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Effect of regulated deficit irrigation and Spanish-style processing on the generation of phytoprostanes in extra virgin olive oil and green table olives

TESIS DOCTORAL

Jacinta Collado González

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Effect of regulated deficit irrigation and Spanish-style processing on the generation of phytoprostanes in extra virgin olive oil and green table olives

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Degree in Chemistry

UNIVERSITAS Miguel Hernández

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Effect of regulated deficit irrigation and Spanish-style processing on the generation of phytoprostanes in extra virgin olive oil and green table olives

Thesis presented by Lcda. Jacinta Collado González to qualify for Doctor (European Mention) degree from Miguel Hernández University of Elche

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APLICADA DEL SEGURA (CEBAS)

Arturo Torrecillas Melendreras, Profesor de Investigación del CSIC y Ángel Gil Izquierdo, Científico Titular del CSIC

Informan

Que la Tesis Doctoral titulada "***Effect of regulated deficit irrigation and Spanish-style processing on the generation of phytoprostanes in extra virgin olive oil and green table olives***", de la que es autora la Licenciada Jacinta Collado González, ha sido realizada bajo nuestra dirección y supervisión en el Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC).

En Murcia, a 1 de Octubre de 2015

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Que la Tesis Doctoral titulada "**Effect of regulated deficit irrigation and Spanish-style processing on the generation of phytoprostanes in extra virgin olive oil and green table olives**" ha sido realizada por la **Lcda. Jacinta Collado González** dentro del programa de doctorado de Recursos y Tecnologías Agroalimentarias.

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1. Doctoral thesis category and structure

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Category

This doctoral thesis corresponds to a **compendium of publications category** selected to qualify for **Doctor** (**European Mention**) degree from Miguel Hernández University of Elche.

To this end, the selected papers are indicated as follows:

- **Collado-González, J.**, Durand, T., Ferreres, F., Medina, S., Torrecillas, A., Gil-Izquierdo, A. 2015. Phytoprostanes. *Lipid Technology* 27: 127-130.
- **Collado-González, J.**, Medina, S., Durand, T., Guy, A., Galano, J. M., Torrecillas, A., Ferreres, F., Gil-Izquierdo, A. 2015. New UHPLC–QqQ-MS/MS method for quantitative and qualitative determination of free phytoprostanes in foodstuffs of commercial olive and sunflower oils. *Food Chemistry* 178: 212-220.
- **Collado-González, J.**, Pérez-López, D., Memmi, H., Gijón, M. C., Medina, S., Durand, T., Guy, A., Galano, J.-M., Ferreres, F., Torrecillas, A., Gil-Izquierdo, A. 2015. Water deficit during pit hardening enhances phytoprostanes content, a plant biomarker of oxidative stress, in extra virgin olive oil. *Journal of Agricultural and Food Chemistry* 63: 3784-3792.
- **Collado-González, J.**, Pérez-López, D., Memmi, H., Gijón, M. C., Medina, S., Durand, T., Guy, A., Galano, J.-M., Fernández, D. J., Carro, F., Ferreres, F., Torrecillas, A., Gil-Izquierdo, A. 2015. Effect of the season on the free phytoprostanes content in Cornicabra extra virgin olive oil from deficit irrigated olive trees. *Journal of the Science of Food and Agriculture* DOI: 10.1002/jsfa.7259.
- **Collado-González, J.**, Moriana, A., Girón, I. F., Corell, M., Medina, S., Durand, T., Guy, A., Galano, J.-M., Valero, E., Garrigues, T., Ferreres, F., Moreno, F., Torrecillas, A., Gil-Izquierdo, A. 2015. The phytoprostane content in green table olives is influenced by Spanish-style processing and regulated deficit irrigation. *LWT-Food Science and Technology* 64: 997-1003.

Structure

The content of this thesis has been prepared in agreement with the internal regulations of the Miguel Hernández University of Elche for the presentation of **Doctoral Thesis (European Mention) as a compendium of publications**. So, this thesis presents structure as follows:

- **Abbreviations and symbols**, where the abbreviations and symbols used in the work are explained.
- **Abstract and Resumen,** where the most significant results and conclusions are exposed.
- **Introduction and Objectives**, where the research performed is presented and all the partial objectives of the research are detailed.
- **Material and methods**, where the agronomic characteristics of the experimental plots are indicated, as well as all the methodologies to determine the plant water status and the physical and chemical characteristics of the raw and processed olives and olive oils.
- **Publications**, where five peer reviewed papers are included
  - The first publication (*Lipid Technology* 27: 127-130) presents a review on the state of the art of the analysis of phytoprostanes (PhytoPs) and its occurrence in plant-derived foodstuffs.
  - In the second publication (*Food Chemistry* 178:212-220) a sensitive, selective and robust method was stablished for the determination and quantification of D_1-, F_1-, B_1- and L_1-PhytoPs and their respective regioisomers.
  - In the third publication (*Journal of Agricultural and Food Chemistry* 63: 3784-3792) the effect of water deficit during

pit hardening on the PhytoPs content in Cornicabra extra virgin olive oil was studied.

- The fourth publication, accepted for publication in the *Journal of the Science of Food and Agriculture* (DOI: 10.1002/jsfa.7259), shows the effect of the season on the free PhytoPs content in Cornicabra extra virgin olive oil.
- The fifth publication (*LWT-Food Science and Technology* 64: 997-1003) was focused on the study of the effect of water deficit and Spanish-style olive processing on the PhytoPs content in Manzanilla de Sevilla olives.

- **Results and Discussion**, where the most interesting and important results are analyzed and discussed.
- **Conclusions**, where the final conclusions are listed.
- **References**, where the references used in sections complementary to the publications are indicated.
- **Appendix**, where quality indexes of each publication are exposed.

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2. Abbreviations and symbols

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Abbreviations

AA	Arachidonic Acid
ALA	α-linolenic acid
BIS-TRIS	Bis (2-hydroxyethyl)amino-tris(hydroxymethyl)methane
cps	Counts per second
cv	Cultivar
DOY	Day of the year
ELISA	Enzyme-Linked ImmunoSorbent Assay
ESI	Electrospray ionization
ETc	Crop evapotranspiration (Crop irrigation requirements)
ETo	Crop reference evapotranspiration
EVOO	Extra Virgin Olive Oil
FAO	Food and Agriculture Organization of the United Nations
FDA	The US Food and Drug Administration
FW	Average fruit weight
GC-MS	Gas Chromatography coupled to Mass Spectrometry
HPLC	High Performance Liquid Chromatography
HPLC–UV	High Performance Liquid Chromatography coupled to UV detector
ICH	International Conference on Harmonization
IS	Internal standard
IsoPs	Isoprostanes
IUPAC	The International Union of Pure and Applied Chemistry
Kc	Crop coefficient
LA	Linoleic acid

LC-MS	Liquid Chromatography coupled to Mass Spectrometry
LOD	Limit Of Detection
LOQ	Limit Of Quantification
MDA	Malondialdehyde
MDS	Maximum Daily Trunk Shrinkage
MPa	Megapascal
MRM	Multiple Reaction Monitoring
MS	Mass Spectrum
MSE	Mean Square Error
MUFA	Monounsaturated Fatty Acid
m/z	Mass/charge ratio
NICI	Negative-Ion Chemical Ionization
nd	Not detected
ns	non-significant
OA	Oleic Acid
OS	Oxidative Stress
PhytoPs	Phytoprostanes
PG	Prostaglandin
PUFA	Polyunsaturated Fatty Acid
QqQ	Triple quadrupole mass spectrometry
RDI	Regulated Deficit Irrigation
ROS	Reactive Oxygen Species
RSD	Relative Standard Deviation
SD	Standard Deviation
SE	Standard Error

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S/N	Signal/Noise ratio
SPE	Solid Phase Extraction
T0	Control treatment
T1	Treatment 1
T2	Treatment 2
T3	Treatment 3
TDF	Trunk Diameter Fluctuations
TGR	Trunk Growth Rate
UHPLC	Ultra-High Performance Liquid Chromatography

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Symbols

b	Stem potential threshold
8-isoPGF2α-d4	8-iso-Prostaglandin $F_{2\alpha}$ containing four deuterium atoms
$[M-H]^-$	Deprotonated molecular ion
n	Number of the days
PPAR-γ	Peroxisome Proliferator-Activated Receptor gamma
RSD	Relative Standard Deviation
r^2	Coefficient of determination
SI ($S_{\Psi_{stem}}$)	Water Stress Integral
T_m	Daily mean Temperature
VPD_m	Mean daily air Vapour Pressure Deficit
$\bar{\Psi}_{stem}$	Average Ψ_{stem} values
Ψ_{stem}	Stem water potential

3. Scientific production during the PhD student period

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3.1. Directly related with the PhD thesis

3.1.1. Peer-reviewed papers

Collado-González, J., Medina, S., Durand, T., Guy, A., Galano, J.-M., Torrecillas, A., Ferreres, F., and Gil-Izquierdo, A. 2015. New UHPLC-QqQ-MS/MS method for quantitative and qualitative determination of free phytoprostanes in foodstuffs of commercial olive and sunflower oils. *Food Chemistry* 178: 212-220

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4. Resumen and Abstract

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Resumen

Este estudio se enfocó hacia el desarrollo de una nueva metodología para la identificación y cuantificación de fitoprostanos (PhytoPs) en materiales de origen vegetal y utilizarla como herramienta de estudio en la respuesta del olivo (*Olea europaea* L.) al déficit hídrico durante el endurecimiento del hueso y más concretamente al contenido de los mencionados compuestos en el aceite de oliva virgen extra (EVOO) Cornicabra y en las aceitunas de mesa Manzanilla de Sevilla. Adicionalmente, se estudió el efecto de la alternancia productiva del olivo y la influencia del procesado de las aceitunas de mesa al estilo español sobre el nivel de PhytoPs libres en los dos productos comerciales estudiados.

Probablemente, debido a la novedad de los fitoprostanos como marcadores de estrés oxidativo no existía ninguna metodología analítica capaz de evaluar estos compuestos de forma individual. Por ello, el primer paso fue el diseño de un método de extracción y la puesta a punto de una plataforma analítica robusta y reproducible para su determinación. La metodología analítica propuesta para la identificación y cuantificación de PhytoPs se basó en la utilización de equipos UHPLC–QqQ-MS/MS. La recuperación proporcionó altas eficiencias de extracción (del orden de 102.90 - 140.64 %) mediante el uso de cartuchos de extracción en fase sólida Strata-XAW. Las variaciones de los compuestos objeto de estudio dentro de un mismo día o entre días consecutivos osciló entre el 2.24 % y el 13.64 % y entre el 0.01 % y el 13.69 %, respectivamente. A este respecto, la precisión osciló entre el 80.33 % y el 119.64 % y entre el 80.34 % y el 119.90 %, respectivamente.

El perfil de PhytoPs en el EVOO se caracterizó por la presencia de 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, 9-D_{1t}-PhytoP, 16-B_1-PhytoP + *ent*-16-B_1-PhytoP, y 9-L_1-PhytoP + *ent*-9-L_1-PhytoP. Las diferencias cualitativas y cuantitativas respecto del contenido de PhytoPs según otros autores podría deberse al efecto decisivo de la variedad, la

metodología de extracción del aceite y/o condiciones de almacenamiento que son susceptibles a la auto-oxidación.

El déficit hídrico durante el endurecimiento del hueso o un periodo algo más prolongado (dos semanas) además de proporcionar el consiguiente ahorro de agua resultó claramente crítico para la composición del EVOO y la pulpa de la aceituna de mesa debido al aumento del contenido de PhytoPs libres, con el potencial efecto beneficioso sobre la salud humana. La variabilidad interestacional en el contenido de PhytoPs en el EVOO puede ser el resultado de una compleja interacción entre múltiples factores no siempre interrelacionados.

El perfil de PhytoPs en la pulpa de la aceituna de mesa se caracterizó por la presencia de 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, 16-B_1-PhytoP y 9-L_1-PhytoP. El procesado 'estilo español' de estas aceitunas aumentó el contenido total de PhytoPs, aunque sólo se detectaron 9-F_{1t}-PhytoP and 9-*epi*-9-F_{1t}-PhytoP. En consecuencia, el riego deficitario durante el endurecimiento del hueso y el procesado 'estilo español' de la aceituna de mesa pueden considerarse acciones complementarias para aumentar el contenido de PhytoPs y consecuentemente sus potenciales efectos beneficiosos en los consumidores.

