos fitoquímicos. Entre los compuestos detectados 21 han sido identificados por primera vez en los frutos de morera.

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11. INVESTIGACIONES FUTURAS

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11. INVESTIGACIONES FUTURAS

Remarcar que en esta Tesis Doctoral, a lo largo de la presente investigación solo se ha abordado el estudio de la caracterización físico-química y las propiedades del fruto y la hoja de la morera.

Esta Tesis deja abiertos varios campos de actuación, cabe destacar:

- Prospección y propagación del material vegetal, para su conservación y estudio en condiciones homogéneas.

- El estudio agronómico y desarrollo de técnicas de cultivo para la explotación de esta especie frutal.

- La utilización de sus frutos y hojas en la industria alimentaria (frutos que se pueden añadir en barritas de muesli y con las hojas se pueden realizar tés, etc.).

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12. REFERENCIAS BIBLIOGRÁFICAS

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