Abstract

The purpose of the present study was to develop an analytical method that allowed the identification and quantification of phytoprostanes (PhytoPs) as a tool to deepen our knowledge of the effect of regulated deficit irrigation on free PhytoP content of Cornicabra extra virgin olive (*Olea europaea* L.) oil (EVOO) and Spanish-style processed Manzanilla de Sevilla table olives. For this, the effect of water deficit during olive pit hardening on the PhytoP profile and content of Cornicabra extra virgin olive oil and Manzanilla de Sevilla green table olives was studied. Also, the effect of the alternate bearing pattern of olive and 'Spanish style' olive processing on free PhytoPs in extra virgin olive oil and olive flesh were studied. Due to the novelty of the free PhytoPs as oxidative stress biomarkers did not exist any previous analytical methodology able to quantify each individual PhytoP. For this, as a preliminary step we propose a new quick and accurate analytical method using UHPLC–QqQ-MS/MS which is able to identify free phytoprostane in plant-derived foodstuffs. The recovery provided high extraction efficiencies ranging from 102.90 % to 140.64 % using a Strata-XAW cartridge. The intra-day and inter-day variations for all target compounds ranged from 2.24 % to 13.64 % and 0.01 % to 13.69 %, respectively, and the accuracies for these parameters varied from 80.33 % to 119.64 % and from 80.34 % to 119.90 %, respectively. The PhytoPs profile in EVOO was characterized by the presence of 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, 9-D_{1t}-PhytoP, 16-B_1-PhytoP + *ent*-16-B_1-PhytoP, and 9-L_1-PhytoP + *ent*-9-L_1-PhytoP. The qualitative and quantitative differences in the PhytoP content with respect to that reported by other authors point to the decisive effect of cultivar, oil extraction technology, and/or storage conditions on autoxidation. Deficit irrigation during pit hardening or during the same period plus an extra two weeks, besides increasing irrigation water saving, is clearly critical for EVOO and raw table olive flesh composition because it enhances the content of free PhytoPs, which have potential beneficial

aspects for human health. An important interseasonal change in the PhytoP content in EVOO was observed, probably due to a very complex multivariate interaction between factors that are not always interrelated. The PhytoP profile in the raw table olive flesh was characterized by the presence of 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, 16-B_1-PhytoP and 9-L_1-PhytyoP. The PhytoPs content in the ‘Spanish style’ processed olive flesh was enhanced. After olive fruit processing only 9-F_{1t}-PhytoP and 9-*epi*-9-F_{1t}-PhytoP with enhanced levels. Consequently, table olive tree culture under deficit irrigation conditions during pit hardening and the processing of its fruits to obtain Spanish-style olives can be considered as complementary actions to enhance the PhytoP content and hence their potential beneficial effects on human health.

5. Introduction. Phytoprostanes

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Feature

Phytoprostanes

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Summary

Phytoprostanes are non-enzymatic lipid peroxidation products derived from α-linolenic acid. Phytoprostanes are not essential for the metabolic activity of living cells but they are considered components of oxidative injury-sensing systems and act as excellent biomarkers of oxidative degradation in plant-derived foodstuffs. They can be quickly and accurately quantified by UHPLC/MS-MS. Specific agronomic and industrial practices can enhance the phytoprostanes content of fruits and seeds and derived vegetable oils. In vitro studies have shown positive biological activities of these compounds and according to existing reports they are orally bioavailable. Further studies are required to comprehensively study the bioavailability and physiological effects of the phytoprostanes.

Introduction

In humans and other mammals, oxidative stress is associated with the pathogenesis of several chronic diseases. High levels of reactive oxygen species (ROS) overwhelm the antioxidant defences in the organism and lead to the oxidative damage of lipids, proteins and nucleic acids. However, these types of ROS are also generated in secondary plants. In particular, one of the free radical attacks is against fatty acids. When the oxidative reaction of ROS is directed against α-linolenic acid (ALA), the predominant polyunsaturated fatty acid (PUFA) in plants, phytoprostanes (PhytoPs) are formed in plant tissues. The PhytoPs levels can increase when plants are subjected to different types of oxidative stress [1]. The most interesting aspect of PhytoPs is that they mimic the structure of other eicosanoids such as isoprostanes (IsoPs) and prostanoids (prostaglandins and thromboxanes), compounds generated by non-enzymatic free radical attack of arachidonic acid in humans. Eicosanoids perform a wide range of biological actions in the human body, from pernicious effects (platelet aggregation, vasoconstriction) to beneficial effects related to the defense-physiological balance of the body [2]. This fact could lead to the discovery of new properties of phytoprostanes related to different physiological effects and disorders of the human body.

Structure and nomenclature

As shown in **Figure 1**, the triene unit is the principal building block for all PhytoP ring systems. The substitutes at the triene unit are the methyl and carboxyl terminus of the fatty acid. The methyl terminal chain at each distinct triene unit can occupy position R_1 or R_2 of the PhytoP, and, thus, two types of regioisomers can be produced per triene unit.

Different nomenclature systems have been proposed to identify the structural PhytoPs isomers unambiguously [3]. Initially, Taber and Roberts [4] suggested a nomenclature which was approved by IUPAC and later another nomenclature was proposed by Rokach [5]. This latter nomenclature has the advantage that the biosynthetic

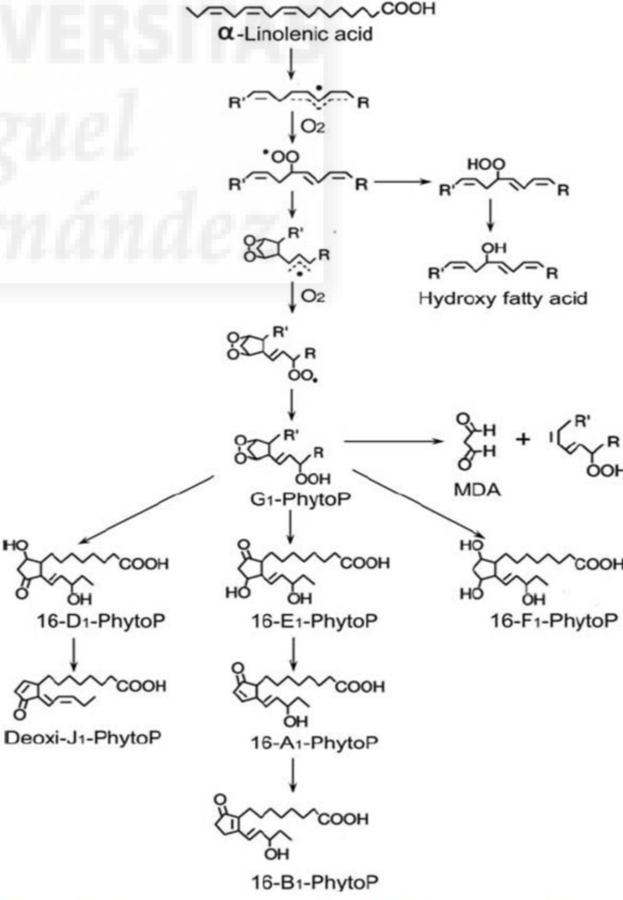

Figure 1. Non enzymatic formation of PhytoPs from ALA.

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Table 1. Denomination of four PhytoPs according to the three nomenclature systems.

PhytoP	Taber/Roberts [3]	Rokach [5]	Mueller [1]
	9-F_{1t}-PhytoP	Dinor-i$PF_{1\alpha}$-II	PPF_1 Type II
	9-D_{1t}-PhytoP	Dinor-i$PE_{1\alpha}$-II	PPE_1 Type II
	16-B_1-PhytoP	Dinor-i$PB_{1\alpha}$-I	PPB_1 Type I
	9-L_1-PhytoP	Dinor-i$PB_{1\alpha}$-II	PPB_1 Type II

heritage of the structure is coded by the type number. The ring substitution patterns are considered in both systems following the established prostaglandin nomenclature. Nevertheless, the main differences between both nomenclatures are based on the different lengths of the side chain, the positions of the side chain hydroxyl group and the relative stereochemistry (**Table 1**). Later, Mueller proposed a modified Rokach nomenclature [1] which does not consider the relative side chain carboxyl group to carbonyl group relationship in the molecule as the classical prostaglandin, Taber/Roberts, IUPAC and Rokach nomenclatures do.

The main characteristic of the Mueller system is that the classification of PhytoPs considers only one common ring substitution pattern (in which the hydroxyl allylic fragment is included) and changes in the resulting side chains. This procedure leads to different names for the same structure with respect to the ring substitution to side chain relationship (**Table 1**). Unfortunately, this is the reason for uncertainty concerning the correct assignment of PhytoP structures which can lead to confusion about structure-activity relationships if different nomenclature systems are used [3].

The importance of correct naming of the individual chemical structures is evident, since the chemical properties of the individual entities may vary considerably. In this sense, although E-, A-, B-, D- and J-PhytoP from Type I can be named systematically and unambiguously, there are important exceptions among Type II phytoprostanes where confusion exists concerning the structures. Here the two nomenclature systems can lead to different structures based on a name, and vice versa to a different name based on the given structure. Following careful analysis, it was found that the PhytoPs with structure PPB_1, Type II according to the Mueller nomenclature, represents a so far unknown ring substitution pattern for PhytoP in the Taber/Roberts framework. It should result from dehydration and base-catalyzed isomerization of D_1-PhytoP. Therefore, the authors Jahn et al. proposed to term this new ring substitution pattern as L_1-PhytoP [3].

Formation mechanisms

The PhytoP pathway in plants involves a non-enzymatic lipid peroxidation, which is initiated by the attack of reactive oxygen species (ROS) on α-linolenic acid (C18:3, n-3, ALA), yielding a linolenate radical that readily oxidizes and cyclizes to complex isomeric mixtures of two regioisomeric classes (9-, and 16-series) of PhytoPs (**Figure 1**). ROS are generated as a consequence of oxidative stress (OS) and are established as plant signals that may induce their defense genes through different mechanisms.

Linolenate radical reacts with an oxygen molecule, forming a linolenate peroxyl radical. The ALA peroxyl radical can also react internally, forming a cyclic peroxyl radical, which spontaneously reacts with a second oxygen molecule and is subsequently reduced to G_1-PhytoP. This PhytoP spontaneously decays, forming MDA (malondialdehyde) and other alkanes and alkenes, or forms other PhytoPs. Lipid peroxides are converted to their corresponding hydroxy fatty acid either enzymatically or spontaneously. Rearrangement to D- and E-classes and subsequently J- and A-classes, respectively, becomes predominant in aqueous medium and when the reducing capacity declines [6]. It is important to realize that PhytoPs are formed as region- and stereoisomeric mixtures, and in addition to this structural manifold PhytoP are produced with diverse ring substitution patterns. Moreover, the only requirement for PhytoP formation is the presence of linolenic acid and molecular oxygen, suggesting that PhytoP formation does not necessarily require the metabolic activity of living cells [6, 7].

PhytoPs are plant analogues of isoprostanes (IsoPs), since both are produced non-enzymatically from PUFAs. PhtoPs and IsoPs

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present some differences in structure and synthesis due to the fact that IsoPs are produced in abundance in humans by the attack of (ROS) on arachidonic acid (C20:4, n-6, AA) and higher plants do not generally possess the enzymatic capacity to synthesize AA that is required for IsoPs formation but they produce ALA which is required for generating PhytoPs.

In earlier reports on the analysis of PhytoPs different analytical methods were employed including gas chromatography coupled to mass spectrometry (GC-MS), high performance liquid chromatography (HPLC) coupled to a fluorescence detector, and nuclear magnetic resonance of ^{1}H or ^{13}C as well as immunological approaches (ELISA) [8]. All these methods of quantification of PhytoPs involved a tedious analytical process and the purification of PhytoPs starting from the autoxidation of α-linolenic acid, which may yield not only a number of different classes, but also high amounts of PhytoP [9]. In addition, the sensitivity and selectivity of these methods were not very high.

Recently, a new quick and accurate analytical method, which is more sensitive and more selective, has been developed. The method is able to identify different classes of PhytoPs, including their regioisomers, in a single injection using ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry (UHPLC/MS-MS). The main advantages of this analysis are that it involves a single assay per sample and only requires 8 minutes for the results to be obtained, meaning that less solvent is used, with the corresponding saving of money [9].

Occurrence

The major targets of free radical attack in plants are membrane lipids. One of the most abundant PUFAs in terrestrial higher plant membranes is ALA from which PhytoPs are produced by autoxidation. Since PhytoPs show some similarity to IsoPs and prostaglandins in humans, there is growing interest in studying PhytoP profile and levels in plant foods and their response to cultural practices and/or environmental conditions.

Since 1998 a large variety of plant species and foodstuffs have been analysed for their PhytoP content. Several classes (A_1-, B_1-, D_1-, E_1-, F_1-and deoxy-J_1-PhytoPs class) of PhytoPs were found in *Arabidopsis thaliana* (leaves), *Betula pendula* (birch; leaves and pollen), *Brassica napus* (rape; seed oil), *Glycine max* (soya bean; seed oil and margarine), *Helianthus annuus* (sunflower; seed oil), *Hypericum perforatum* (saint johnswort), *Juglans regia* (walnut; seed oil), *Linum usitatissimum* (flax; seed oil), *Lycopersicon esculentum* (tomato; leaves), *Mentha piperita* (peppermint, leaves), *Nicotiana tabacum* (tobacco; leaves), *Olea europeae* (olive; fruit, flesh oil), *Prunus dulcis* (almond; kernel), *Rauvolfia serpentina* (Indian snakeroot; cell culture), *Salix alba* (white willow; leaves), *Tilia cordata/platyphyllos* (lime; tree flowers), *Valeriana officinalis* (valerian; root), and *Vitis vinifera* (grape; seed oil) [10].

Data collected during the early years suggested that D_1-PhytoP and J_1-PhytoP were the most prominent PhytoPs in many plant species. However, it has recently been shown that this is not always the case since in other fruits (e.g. olives or almonds), F_1-PhytoPs are the predominant PhytoPs. Moreover, it has been demonstrated that some PhytoPs can be transformed into others PhytoPs during storage or processing. Immediately after harvest, table olive fruits are processed to obtain the so called 'Spanish-style' or 'Seville-style' olives. During processing the PhytoPs profile changes, favouring the formation of 9-F_{1t}-PhytoP and 9-*epi*-9-F_{1t}-PhytoP and the degradation of the 16-B_1-PhytoP and 9-L_1-PhytoP.

It is important to take into account the effect of cultural practices management on the PhytoPs content of the crop. As an example, deficit irrigation during olive pit hardening (phase II of fruit growth) is not critical for yield or fruit size, but is clearly critical for the PhytoPs content which increases in the olive flesh. In this sense, the increase in PhytoPs depend more on the duration of water stress than on the level of stress achieved. Moreover, the pit hardening period is also clearly critical for extra virgin olive oil composition because water deficit enhances the PhytoPs content. However, the response of each PhytoP to water deficit is not homogeneous. For this reason, 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP and the 16-B_1-PhytoP have been proposed as early biomarkers of water stress in olive tree.

Important qualitative and quantitative differences in the PhytoP content are found when freshly extracted vegetable oils are compared, due to the effect of plant species, cultivar, extraction technology and/or the storage conditions, which may induce autoxidation. For example, refined sunflower oil contains a higher amount and a larger number of PhytoPs than two types of olive oil (extra virgin olive oil and olive oil). The manufacturing process used could be the key for these different PhytoPs levels since most plant oils are subjected to a refining treatment. Also, the characteristic alternate bearing pattern of olive tree may be responsible for the inter-seasonal variability of the PhytoPs content in extra virgin olive oils.

In short, some agronomic and industrial practices may affect the PhytoPs content of fruits and derived vegetable oils, and hence their potential beneficial effects on human health.

Functions in plants and humans

Traditionally, the products of non-enzymatic lipid peroxidation have been considered as toxic waste or as by-products of aerobic metabolism. Nevertheless, PhytoPs are formed continuously, accumulate during oxidative stress, are highly biologically active and can be rapidly inactivated.

Although it has been reported that PhytoPs have a wide range of biological activities in plant species, their exact role and function in plant physiology remains to be elucidated in detail. Thoma et al. [10] proposed a model in which PhytoPs are components of an oxidant injury-sensing, archaic signaling system that serves to protect plants from stress associated with increased free radical production. Moreover, PhytoPs are not only considered as components of an oxidant injury-sensing system but also excellent biomarkers of oxidative degradation in plant derived foodstuff.

The best studied compounds are the cyclopentenone-PhytoP of the A and B classes. Exogenous application of B_1-PhytoP regulates gene expression in *Arabidopsis thaliana* and *Nicotiana tabacum*. Some of these genes have been related to an adaptive response that would provide the plant with a survival advantage through detoxification, stress responses and pathways not associated with chloroplasts or photosynthesis. It is necessary to highlight the fact that a stimulatory effect of B_1-PhytoP on phytoalexin accumulation has been found for multiple classes of phytoalexins in a variety of plant species [6, 10].

As regards the bioavailability, metabolism and biological effects of these compounds in humans, it should be noted that they can reach the gastrointestinal tract and interact with the gut microflora after the intake of the foodstuff. However, the most important thing prior to *in vivo* assays is to evaluate their potential bioactivities, bioavailability, absorption and metabolism. A clinical trial with healthy volunteers whose diet was supplemented with flaxseed and olive oils was able to detect PhytoPs in plasma and urine [11].

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Their concentration was relatively high and derived from their presence in the oils, although some authors also suggest that they may also be generated at a systemic level in the body from ALA. Regarding the circulating forms of phytoprostanes, they are esterified in plasma [11] and probably they can follow the same metabolic pathway as eicosanoids, that means, conjugation with glucuronic acid and/or sulphate. However, this point has not been confirmed in any study yet.

Little is known of their biological effects, due to the fact that they are not commercially available, although there is some evidence about the role of some PhytoPs in the regulation of the immune function in humans. E_1-PhytoP is one of the most abundant PhytoP in pollen and is able to favour the immune response by the modulation of dendritic cells [12]. A subsequent work corroborated the immunomodulatory effects of E_1-PhytoPs and also added new information about the activity of the F_1-PhytoPs in *in vitro* studies [12]. Recently, Minghetti et al [13] reported that the 16-B_1-PhytoPs, through novel mechanisms involving PPAR-γ, can specifically affect immature brain cells, such as neuroblasts and oligodendrocyte progenitors [13]. While research into cardiovascular and circulation diseases involving PhytoPs is quite limited, one promising *in vitro* study demonstrated the reversible aggregation of 16-F_{1t}-PhytoP caused by a stable analog of the endoperoxide prostaglandin H_2 exhibiting properties similar to thromboxane A_2 (TXA_2) (U46619) [14]. However, further studies are required, especially with animal models, and clinical trials in humans using the natural dietary sources of PhytoPs, including processed food products like vegetable oils, to evaluate the real effect of these plant compounds on human health.

Conclusions

PhytoPs are non-enzymatic lipid peroxidation products derived from ALA that are not necessary for the metabolic activity of living plant cells. They are not only considered as components of oxidant injury-sensing systems but can also act as excellent biomarkers of oxidative degradation of plant-derived foodstuffs. It is important to note that certain agronomic and industrial procedures may enhance the PhytoPs content of fruits and derived vegetable oils. In this way, it may be possible to "functionalize" plant products with higher concentrations of these compounds, which are readily bioavailable and absorbed by the human body. Little is known of their biological effects, although there is some *in vitro* evidence about the role of some PhytoPs in the regulation of the immune function, against certain type of cancers and of vascular effects. However, further studies are required in animal models and clinical trials in humans, using the natural dietary sources of PhytoPs and processed food products like vegetable oils to evaluate the circulating forms in the human body after absorption and metabolism. Their pharmacokinetic behaviour also needs to be studied, along with the real importance of these plant compounds in human health.

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6. Objectives

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The overall objective of the present study aimed to develop an analytical method that allows the **identification and quantification of phytoprostanes (PhytoPs)** to be used as a tool to deepen our knowledge of the **effect of regulated deficit irrigation on the free PhytoP content of extra virgin olive oil and Spanish-style processed olives**

The following partial objectives were addressed:

- *To develop a rapid, reliable and efficient analytical method that allows the identification and quantification in the same chromatogram of F_1-, D_1-, B_1-, L_1-PhytoPs and their regioisomers in olive oil, using ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry (UHPLC/MS-MS).*

- *To increase knowledge of the PhytoP profile present in Cornicabra extra virgin olive oil, as a plant biomarker of oxidative stress in the olive tree.*

- *To study the effect of different water deficit levels during olive pit hardening on free PhytoP levels, and assess whether a longer water deficit during olive oil accumulation (just after pit hardening) is able to enhance free PhytoPs accumulation.*

- *To evaluate the effect of the olive alternate bearing pattern on the profile and contents of free PhytoPs in Cornicabra extra virgin olive oil.*

- *To look at the effect of deficit irrigation during early fruit growth (phase I) or pit hardening (phase II) on the qualitative and quantitative profile of PhytoPs in green table olive flesh.*

- *To evaluate whether the elaboration process of Manzanilla de Sevilla Spanish style green table olives modifies the PhytoPs content.*

7. Materials and Methods

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In this section the main characteristics of the experimental conditions, irrigation treatments, analytical methodologies, statistical design and analysis, etc. used in this thesis are included. For more detailed aspects the papers that constitute this thesis can be consulted.

Plant Material, experimental conditions and treatments

El Chaparrillo experimental farm

El Chaparrillo is located in Ciudad Real (Spain) (39º00'N, 3º56'W; altitude 640 m). The soil is an alkaline (pH 8.1) and shallow Petrocalcic Palexeralfs with a clay loam texture, low electrical conductivity (0.2 dS m^{-1}), organic matter (1.05 %), nitrogen (0.12 %) and potassium (17×10^{-4} mol kg^{-1}) levels and high cationic exchange capacity (0.186 mol kg^{-1}) level (Picture 7.1) . A discontinuous petrocalcic horizon is located between 0.75 and 0.85 m. The soil volumetric water content for the first 0.3 m depth is 22.8 % at field capacity (soil matric potential - 0.03 MPa) and 12.1 % at permanent wilting point (soil matric potential - 1.5 MPa), and from 0.3 to 0.75 m it was 43.0 and 21.1 %, respectively.

Picture 7.1. Soil of the El Chaparrillo experimental plot

The orchard was managed under *no tillage* conditions; weeds were controlled with post-emergence herbicides. Pest control and fertilization practices were those usually used by local growers.

The plant material consisted of olive trees (*Olea europaea* L., cv Cornicabra) planted in 1998 and spaced 7 m x 4.76 m (300 trees ha^{-1}) (Picture 7.2). The first crop yield (more than 5 kg per tree) was obtained in 2003.

Picture 7.2. Olive (*Olea europaea* L., cv Cornicabra) trees at El Chaparrillo experimental plot

Irrigation was carried out daily and during the night using a drip irrigation system with four emitters (each delivering 8 l h^{-1}) per tree and irrigation water with an electrical conductivity of 2.6-2.9 dS cm^{-1}. Crop irrigation requirements (ETc) were estimated according to daily crop reference evapotranspiration (ETo), calculated using the Penman–Monteith equation (Allen et al., 1998), and a crop factor based on the time of the year (Orgaz and Fereres, 2004) and taking into consideration canopy size (Fereres and Castel, 1981).

Control plants (T0 treatment) were irrigated at 100 % ETc of the previous week. When midday stem water potential (Ψ_{stem}) values of that week were below -1.2 MPa before pit hardening (phase I of fruit growth) or -1.4 MPa during and after pit hardening (phases II and phase III of fruit growth, respectively) irrigation amounts were increased by 10 % in order to prevent irrigation-related stress (Moriana et al., 2012). In addition to T0, two regulated deficit irrigation (RDI) treatments (T1 and T2) were applied, based on avoiding water deficit during phase I and III of fruit growth, but maintaining Ψ_{stem} values around the threshold values indicated for T0 plants. This saved irrigation water during the non-critical phenological period of pit hardening (phase II), developing different situations of water deficit (threshold Ψ_{stem} values of -2.00 and -3.00 MPa in T1 and T2 plants, respectively). In 2013, a fourth treatment (T3 treatment) was also performed, which was based on an irrigation protocol similar to that used for T2, except that the Ψ_{stem} threshold value of -3.00 MPa was used from the beginning of phase II to 15 days after the end of phase II.

The irrigation protocol in T1, T2 and T3 plants was based on that proposed by Moriana et al. (2012). Irrigation requirements were determined weekly based on the Ψ_{stem} measurements and irrigation began when measured Ψ_{stem} values were lower than the threshold values suggested.

During the 2012 and the 2013 irrigation seasons (day of the year, DOY, 134-273 and DOY 148-273, respectively), ETc were 202 and 183

mm, respectively. Cumulative amounts of applied water in T0, T1 and T2 treatments, measured by means of flow meters integrated in the irrigation system, were 407, 196 and 141 mm in the 2012 season and 338, 164 and 112 mm in the 2013 season, whereas irrigation water amounts applied in T3 treatment (2013) was 88 mm.

La Hampa experimental farm

La Hampa is the experimental farm of the IRNAS-CSIC (Sevilla), which is located at the southwest of Spain (37º 17' N, 6º 3' W, 30 m a.s.l). The soil is a sandy loam (Xerochrept) of a depth varying between 0.9 – 2 m with a quite homogeneous root zone with average values of 73.5 % coarse sand, 4.7 % fine sand, 7 % silt and 14.8 % clay. The organic matter content is between 0.6 and 1 %. The soil water contents at field capacity (-0.01 MPa) and wilting point (- 1.5 MPa) were 0.21 and 0.10, respectively. The orchard was managed under no tillage conditions; weeds were controlled with post-emergence herbicides. Pest control and fertilization practices were those usually used by local growers.

The plant material consisted of adult olive trees (*Olea europaea* L., cv Manzanilla de Sevilla) spaced 7 m x 5 m (Picture 7.3). Plants were irrigated daily during the night from mid-April to mid-September using a drip irrigation system with 5 emitters (each delivering 8 l h^{-1}) per tree and irrigation water with an electrical conductivity of 2.0 dS m^{-1}. Crop water requirements (ETc) were estimated taking into account crop reference evapotranspiration (ETo), crop coefficient (Kc) and the percentage of ground covered by the crop (Kr) (Fernandez et al., 2006).

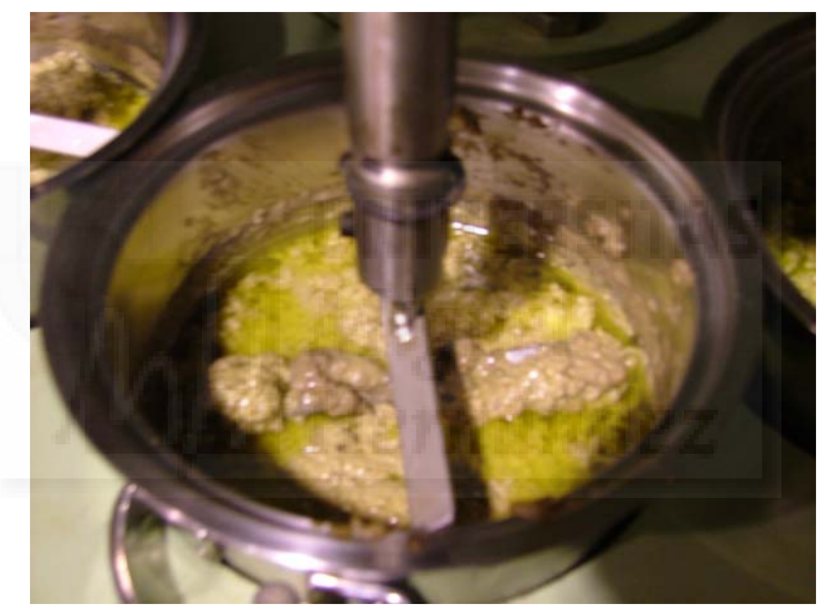
Control treatment trees (T0) were over-irrigated (125% ETc). T1 and T2 treatment trees were submitted to RDI according to trunk diameter fluctuations data (Moriana et al., 2013). In 2012 and 2013 seasons, before and after pit hardening, T1 plants were irrigated in order to maintain trunk growth rate (TGR) near to that of T0 trees. During 2012 pit hardening, T1 plants were irrigated to obtain maximum daily trunk shrinkage (MDS) values of around 90% of the MDS values observed in T0 plants. During

2013 pit hardening, T1 plants were irrigated only when TGR was lower than -10 µm day^{-1}. In both seasons, before the pit hardening period, T2 plants were irrigated in order to maintain TGR values at around the 50% of those observed in T0 plants. During pit hardening, T2 plants were irrigated in order to obtain MDS values at around the 75% of those observed in T0 plants. After pit hardening, T2 plants were irrigated in order to obtain TGR values similar to those observed in T0 plants.

Picture 7.3. Olive (*Olea europaea* L., cv Manzanilla de Sevilla) trees at La Hampa experimental plot.

Extra virgin olive oil extraction

Olive fruits were harvested in mid-December (DOY 345 in 2012 and DOY 346 in 2013). Extra virgin olive oil (EVOO) was extracted using an Abencor system and the oil obtained was separated by decanting (Picture 7.4). Samples for analysis were filtered and stored at -18 ºC in darkness using amber glass bottles without headspace until analysis.



Picture 7.4. Abencor system for extracting extra virgin olive oil.

Processing of green olives

In mid-September, when the olive fruits skin from each irrigation treatment had reached a yellowish green color (Hermoso et al., 2006) (Picture 7.5), the fruits were harvested and processed to obtain so-called ‘Spanish-style’ or ‘Seville-style’ olives (Sánchez et al., 2006).

Fruit samples were dressed in a table olive processing company (Cooperativa Las Virtudes, La Puebla de Cazalla, Seville, Spain) following the usual processing steps. Fruit processing began with a lye treatment (NaOH, 2-4 % (w/v)) for 6-12 h until 75% of the fruit flesh was dyed (IOOC,

1990) (Tarrado-Castellarnau et al., 2013). The degree of lye penetration in the fruit was checked by dying longitudinal fruits cuts with a solution of phenolphthalein. Then the fruits were soaked for 15 h to eliminate the lye and placed in brine (NaCl 10-12 % (w/v)) where natural lactic fermentation took place for around 7 months. This fermentation was controlled by periodically measuring pH, free acidity and the salt content and by the addition of acid (HCl) or lye (NaOH). The concentration of salt was increased when the lactic fermentation was completed (pH < 4) to improve the preserving process (IOOC, 1990). Then, the fruits (Picture 7.6) were packed with covering liquid (IOOC, 2004) in 2 kg plastic containers and stored at 4 ºC in darkness until analysis. PhytoP analyses were performed in the flesh of raw and treated olives and in the treated olive covering liquid from 2012 and 2013 harvests.

Picture 7.5. Manzanilla de Sevilla olive fruits at harvest.

Measurements

Climate and plant water status

Meteorological data, namely air temperature, solar radiation, air relative humidity, rainfall, and wind speed 2 m above the soil surface, were collected in a nearby automatic weather station. Mean daily air vapour

pressure deficit (VPDm) was calculated according to Allen et al. (1998). Daily ETo was estimated using the Penman-Monteith equation (Allen et al., 1998).

Picture 7.6. 'Spanish-style' or 'Seville-style' processed table olives

Midday (12 h solar time) stem water potential (Ψ_{stem}) was measured on the middle third of the trees, in fully developed leaves near the main trunk from two trees of each replicate, enclosing leaves in small black plastic bags covered with aluminium foil for at least 2 h before measurements in the pressure chamber. In order to describe the cumulative effect of the water deficit, the water stress integral ($S_{\Psi_{stem}}$) was calculated from the Ψ_{stem} data, using the expression proposed by Myers (1988). Moriana et al. (2012) suggested the use of stem potential threshold values of -1.2 MPa before the beginning of pit hardening and of -1.4 MPa from that moment to harvesting. This change improves the capacity of comparison between different experiments and/or locations. The expression used was:

$$S_{\Psi_{stem}} = |\sum(\overline{\Psi}_{stem} - b) \times n|$$

where $\overline{\Psi}_{stem}$ is the average Ψ_{stem} values for any interval, b is the stem potential threshold value and n is the number of the days in the interval.

Micrometric trunk diameter fluctuations (TDF) were measured using a set of linear variable displacement transducers (LVDT) (model DF ± 2.5 mm, accuracy ± 10 µm, Solartron Metrology, Bognor Regis, UK) attached to the main trunk of 6 trees per treatment (Moriana et al., 2013) with a special bracket made of invar and aluminium. Sensors were placed on the north side and measurements were taken every 2 s and the datalogger (model CR10X with AM25T multiplexer, Campbell Scientific Ltd, Logan, USA) was programmed to report 15 min means. MDS was calculated as the difference between maximum and minimum daily trunk diameter and TGR was calculated as the difference of two consecutive maximum daily trunk diameter values.

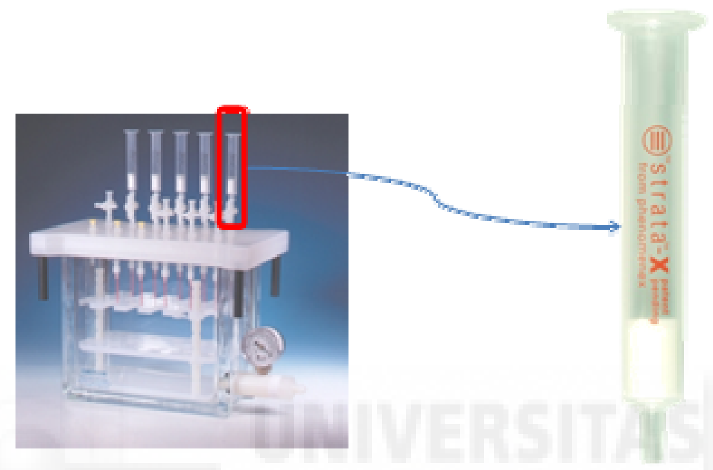
Fruit yield

Marketable olive fruits were harvested and the mean fruit weight was determined according to the weight and number of fruits per box in randomly selected boxes per replicate

Phytoprostanes extraction

PhytoPs were extracted from olive flesh, olive oil and brine. For this, 10 treated or raw olives were pressed for 30 s and 2 g of resulting material was crushed in a mortar containing 10 mL of a solution of MeOH and BHA (1 g butylhydroxyanisole/ 1 L MeOH) and transferred to a polypropylene tube. Then the sample was vortexed for 5 min and centrifuged for 10 min at 2000 x g and 4º C. The supernatant was collected and filtered through a Sep-pak. The PhytoPs in olive oil were extracted following a dilution and a solid-phase extraction (SPE). 1 mL of EVOO sample was dissolved in 10 mL of n-hexane and this solution was diluted in 2 mL of methanol, stirred and then rediluted in 2 mL of BIS-TRIS buffer (0.02M, HCl, pH=7) to

develop the liquid-liquid extraction. Immediately after, the emulsion of each sample (oil+hexane+BIS-TRIS buffer) was subjected to SPE using a Strata X-AW cartridge (100mg /3mL) (Picture 7.7), which was conditioned and equilibrated with 2 mL of hexane, 2mL of methanol and 2 mL of milliQ water prior to loading the oil samples.



Picture 7.7. SPE extraction using Strata X-AW cartridge

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Then, in order to remove undesired compounds, the cartridge was washed with a series of solvents in the following sequence: 2 mL of hexane, 2 mL of milliQ water, 2 mL of a solution of methanol/ milliQ water (1/3) and 2 mL of acetonitrile. Retained compounds were eluted with 1 mL of methanol, which was evaporated by using a SpeedVac concentrator (Savant SPD121P, Thermo Scientific, MA, USA). The residue of each sample was redissolved in 200 µL of a mixture of A/B solvents (90:10, v/v), using a mixture of milliQ water/ 0.01% acetic acid as solvent A and a solution of methanol/ 0.01% acetic acid as solvent B. Reconstituted samples were sonicated for 10 minutes and passed through a 0.45-µm filter (Millipore, MA, USA) before twenty microlitres of each were analyzed by UHPLC–QqQ-MS/MS (Agilent Technologies, Waldbronn, Germany).

Phytoprostanes identification and quantification

PhytoPs were analysed by reversed phase using UHPLC coupled

with a 6460 triple quadrupole-MS/MS (Agilent Technologies, Waldbronn, Germany) (Picture 7.8).

Picture 7.8. UHPLC coupled with a 6460 triple quadrupole-MS/MS (Agilent Technologies, Waldbronn, Germany)

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Chromatographic separation was carried out on a BEH C18 column (2.1x50 mm, 1.7 µm) (Waters, Milford, M. A.), the temperature of which was 6 °C (both, left and right sides of the column). The solvents used as mobile phase were water/acetic acid (99.99:0.01, v/v) (A) and methanol/acetic acid (99.99:0.01, v/v) (B). The MS analysis was applied in the multiple reaction monitoring (MRM negative) ESI mode. The injection volume was 20 µL and elution was performed at a flow rate of 0.2 mL/min. The linear gradient started with 60 % B at 0 min, 62 % B at 2 min, 62.5 % B at 4 min to reach 65 % B at 8 min, returning to the initial conditions at 8.01 min. In this way, the acquisition time was 8.01 min for each sample, with a post-run of 1.5 min for column equilibration. ESI conditions, and ion optics were as follows: gas temperature: 325 ºC, gas flow: 8 L min^{-1}, nebulizer: 30 psi, sheath gas temperature: 350 ºC, jetstream gas flow: 12 L min^{-1}, capillary voltage: 3000 V, nozzle voltage: 1750 V. Data acquisition and processing were performed using the MassHunter software version B.04.00 from Agilent Technologies. The MS analysis was applied in the multiple reaction monitoring negative ESI mode.

Statistical design and analysis

The design of the experiments at El Chaparrillo and La Hampa experimental farms were completely randomized with four replications, each replication consisting of five adjacent tree rows, each of nine trees. Measurements were taken on the inner trees of the central row of each replicate, which were very similar in appearance (leaf area, trunk cross sectional area, height, ground shaded area, etc.), while the other trees served as border trees. Data were analyzed using SPSS software (SPSS, 2002). Analysis of variance was performed and mean values were compared by $Tukey_{0.05}$ test. Values for each replicate were averaged before calculating the mean and the standard error of each treatment.

To check the regression model hypothesis (linearity, homoscedasticity, normality and independency) Kolmogorov–Smirnov was used with the Liliefors correction and the Shapiro–Wilk tests for normality and the Levene test for homoscedasticity on the typified residuals. For the Levene test, data were divided into two groups according the data for the median of the abscissa.

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8. Publications

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Food Chemistry

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Analytical Methods

New UHPLC–QqQ-MS/MS method for quantitative and qualitative determination of free phytoprostanes in foodstuffs of commercial olive and sunflower oils

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ABSTRACT

In this work, we propose a new quick and accurate analytical method by UHPLC–QqQ-MS/MS which is able to identify free phytoprostanes in olive and refined sunflower oils. The recovery provided high extraction efficiencies ranging from 102.90% to 140.64% using Strata-XAW cartridge. The intra-day and inter-day variations for all target compounds ranged from 2.24% to 13.64% and 0.01% to 13.69%, respectively, and the accuracies for these parameters varied from 80.33% to 119.64% and from 80.34% to 119.90%, respectively. Results obtained reflect that refined sunflower presented more series of phytoprostanes and a 20 and 8-fold higher quantity than two types of olive oil: Extra virgin olive oil and olive oil, (containing half virgin extra olive oil and half refined olive oil). The manufacture process could be the key for the different phytoprostane production since most of the plant oils are subjected to a refining treatment.

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1. Introduction

In the middle of the 60s and in the decade of the 70s Nugteren, Vonkeman, and Vandorp (1967) and Pryor, Blair, and Stanley (1976) shown that C18-“prostaglandin-like” compounds could be formed by *in vitro* autoxidation of purified polyunsaturated fatty acids (PUFAs). However, in 1990, *in vivo* study by Morrow et al. (1990) showed a family of novel prostaglandin-like compounds termed isoprostanes (IsoPs). They are produced in abundance in humans and are considered biomarkers of oxidative stress in mammals (Jahn, Galano, & Durand, 2008). They are produced non-enzymatically through free-radical inducing peroxidation of arachidonic acid (AA, C20:4, ω-6) (Imbusch & Mueller, 2000a, 2000b; Medina et al., 2012; Morrow et al., 1990; Nugteren et al., 1967; Parchmann & Mueller, 1998; Pryor et al., 1976). However, higher plants generally do not possess the enzymatic capacity to synthesize the precursor of AA required for IsoP formation, but in 1998 Parchmann and Mueller (Parchmann & Mueller, 1998) discovered that α-linolenic acid (ALA, C18:3, ω-3), which is the main PUFA present in higher plants, is used for the synthesis of the C18-IsoPs in plants (Dinor-IsoPs) termed phytoprostanes (PhytoPs) (Durand et al., 2009; Imbusch & Mueller, 2000b; Medina et al., 2012; Thoma et al., 2003) The PhytoP pathway in plants is a non-enzymatic lipid peroxidation, similar to AA, which is initiated by the attack of reactive oxygen species (ROS) to ALA, yielding a linolenate radical that readily oxidizes and cyclizes to complex isomeric mixtures of two series (16-series/type I and 9-series/type II). They are classified according to a nomenclature system that conforms with PG convention (Imbusch & Mueller, 2000a, 2000b;

Abbreviations: ALA, α-linolenic acid; AA, Arachidonic Acid; GC–MS, gas chromatography coupled to mass spectrometry; HPLC, high performance liquid chromatography; ICH, International Conference on Harmonization; LOD, limit of detection; LOQ, limit of quantification; MRM, multiple reaction monitoring; MUFA, monounsaturated fatty acid; OS, oxidative stress; PG, prostaglandin; PhytoP, phytoprostane; PUFA, polyunsaturated fatty acid; RSD, relative standard deviation; ROS, reactive oxygen species; SPE, solid-phase extraction; UHPLC–QqQ-MS/MS, ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry.

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Jahn, Galano, & Durand, 2010; Thoma, Krischke, Loeffler, & Mueller, 2004; Thoma et al., 2003). In this paper we will use the Taber/Roberts nomenclature, approved by the International Union of Pure and Applied Chemistry (IUPAC) which differs from Mueller reports. ROS are generated as a consequence of oxidative stress (OS) and are established as plant signals that may induce its defense genes through different mechanisms (Imbusch & Mueller, 2000a; Loeffler et al., 2005; Thoma et al., 2003, 2004). Thus, a consequence to a greater exposure to ROS is that PhytoPs activate the expression of plant genes in response to stress which leads to higher protection of the plant to OS (Eckardt, 2008). For this reason, other authors have proposed that PhytoPs are not only excellent biomarkers of oxidative degradation of plant derived foodstuff but also biologically active molecules because they are components of an oxidant-injury-sensing, archaic signaling system that induces several plant defense mechanisms (Loeffler et al., 2005; Thoma et al., 2003; Vazquez-Romero, Verdaguer, & Riera, 2013). However, despite the physiological role found of PhytoPs in plants they are beginning to emerge as interesting compounds to be studied in the area of plant physiology. Little is known of their biological effects, although there is some evidence that they play a role in regulation of immune function in humans (Barden, Croft, Durand, Guy, & Mueller, 2009; Gutermuth et al., 2007).

Olive oil is the principal source of fat of the Mediterranean diet, and the production of olive oil for 2012–2013 in the European Community is estimated to be 1,739,200 tons, which represents 64% of the world production. Spain, Italy and Greece add up 95% of olive oil from EU, from which 47% is from Spain (Fki, Allouche, Sayadi, Fki, & Allouche, 2005; International Olive Oil Council, 2012). Evidence suggests that olive oil shows health benefits including a lower incidence of coronary heart disease, prevention of certain types of cancers, and modification of immune and inflammatory responses (Abuznait, Qosa, Busnena, El Sayed, & Kaddoumi, 2013; Beauchamp et al., 2005; Fki et al., 2005; Harwood & Yaqoob, 2002; Stark & Madar, 2002). To date, these effects have been mainly attributed to the high content of oleic acid (OA, C18:1, ω-9) that is a monounsaturated fatty acid (MUFA) (55–83%) and a low content of PUFAs like linoleic acids (LA, C18:2, ω-6) and ALA, respectively ranging between 3.5% to 21.0% and between 0.0% to 0.9%, respectively in this type of oil (Codex Alimentarius, 2009; De la Lastra, Barranco, Motilva, & Herrerias, 2001; Kris-Etherton, Hecker, & Binkoski, 2004; Ruxton, Reed, Simpson, & Millington, 2004; Stark & Madar, 2002). In order to preserve the maximum content of the ALA owing to its great importance in olive from a nutritional and health benefits, the purpose of this study was to evaluate the PhytoP content present in olive oil.

Earlier reports focused on the analysis of PhytoPs indicate that different analytical methods were followed such as gas chromatography coupled to mass spectrometry (GC–MS) (Barden et al., 2009; Imbusch & Mueller, 2000a, 2000b; Thoma et al., 2003, 2004), high performance liquid chromatography (HPLC) coupled to a fluorescence detector (Loeffler et al., 2005), nuclear magnetic resonance of 1H or 13C (Vazquez Romero, Verdaguer, & Riera, 2013) as well as immunological approaches (ELISA) (Gutermuth et al., 2007). These methods either have a very slow processing or are not very sensitive. Therefore, the aim of this experiment was to develop a rapid, reliable and efficient analytical method that allows identification and quantification in a same chromatogram of F_1-, D_1-, B_1-, L_1-PhytoPs and their regioisomers in olive oil, using ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry (UHPLC/MS–MS), due to its higher speed of analysis, robustness, selectivity, and sensitivity (Medina et al., 2012), operating in multiple reaction monitoring (MRM) mode, as the acquisition and detection system. In the same way, PhytoP profile and content were provided by first time in commercial olive and sunflower oils.

2. Materials and methods

2.1. Chemicals and reagents

PhytoPs, including 9-F_{1t}-PhytoP (SE 139 A), 9-epi-9-F_{1t}-PhytoP (SE 139 B), Ent-16-F_{1t}-PhytoP (SE 126 B), Ent-16-epi-16-F_{1t}-PhytoP (SE 126 A), 9-D_{1t}-PhytoP (AG 441 B), 9-epi-9-D_{1t}-PhytoP (AG 441 A), Ent-16-B_1-PhytoP (SE 175 A), 16-B_1-PhytoP (SE 175 B), Ent-9-L_1-PhytoP (SE 194 R), 9-L_1-PhytoP (SE 194 S), were synthesized according to our published procedures (El Fangour et al., 2004; El Fangour, Guy, Vidal, Rossi, & Durand, 2005; Pinot et al., 2008). The synthetic isoprostane 8-isoPGF2α-d4 (containing four deuterium atoms at positions 3, 3′, 4, and 4′) used as internal standard (Fig. 2) was purchased from Cayman Chemicals (Ann Arbor, Michigan, USA). Hexane was obtained from Panreac (Castellar del Vallés, Barcelona, Spain), Bis–Tris (bis(2-hydroxyethyl)amino-tris (hydroxymethyl)methane) were purchased from Sigma–Aldrich (St. LOUIS, MO USA) and all LC–MS grade solvents, methanol and acetonitrile, were purchased from J.T. Baker (Phillipsburg, New Jersey, USA). The SPE cartridges used was Strata cartridge (Strata X-AW, 100 mg/3 mL), which was acquired from Phenomenex, (Torrance, CA, USA).

2.2. Preparation of standards

Stock solutions of PhytoP were prepared in methanol/water (50:50, v/v) to facilitate the ionization process in the mass spectrometer at a concentration of 1000 nM for each compound and stored in Eppendorf tubes at −80 °C. Twelve successive dilutions were prepared for producing the standard solutions as follows: 1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.9, 0.95, and 0.47 nM, which were required for following determination of calibration curve. The isoprostane 8-isoPGF2α-d4, (molecular weight: 358.2; $C_{20}H_{30}D_4O_5$), was used as internal standard (IS) (Fig. 2) due to be an IsoP containing four deuterium atoms, makes that this standard cannot be found in humans or plants. This IS has been used in the absence of the commercial availability of a deuterated standard of PhytoPs. IS was prepared in commercial oil at a concentration of 1000 nM and same successive dilutions were prepared like the above standards to determinate the range of linearity of this compound.

2.3. Sample preparation and sample extraction

The analyzed samples were three types of commercial oils: 0.8° Virgin extra olive oil (A) (Oro Ibérico, Jomipsa, Alicante, Spain), 0.4° olive oil (virgin extra olive oil/refined olive oil (50/50, v/v), La Española, Acesur, Sevilla, Spain) (B), and 0.2° refined sunflower oil (Sovena, Sevilla, Spain) (C). Oils were stored at room temperature until extraction and posterior analysis.

PhytoPs presents in oils were isolated using a dilution followed by a solid-phase extraction (SPE) according to the following protocol: 1 mL of olive oil was dissolved in 10 mL of hexane (Del Carlo, Ritelli, Procida, Murmura, & Cichelli, 2006). The extract (11 mL) was spiked with the stock solution of PhytoPs to achieve a concentration of 0.1 and 0.05 μM for each compound including the IS. The solution was diluted in 2 mL of methanol, stirred and then rediluted in 2 mL of BIS–TRIS buffer (0.02 M, HCl, pH = 7). Then, the emulsion (oil+hexane+BIS–TRIS buffer) was subjected to SPE using a Strata X-AW cartridge (100 mg/3 mL), according to a procedure previously described for IsoPs (Medina et al., 2012) with minor modifications. Briefly, the cartridge were conditioned and equilibrated with 2 mL of hexane, 2 mL of methanol and 2 mL of milliQ water prior to load the oil samples. Each sample was loaded at flow rate of about 1 drop per 2 s. After this step, and to remove the

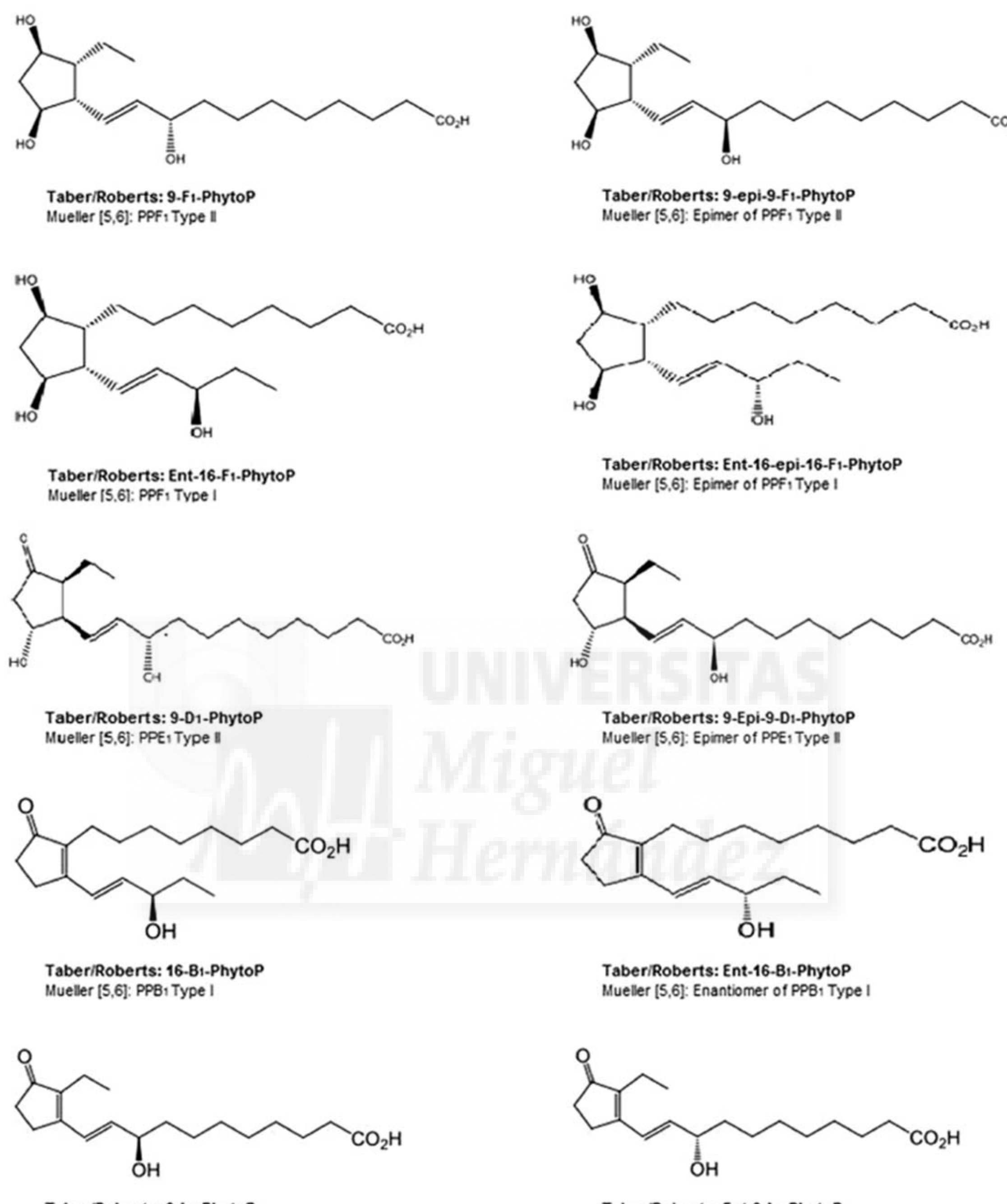

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Fig. 1. Chemical structures of PhytoPs. PhytoPs are named according to the Taber/Roberts, and Mueller nomenclature.

undesired compounds, the cartridge was washed with 2 mL of hexane, 2 mL of milliQ water, 2 mL of a solution of methanol/milliQ water (1/3) and last, with 2 mL of acetonitrile. The target compounds were eluted with 1 mL of methanol and dried using a SpeedVac concentrator (Savant SPD121P, Thermo Scientific, MA, USA). The dry extracts were reconstituted with 200 µL of A/B solvents (90:10, v/v), being the solvent A a mixture of milliQ water/0.01% acetic acid and a solution of methanol/0.01% acetic acid was used as solvent B. After that, samples were sonicated for 10 min and filtered through a 0.45-µm filter (Millipore, MA, USA). Twenty microliters of each sample were injected and analyzed in a UHPLC–QqQ-MS/MS (Agilent Technologies, Waldbronn, Germany).

2.4. UPLC–QqQ-MS/MS analyses

Separation of PhytoPs was performed using a UHPLC coupled with a 6460 triple quadrupole-MS/MS (Agilent Technologies, Waldbronn, Germany). The analytical column was a BEH C18 (2.1 × 50 mm, 1.7 µm) (Waters, Milford, M.A.). The column

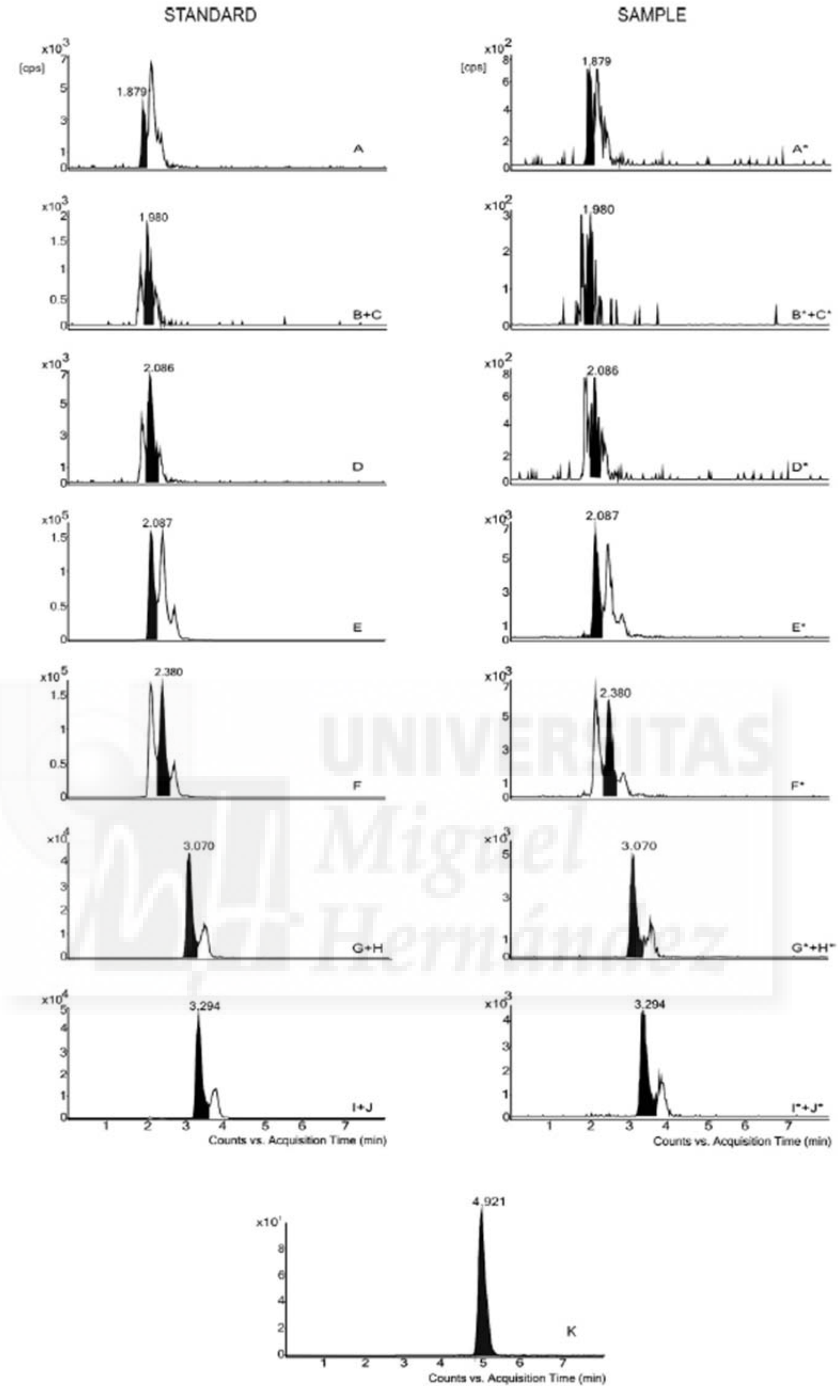

Fig. 2. UHPLC–MS/MS chromatograms of the used internal standard (8-iso-$PGF_{2\alpha}$-d_4) (K) and PhytoPs standards and PhytoPs present in sunflower oil at the MRM transitions for quantification. PhytoPs standards are identified by a capital letter whereas those PhytoPs found in the sample are identified by a capital letter with asterisk. In this sense, 9-F_{1t}-PhytoP (A, A*); the pair of epimers Ent-16-epi-16-F_{1t}-PhytoP + Ent-16-F_{1t}-PhytoP (B + C, B* + C*); 9-epi-9-F_{1t}-PhytoP (D, D*); 9-epi-9-D_{1t}-PhytoP (E, E*); 9-D_{1t}-PhytoP (F, F*); the enantiomers 16-B_1-PhytoP + Ent-16-B_1-PhytoP (G + H, G* + H*); and the pair of enantiomers 9-L_1-PhytoP + Ent-9-L_1-PhytoP (I + J, I* + J*).

temperatures were 6 °C (both, left and right). The mobile phases consisted of water/acetic acid (99.99:0.01, *v/v*) (A) and methanol/acetic acid (99.99:0.01, *v/v*) (B). The injection volume was 20 µL and elution was performed at a flow rate of 0.2 mL/min. The linear gradient was as follow: 60% B at 0 min, 62% B at 2 min, 62.5% B at 4 min, 65% B at 8 min and 60% B at 8.01 min. The acquisition time was 8.01 min for each sample, with a post-run of 1.5 min for the column equilibration. Analysis were performed by multiple reaction monitoring (MRM) in the negative mode. The MS parameters fragmentor (ion optics: capillary exit voltage) and collision energy were optimized for each analyte (Table 1). The allocation of these parameters, along with preferential MRM transition of the corre-

Table 1
Multiple reaction monitoring (MRM) parameters and analytical features of the method: linearity, limit of detection (LOD) and limit of quantification (LOQ) for each PhytoP by UHPLC–QqQ-MS/MS.

Analyte	Retention time (min)	Quantification transition	Calibration equation	Correlation (R^2)	LOD (ng/mL)	LOQ (ng/mL)	Fragmentor (V)
9-F_{1t}-PhytoP	1.879	327.2 → 171.2	$y = 17{,}517X - 31.84$	0.9999	2.56	5.12	80
Ent-16-epi-16-F_{1t}-PhytoP	1.982	327.2 → 251.2	$y = 2935.1X - 1.2917$	0.9954	2.56	5.12	80
Ent-16-F_{1t}-PhytoP	1.982	327.2 → 251.2	$y = 9085X - 30.63$	0.9982	2.56	5.12	80
9-epi-9-F_{1t}-PhytoP	2.086	327.2 → 171.2	$y = 43{,}350X - 56.57$	0.9996	2.56	5.12	80
9-epi-9-D_{1t}-PhytoP	2.087	325.2 → 307.2	$y = 400{,}046X + 621.21$	0.9993	0.31	0.62	80
9-D_{1t}-PhytoP	2.380	325.2 → 307.2	$y = 483{,}633X + 757.29$	0.9998	0.31	0.62	80
16-B_1-PhytoP	3.070	307.2 → 235.2	$y = 261{,}491X - 230.58$	0.9999	0.29	0.59	70
Ent-16-B_1-PhytoP	3.070	307.2 → 235.2	$y = 100{,}576X - 121.21$	0.9991	0.29	0.59	70
9-L_1-PhytoP	3.294	307.2 → 185.2	$y = 300{,}631X + 726.06$	0.9999	0.29	0.59	70
Ent-9-L_1-PhytoP	3.294	307.2 → 185.2	$y = 110{,}317X + 39.21$	0.9996	0.59	0.59	70

sponding analytes, generated the most abundant product ions (Table 1). After optimization, the source parameters used were: gas temperature: 325 °C, gas flow: 8 L/min, nebulizer: 30 psi, sheath gas temperature: 350 °C, jetstream gas flow: 12 L/min, capillary voltage: 3000 V, nozzle voltage: 1750 V. Data acquisition and processing were performed using MassHunter software version B.04.00 (Agilent Technologies).

2.5. Validation parameters

Calibration curves were obtained using a series of dilutions of ten calibration standards. A useful parameter to evaluate the linearity of an analytical method is the sensitivity because it is established as the ability to detect any change produced in the instrumental response due to differences in concentration of the analyte. To assess the sensitivity of this analytical method, lower limits of detection and quantification (LOD and LOQ, respectively) were measured, considering the LOD as the lowest detected concentration of analyte but not quantified and the LOQ as the lowest concentration that is sensitive to be determined quantitatively. These parameters were established with an S/N ratio of 3:1 for LOD and 10:1 for LOQ.

The assessment of precision and repeatability of the assay was carried out by determining the relative standard deviation (% RSD) of intra- and inter-day variations and accuracy of target PhytoPs. These variations were evaluated by analyzing three concentrations of the objective compounds within the linear range (5.1, 20.5 and 164.2 ng/mL for the least sensitive compounds and 1.2, 10 and 160 ng/mL for most sensitive compounds) in three replicates on the same day for intra-day measure and on two consecutive days for inter-day measure. % RSD is calculated as the standard deviation divided by mean and multiplicated by 100 and the accuracy is calculated as the percentage ratio of the measured concentration to the nominal concentration.

Recovery may be affected by several factors including chromatography, ionization at the source, and selectivity of the SPE cartridges. It involves the efficiency of the extraction procedure and is determined by spiking known amounts of standards to the baseline urine samples. Recovery was calculated as previously described (Cavalca et al., 2010; Medina et al., 2012). The ratio '[(final concentration – initial concentration)/added concentration]' was determined in two different levels of concentrations (15–16 ng/mL and 30–32 ng/mL depending on the metabolite) between the lower and upper limits of the previously estimation of the standards linearity. It was performed by triplicate ($n = 3$) by addition of the standard working solution before and after sample extraction, and the resultant peak area ratio was compared (Matuszewski, Constanzer, & Chavez-Eng, 2003).

3. Results and discussion

For the validation of this method, the determination of fundamental parameters such as linearity, sensitivity, precision, accuracy and recovery were required according to the The US Food and Drug Administration (FDA) Guidance for Industry-Bioanalytical Method Validation (FDA, 2001) and International Conference on Harmonization (ICH) (ICH, 1994) for the validation of analytical methods.

3.1. Optimization of the LC/MS/MS method

The structure of the analyzed PhytoPs displays a common carboxylic acid moiety, therefore, all of them generated a deprotonated molecular ion $[M-H]^-$ in the negative ion mode (Fig. 1). The most abundant fragment ion produced was considered as product ion for MRM. To optimize the resolution of chromatographic peak definition, the fragmentor voltage and the collision energy were analyzed for each standard. Both of them, the precursor and selected product ion for the quantification of each analyte, as well as the different optimized parameters are presented in Table 1.

3.2. Linearity and sensitivity

The quantification of PhytoPs was carried out by preparation of the corresponding calibration curves. They were fitted using the linear regression equation '$y = ax + b$'. The linearity of the method was assessed for each metabolite by evaluating the correlation coefficient (r^2) being higher than 0.995, indicating that this analytical method present an adequate linearity. The LOD and LOQ of our method were evaluated, being both of these values metabolite-dependent. As shown in Table 1, the LOD values of our method ranged from 0.29 to 2.56 ng/mL and the LOQs from 0.59 to 5.12 ng/mL. The linearity range established for these PhytoPs measured by this method is from 5.12 to 328.44 ng/mL for Ent-16-epi-16-F_{1t}-PhytoP, 9-F_{1t}-PhytoP, Ent-16-F_{1t}-PhytoP and 9-epi-9-F_{1t}-PhytoP, oscillating between 0.62 and 326.43 ng/mL for 9-epi-9-D_{1t}-PhytoP and 9-D_{1t}-PhytoP, and finally for 16-B_1-PhytoP, 9-L_1-PhytoP, Ent-16-B_1-PhytoP and Ent-9-L_1-PhytoP the linearity range reached from 0.59 to 308.41 ng/mL (Table 1).

3.3. Precision and recovery

The intra- and inter-day variations for the target PhytoPs determinations in olive oil are shown in Table 2. The intra-day variations for all target compounds ranged from 2.24% to 13.64% and the accuracies varied from 80.33% to 119.64%. The

Table 2
Intra-day and inter-day coefficients of variation for the determination of PhytoPs.

Analyte	Nominal conc. (ng/mL)	Intra-day			Inter-day		
		Measured	RSD (%)	Accuracy (%)	Measured	RSD (%)	Accuracy (%)
9-F_{1t}-PhytoP	5.12	5.35 ± 0.46	8.61	104.43	4.98 ± 0.55	11.11	97.35
	20.52	23.51 ± 2.10	8.90	114.57	24.54 ± 3.02	12.30	119.59
	164.22	179.15 ± 19.28	10.76	109.10	192.21 ± 24.84	12.92	117.04
Ent-16-epi-16-F_{1t}-PhytoP	5.12	5.55 ± 0.34	6.16	108.46	6.11 ± 0.39	6.43	119.39
	20.52	21.14 ± 1.53	7.22	103.04	19.39 ± 2.07	10.69	94.50
	164.22	174.75 ± 23.83	13.64	106.42	157.70 ± 18.31	11.61	96.04
Ent-16-F_{1t}-PhytoP	5.12	5.24 ± 0.59	11.22	102.35	5.34 ± 0.49	9.16	104.24
	20.52	17.73 ± 1.07	6.02	86.35	17.79 ± 2.09	11.75	86.67
	164.22	148.22 ± 18.80	12.68	90.27	144.73 ± 19.62	13.56	88.13
9-epi-9-F_{1t}-PhytoP	5.12	5.10 ± 0.67	13.14	99.43	4.60 ± 0.51	11.18	89.86
	20.52	24.43 ± 1.94	7.94	119.07	22.97 ± 2.92	12.73	111.94
	164.22	156.26 ± 16.64	10.65	95.17	155.29 ± 20.49	13.19	94.56
9-epi-9-D_{1t}-PhytoP	1.27	1.02 ± 0.10	10.13	80.33	1.04 ± 0.14	13.69	82.23
	10.18	9.59 ± 1.22	12.69	94.19	9.12 ± 1.01	11.10	89.57
	163.22	172.00 ± 12.97	7.51	108.09	160.72 ± 15.00	9.33	98.47
9-D_{1t}-PhytoP	1.27	1.03 ± 0.11	10.61	81.44	1.02 ± 0.12	12.10	80.34
	10.18	10.23 ± 1.21	11.79	100.51	9.95 ± 0.57	5.71	97.77
	163.22	176.43 ± 13.51	7.66	108.09	167.28 ± 0.02	0.01	102.49
16-B_1-PhytoP	1.20	1.44 ± 0.11	7.69	119.64	1.27 ± 0.09	6.75	105.61
	9.62	9.39 ± 0.25	2.68	97.81	9.52 ± 0.70	7.39	99.13
	154.21	157.18 ± 8.94	5.69	101.92	150.54 ± 2.17	1.44	97.62
Ent-16-B_1-PhytoP	1.20	1.34 ± 0.04	2.87	111.89	1.41 ± 0.13	9.29	117.52
	9.62	9.29 ± 1.20	12.89	96.83	7.74 ± 0.79	10.19	80.58
	154.21	178.02 ± 19.71	11.07	115.44	155.39 ± 5.34	3.44	100.75
9-L_1-PhytoP	1.20	1.44 ± 0.07	4.86	119.59	1.44 ± 0.09	6.58	119.90
	9.62	8.89 ± 0.28	3.12	92.65	8.12 ± 0.53	6.58	84.62
	154.21	166.14 ± 6.67	4.01	107.73	165.65 ± 6.65	4.01	107.42
Ent-9-L_1-PhytoP	1.20	1.32 ± 0.03	2.24	109.68	1.31 ± 0.12	9.10	109.29
	9.62	8.18 ± 0.97	11.88	85.19	7.77 ± 0.56	7.20	80.98
	154.21	169.12 ± 9.15	5.41	109.67	156.66 ± 6.82	4.35	101.59

Values shown are means ± SD of three parallel measurements (n = 3).

inter-day variations for the same compounds were from 0.01% to 13.69% and the accuracies from 80.34% to 119.90% (Table 2). The RSD values for three injections were lower than 15%, indicating that this method is very reproducible for the PhytoPs analyzed.

The efficiency of the extraction is shown in Table 3. Results of the absolute recovery provided high extraction efficiencies ranging from 102.90% to 140.64%, which reflect an adequate and good efficiency of the SPE. In addition, of note is the fact that low standard deviations have been obtained, highlighting the good repeatability of the process.

3.4. Qualitative and quantitative profile of PhytoPs in olive and sunflower oils

The developed method was used to analyze PhytoPs in oil samples of three types of commercial oil: 0.8° extra virgin olive oil (A), 0.4° olive oil (B) and 0.2° refined sunflower oil (Table 4).

This new method developed in this trial allowed the detection and quantification of four PhytoPs, in both olive oil samples (Table 4) and the whole of them (10) in the sunflower oil. The total/analyzed content of PhytoPs were 14.97, 39.35 and 297.45 ng/mL in virgin extra olive oil (A), 0.4° olive oil (B) and sunflower oil (C), respectively. As for the individual PhytoPs content in these oils ranged from 0.35 to 11.29 ng/mL and 3.70 to 24.86 ng/mL in olive oils A and B, respectively, and varied from 19.34 to 67.60 ng/mL in sunflower oil (Table 4). These PhytoPs have been identified and quantified according to their specific ionic transitions (Table 1).

The 'Oxygen Paradox' outlined that plants and all living organisms cannot exist without oxygen, nevertheless, oxygen is inherently dangerous to their existence. Under normal conditions, due to their reductive environment, cell plants are promoting continuously the reduction of oxygen, fostering endogenous production of ROS (Mueller, 2004). The production of these ROS is increased as a

Table 3
Recovery of PhytoPs.

Analyte	Added (ng/mL)	Absolute recovery (%)
Ent-9-F_{1t}-PhytoP	16.42	108.50 ± 11.05
	32.84	103.43 ± 9.77
Ent-16-epi-16-F_{1t}-PhytoP	16.42	106.73 ± 4.06
	32.84	128.74 ± 16.22
Ent-16-F_{1t}- PhytoP	16.42	122.16 ± 7.34
	32.84	137.60 ± 17.95
9-epi-9-F_{1t}-PhytoP	16.42	109.07 ± 7.98
	32.84	113.74 ± 19.35
9-epi-9-D_{1t}-PhytoP	16.32	118.11 ± 11.30
	32.64	125.76 ± 4.95
9-D_{1t}-PhytoP	16.32	123.95 ± 14.28
	32.64	140.64 ± 11.86
16-B_1-PhytoP	15.42	107.91 ± 10.52
	30.84	116.31 ± 12.13
Ent-16-B_1-PhytoP	15.42	111.60 ± 10.97
	30.84	120.43 ± 16.13
9-L_1-PhytoP	15.42	102.90 ± 13.74
	30.84	110.67 ± 6.55
Ent-9-L_1-PhytoP	15.42	119.70 ± 19.68
	30.84	125.93 ± 22.01

Values shown are means ± SD of three parallel measurements (n = 3).

Table 4
Concentration of phytoprostanes (ng/mL) determined in oil samples.

Analyte	Phytoprostanes		
	0.8° Extra virgin oil (A)	0.4° Olive oil (B)	Refined sunflower oil (C)
9-F_{1t}-PhytoP	n d.	n d.	25.14 ± 0.95
Ent-16-epi-16-F_{1t}-PhytoP + Ent-16-F_{1t}-PhytoP	2.13 ± 0.50	3.70 ± 0.49	24.40 ± 2.89
9-epi-9-F_{1t}-PhytoP	n d.	n d.	19.34 ± 0.32
9-epi-9-D_{1t}-PhytoP	1.20 ± 0.07	9.33 ± 2.16	57.13 ± 2.30
9-D_{1t}-PhytoP	11.29 ± 0.57	24.86 ± 6.28	46.47 ± 6.61
16-B_1-PhytoP + Ent-16-B_1-PhytoP	0.35 ± 0.02	n d.	67.60 ± 4.87
9-L_1-PhytoP + Ent-9-L_1-PhytoP	n d.	n d.	56.47 ± 5.95
Total Content	14.97 ± 0.29	39.35 ± 9.02	297.45 ± 23.43

Values shown are means ± SD of three parallel measurements ($n = 4$); n.d., not detected.

consequence of almost any stress (abiotic and biotic) to which the plant is subjected. These ROS readily attack ALA in membrane lipids that are non-enzymatically oxidized to a PhytoP family (Cruz de Carvalho, 2008; Mueller, 2004). The importance of PhytoPs is not only based on their biological activity, but also on their use as biomarkers of oxidative degradation of plant derived foodstuff (Barden et al., 2009). Further, previous studies have reported that the intake of these PhytoPs appear to be related to the regulation of immune function in humans (Barden et al., 2009; Gutermuth et al., 2007).

To perform subsequent study of PhytoP content in plant material is necessary to develop a good extraction and purification process. Therefore, a solid phase extraction (SPE) was carried out, from which very good recoveries were obtained ranging from 102.90% to 140.64% (Table 3) depending on the PhytoP. In this purification procedure cartridges X-AW Strata anion-exchange were employed. This type of cartridge was chosen as a consequence of a previous study carried out (Medina et al., 2012) with similar compounds in biological samples. That study was based on the analysis of IsoPs, which are similar to PhytoPs but obtained from AA, in urine samples. In that study the efficiency of various types of cartridges (Strata X-AW cartridge, C18 Sep-Pack and Oasis HLB) were assayed for the extraction of IsoPs, showing that Strata X-AW cartridge gave the best results due its higher recovery rates.

Analysis of PhytoPs in plant material is challenging due to the presence of several structural classes and isomers and to the low abundance of these compounds in comparison to the high levels of primary and secondary metabolites in plant materials (Thoma et al., 2004). Therefore, for their characterization, analysis and quantification in plants, several techniques such as ^{1}H or ^{13}C nuclear magnetic resonance, gas chromatography coupled to mass spectrometry (GC–MS), high performance liquid chromatography (HPLC) coupled to fluorescence detector, and ESI-MS have been developed. Although the HPLC–UV analysis method is rapid, its sensitivity and specificity are not high enough to analyze these compounds in plants. So far, the most sensitive method developed for quantifying these compounds is a NICI GC–MS method (Göbel & Feussner, 2009; Imbusch & Mueller, 2000a, 2000b; Karg et al., 2007; Parchmann & Mueller, 1998; Thoma et al., 2003, 2004). The drawback of this method is, however, that PhytoPs must be subjected to reactions of derivatization to become their corresponding pentafluorobenzyl ester and trimethylsilyl ether and thereby the different PhytoPs and their regioisomers can be quantified. This handicap can be solved by employing of LC/MS because the derivatization is not required. LC/MS would be more specific and sensitive because it would allow the separation of these compounds and identification of their regioisomers. However, LC/MS present limitations such as the required time for the analysis and the restricted number of transitions that can be monitored. For this reason in this work we propose for the first time a faster, more sensitive and more specific method than GC–MS for the determination and quantification of D_1-, F_1-, B_1-and L_1-PhytoPs and their respective enantiomers by using ultra high performance liquid chromatography coupled to a triple quadrupole (UHPLC–QqQ-MS/MS). The separation of the target compounds and the internal standard, using the chromatographic conditions, was satisfactory except for three pairs of PhytoP, since they were not completely differentiated by their retention times due to the fact that a pair of them co-elute and the others two pairs correspond to two pairs of enantiomers, which present identical physical and chemical properties, yielding same fragmentation efficiency and they can be only separated by using chiral additives and/or columns (Fig. 2). For the unequivocal identification and quantification of the target compounds the most intense MRM transition was selected. None of the MRM transitions corresponded solely to a single PhytoP, but rather to a pair of diastereoisomers of these compounds (Fig. 2), for example in the transition of 327.2→251.2 and 327.2→171.2 corresponded to two pairs of diastereoisomers of F_{1t}-PhytoP, being Ent-16-epi-16-F_{1t}-PhytoP and Ent-16-F_{1t}-PhytoP and 9-F_{1t}-PhytoP and 9-epi-9-F_{1t}-PhytoP, respectively. In the same way, the transition 325.2→307.2 matched to a pair of diastereoisomers of D_{1t}-PhytoP, being 9-epi-9-D_{1t}-PhytoP and 9-D_{1t}-PhytoP. Concerning the transition 307.2→235.2 and 307.2→185.2, which is characteristic of the B_1-PhytoPs enantiomers (16-B_1-PhytoP and Ent-16-B_1-PhytoP) and L_1-PhytoPs enantiomers (9-L_1-PhytoP and Ent-9-L_1-PhytoP), respectively. Some of these compounds were differentiated by their distinct retention times (Table 1) except for the three pairs of compounds (Fig. 2, pairs B + C, G + H, and I + J), they were quantified in oil samples as pairs of PhytoPs (Table 4).

To the best of our knowledge, little is known about the content of PhytoPs present in edible oils. Therefore, in this study we analyzed ten of these compounds in three edible oils, from which two are from olive, 0.8° Virgin extra olive oil (A) and 0.4° olive oil (virgin extra olive oil/refined olive oil (50/50, *v/v*) (B) and the third is a 0.2° refined sunflower oil (C). Previous studies have measured concentration of PhytoPs by GC–MS in tissues of several plants, for instance in tomato leaves, peppermint leaves, lime tree flowers, birch pollen, birch leaves, and valerian root (Imbusch & Mueller, 2000a, 2000b) and other studies have also detected them in cell cultures of different types of plants such as *Nicotiana tabacum* (Solanaceae), *Glycine max* (Fabaceae), *Rauvolfia serpentina* (Apocynaceae), *Agrostis tenuis* (Poaceae), *Salix alba*, *Arabidopsis thaliana*, *Tilia cordata* and *Betula pendula* (Parchmann & Mueller, 1998; Thoma et al., 2003). Furthermore, by employing GC–MS several PhytoPs were also measured in edible oils and in biological samples upon the administration of them to human volunteers (Barden et al., 2009; Karg et al., 2007). Sensitivity and selectivity of our method was reflected by LOQ values, which ranged from 0.0019 μM (0.59 ng/mL) to 0.0156 μM (5.12 ng/mL), depending on the compound analyzed (Table 1). These values were quite better than those found by other author (Karg et al., 2007) for determination of different PhytoPs in vegetable oils by GC–MS, whose LOQ values varying depending on the type of edible oil and the compound tested from 0.01 μM (3.08 ng/mL) to 0.56 μM

(182.80 ng/mL). This fact highlights the higher sensitivity showed by our method using this technique (UHPLC–QqQ-MS/MS) compared to previous methods by GC–MS.

In connection with the total content of PhytoPs in different oils, olive oils (A and B) only four PhytoPs have been obtained, being the dominant PhytoP class the D_1-PhytoP, which agree with results exhibited by Karg et al. (2007), who reported that in all studied oils either D_1-PhytoP or F_1-PhytoP was the dominant PhytoP class, being B_1-PhytoPs (both enantiomers) the minor components. However, in case of the refined sunflower oil the major PhytoP class was the both B_1-PhytoPs. Besides, the results from this oil showed a higher amount and a larger number of PhytoPs than those obtained by olive oil. This could be due for both olive oils to the softer extraction treatment compared to the harsh treatment for refined sunflower oil. The radical temperature treatment for sunflower oil could induce higher cross oxidation reactions of ALA at 100 °C or 240 °C.

Table 4 shows that sum of the PhytoP content (4 compounds detected) for 0.8° extra virgin olive oil and olive oil (virgin extra olive oil/refined olive oil (50/50, *v/v*) was 14.97 and 39.35 ng/mL, respectively, whereas 0.2° refined sunflower oil PhytoP was 297.45 ng/mL. In other words, refined sunflower oil (C) provided a 20-fold higher concentration of PhytoPs than olive oil A and 8-fold higher concentration of PhytoPs than olive oil B. Karg et al. (2007) studied the total PhytoP contents for the olive oil (vs Brändle vita) and reported that was approximately 1057 ng/mL. It is important to note that in their assay they obtained the total amount of each of the different class of PhytoPs starting from ALA, whose *in vitro* and *in vivo* autoxidation may yield not only a large number, but also high amounts of PhytoPs (Imbusch & Mueller, 2000a, 2000b; Karg et al., 2007; Parchmann & Mueller, 1998; Sattler et al., 2006; Thoma et al., 2003, 2004). In contrast, in our study we work with individual synthetic standards (ten in total). This fact makes that our results are lower than those found for other people. In addition, in their study, Karg et al. (2007) did not study the total content of PhytoPs in sunflower oil, showing our results that sunflower oil displays higher qualitative profile and content than olive oils (20-fold higher than olive oil A and 8-fold higher than olive oil B) (Table 4). Nevertheless, they studied them in other edible oils, reporting that these compounds not only vary depending on their type of oil, but also the cultivar of the fruit from which the oil is obtained, being the absolute PhytoP content of the oil declined in the order as follows: Linseed oil ≈ soybean oil > olive oil > walnut oil > rapeseed oil ≫ grape seed oil. Although it is remarkable that our results obtained for the refined sunflower oil are not comparable with the results shown by Karg et al. (2007) for the reasons described above, in this classification, for some edible oils, the refined sunflower oil is ahead in the olive oil, but it cannot tell whether the total content of PhytoPs present in refined sunflower oil would be around the PhytoP content present in soybean oil or linseed oil. The fact that refined sunflower oil possessed a higher concentration of PhytoPs than olive oil could be related to a higher initial concentration of ALA than the second one although Karg et al. (2007) reported that the total amount of PhytoPs did not correlate well with the ALA content. Regardless the initial ALA content of the oils, the manufacture process could be the key for the different PhytoP production since most of the plant oils are subjected to a refining treatment which includes dramatic temperature procedures to whitening (100 °C/15 min) and remove the flavors (240 °C at 2–3 mm pression), that could increase the oxidation of this fatty acid. However, the extra virgin olive oil only requires a soft mechanical extraction (De la Lastra et al., 2001). The PhytoP value for oil B is 2.6-fold higher than oil A since it is half virgin extra olive oil and half refined olive which suffered the same dramatic oxidative temperature procedures than those described for sunflower oil (Food and Agriculture Organization of the United Nations (FAO), 1997, chap. 5; Melgarejo, 2003).

Despite the negative connotation of the PhytoP production linked to co-lateral oxidation processes of ALA, Barden and collaborators (Barden et al., 2009) measured by GC–MS the total content of F_1-PhytoPs in plasma samples and 24 h urine samples of humans during four weeks after consuming olive and flaxseed oil, founding that the flaxseed oil supplementation significantly increased the concentration of plasma F_1-PhytoPs relative to olive oil. Therefore, these compounds are absorbed at physiological level in humans. However, the pharmacokinetic curve and the physiological effects in the human body is unknown yet.

4. Conclusion

In summary, recently some researchers have studied the relationship of OS and the content of compounds called PhytoPs in plants. So far the methods of quantification of these compounds involved a tedious analytical process and a purification of these compounds starting from autoxidation of ALA that may yield not only a large number, but also high amounts of PhytoPs. In addition, the sensitivity and selectivity of those methods were not very high. Therefore, in this work, we propose a new quick and accurate analytical method, which is more sensitive and more selective and is able to identify different classes of PhytoPs, in a single injection in olive and refined sunflower oils. The advantages of this analysis are that is performed in a single assay per sample and only require 8 min for obtaining the results, which are linked to lower expenses of solvents. Results obtained by this new method reflect that refined sunflower presented higher amount and a larger number of PhytoPs than those obtained by two types of olive oil: Extra virgin olive oil (A) and olive oil (B), which exclusively contains half virgin extra olive oil and half refined olive. In other words, refined sunflower oil (C) provided 20-fold higher than olive oil A and 8-fold higher than olive oil B. This fact could be probably related to the extraction process of these compounds. The manufacture process could be the key for the different PhytoP production since most of the plant oils are subjected to a refining treatment.

From agronomical point of view in connection with food science and technology, future research is required to study how the production of PhytoPs is affected by different types of abiotic stress on olive oil and other plant oils. Besides and nutritionally, additional studies would be necessary to know the physiological effects of the PhytoPs in humans, since they show very similar structures than IsoPs and PGs, relevant bioactive compounds at physiological level.

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Article

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Water Deficit during Pit Hardening Enhances Phytoprostanes Content, a Plant Biomarker of Oxidative Stress, in Extra Virgin Olive Oil

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ABSTRACT: No previous information exists on the effects of water deficit on the phytoprostanes (PhytoPs) content in extra virgin olive oil from fruits of mature olive (*Olea europaea* L. cv. Cornicabra) trees during pit hardening. PhytoPs profile in extra virgin olive oil was characterized by the presence of 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, 9-D_{1t}-PhytoP, 16-B_1-PhytoP + *ent*-16-B_1-PhytoP, and 9-L_1-PhytoP + *ent*-9-L_1-PhytoP. The qualitative and quantitative differences in PhytoPs content with respect to those reported by other authors indicate a decisive effect of cultivar, oil extraction technology, and/or storage conditions prone to autoxidation. The pit hardening period was critical for extra virgin olive oil composition because water deficit enhanced the PhytoPs content, with the concomitant potential beneficial aspects on human health. From a physiological and agronomical point of view, 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, and 16-B_1-PhytoP + *ent*-16-B_1-PhytoP could be considered as early candidate biomarkers of water stress in olive tree.

KEYWORDS: *phytoprostanes, mass spectrometry, UHPLC, olive oil, water deficit*

■ INTRODUCTION

Olive tree (*Olea europaea*) is the second most widely cultivated tree around the world, exceeded only by coconut (*Cocos nucifera*), with a cultivation area around 10 million hectares, of which 97.8% is located in the Mediterranean Basin, mainly in Spain, Italy, and Tunisia (25%).[1]

Extra virgin olive oil is a marketable subcategory of olive oil that has the best organoleptic features with tastes and aromas which perfectly reproduce the fruit of origin. It must also maintain a free acidity of not more than 0.8 g 100 g^{-1} oleic acid, the highest unsaponificable fraction of all olive oils and therefore also the one with the highest amount of antioxidants.[2] This subcategory of olive oil is extracted from the olive fruits solely by mechanical procedures under thermal conditions that avoid any chemical alteration in the oil.[3] The health-promoting effects of olive oil include a lower incidence of coronary heart disease, prevention of certain types of cancers, and modification of immune and inflammatory responses.[4−8] For this reason, and taking into consideration that there is a direct relationship between olive oil consumption and health benefits,[9] the irrigated olive area in Spain has increased from 122.534 ha in 1990 to 435.811 ha in 2011.[10] This great increase of irrigated olive area has been due to the fact that olive yield is highly correlated with applied irrigation water amounts,[11,12] and it is possible to raise olive crop productivity with very low volumes of applied water.[13] Nevertheless, the aridity of the climate and the persistent shortage of water resources in the Mediterranean agrosystems are aggravated by strong competition for the water that is available with other nonagricultural users that has arisen in recent years. Therefore, to cope with this water scarcity, efficient strategies for deficit irrigation management are needed.[14,15] Regulated deficit irrigation (RDI) is an irrigation strategy designed to save water with a minimum impact on yield and fruit quality.[15,16] RDI strategies require precise knowledge of the crop response to drought stress during different phenological phases, to identify phenological periods in which adverse effects on productivity are minimized (noncritical phenological periods). In this sense, studies in olive trees report that flowering and fruit set and fruit oil assimilation phases are the most critical phenological periods sensitive to drought,[12,17] whereas pit hardening is the most resistant (noncritical).[18]

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9-F_1-PhytoP

9-*epi*-9-F_1-PhytoP

9-D_1-PhytoP

9-*epi*-9-D_1-PhytoP

16-B_1-PhytoP

Ent-16-B_1-PhytoP

9-L_1-PhytoP

Ent-9-L_1-PhytoP

Figure 1. Chemical structures of 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, 9-D_{1t}-PhytoP, 16-B_1-PhytoP, *ent*-16-B_1-PhytoP, 9-L_1-PhytoP, and *ent*-9-L_1-PhytoP.

Parchmann and Mueller[19] showed that α-linolenic acid (ALA, C18:3, ω-3), which is the main polyunsaturated fatty acid (PUFA) present in higher plants, is used for the synthesis of the C18-isoprostanes (IsoPs) in plants termed phytoprostanes (PhytoPs).[20−22] PhytoPs are classified according to a nomenclature system that conforms with the prostaglandins (PG) convention.[22−24] In this paper we will use the Taber/Roberts nomenclature (IUPAC), which is different from the one described by Mueller, so care must be taken by the reader to acknowledge the structure associated with the name.[23]

PhytoPs are not only excellent biomarkers of oxidative degradation of plant-derived foodstuffs but also biologically active molecules because they are components of an oxidant-injury-sensing, archaic signaling system that induces several plant defense mechanisms.[24,25] The levels of PhytoPs in plants increase under a variety of conditions, especially when free radical generation is enhanced.[26] Despite little being known of the biological effects of PhytoPs, they have been identified in urine and are found esterified to lipids in plasma in healthy men consuming vegetable oil.[27] Nevertheless, there is some evidence that they play a role in regulation of the immune function,[28] and according to Durand et al.[29] PhytoPs may contribute to the benefical effects of the Mediterranean diet, because they display potent anti-inflammatory and apoptosis-inducing activities similar to those of other prostanoids.

For these reasons, the aim of the experiment described was to increase our understanding of the PhytoPs profile present in Cornicabra extra virgin olive oil, as plant biomarker of oxidative stress in the olive tree. Moreover, special attention was paid to analyzing for the first time if the pit hardening phenological period is really a noncritical period to water deficit for olive fruit, because some individual PhytoP content in the extra virgin olive oil extracted could be affected and, subsequently, the oxidative stress of the tree.

MATERIALS AND METHODS

Plant Material, Experimental Conditions, and Treatments. The experiment was carried out in 2012 at the El Chaparrillo experimental farm, located in Ciudad Real (Spain) (39°00′ N, 3°56′ W; altitude 640 m). The plant material consisted of olive trees (*O. europaea* L. cv Cornicabra) planted in 1998 and spaced 7 m × 4.76 m (300 trees ha^{-1}). The first crop yield (>5 kg per tree) was obtained in 2003.

The soil is an alkaline (pH 8.1) and shallow Petrocalcic Palexeralfs with a clay loam texture, low electrical conductivity (0.2 dS m^{-1}), organic matter (1.05%), nitrogen (0.12%), and potassium (17 × 10^{-4} mol kg^{-1}) levels, and high cationic exchange capacity (0.186 mol kg^{-1}) level. A discontinuous petrocalcic horizon is located between 0.75 and 0.85 m. The soil volumetric water content for the first 0.3 m depth is 22.8% at field capacity (soil matric potential −0.03 MPa) and 12.1% at permanent wilting point (soil matric potential −1.5 MPa), and from 0.3 to 0.75 m it was 43.0 and 21.1%, respectively.

Irrigation was carried out daily and during the night using a drip irrigation system with four emitters (each delivering 8 L h^{-1}) per tree and irrigation water with an electrical conductivity of 2.9 dS cm^{-1}. Crop irrigation requirements (ETc) were estimated according to daily crop reference evapotranspiration (ETo), calculated using the Penman−Monteith equation,[30] and a crop factor based on the time of the year[31] and taking into consideration canopy size.[32]

Plants were irrigated from mid-May (day of the year, DOY, 134) to the end of September (DOY 273). Control plants (T0 treatment) were irrigated at 100% ETc of the previous week. When midday stem water potential (Ψ_{stem}) values of that week were below −1.2 MPa before pit hardening (phase I of fruit growth) or −1.4 MPa during and after pit hardening (phases II and III of fruit growth, respectively), irrigation amounts were increased 10% in order to ensure no irrigation-related stress.[33]

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In addition to T0, two regulated deficit irrigation treatments (T1 and T2) were applied, which were calculated to avoid water deficit during phase I and III of fruit growth, maintaining Ψ_{stem} values around the threshold values indicated for T0 plants, and save irrigation water during the noncritical phenological period of pit hardening (phase II) developing different situations of water deficit (threshold Ψ_{stem} values of −2.00 and −3.00 MPa in T1 and T2 plants, respectively). The irrigation protocol in T1 and T2 plants was based on that proposed by Moriana et al.[33] Irrigation requirements were determined weekly on the basis of the Ψ_{stem} measurements, and irrigation was begun when measured Ψ_{stem} values were lower than the threshold values suggested. The first irrigation event was always 1 mm in both treatments. After each requirement determination the increase or decrease in the irrigation rate was related to the differences between the Ψ_{stem} measured and the threshold suggested as follows:

- When deviations were <10%, the variation in the irrigation was 0.25 mm day^{-1}.
- When deviations were between 10 and 20%, the variation in the irrigation was 0.5 mm day^{-1}.
- When deviations were between 20 and 30%, the variation in the irrigation was 1 mm day^{-1}.
- When deviations were >30%, the variation in the irrigation was 2 mm day^{-1}.

Irrigation was stopped if this approach indicated a negative application.

During the irrigation season (DOY 134−273), ETc was 202 mm and cumulative amounts of applied water in T0, T1, and T2 treatments, measured by means of flow meters integrated in the irrigation system, were 407, 196, and 141 mm, respectively. The orchard was managed under no-tillage conditions; weeds were controlled with postemergence herbicides. Pest control practices were those usually used by local growers.

Climate and Plant Water Status. Meteorological data, namely, air temperature, solar radiation, air relative humidity, rainfall, and wind speed 2 m above the soil surface, were collected in a nearby automatic weather station. Mean daily air vapor pressure deficit (VPD_m) was calculated according to the method of Allen et al.[30] Daily ETo was estimated using the Penman−Monteith equation.[30]

From DOY 173 to 276, midday (12 h solar time) stem water potential (Ψ_{stem}) was measured on the middle third of the trees, in fully developed leaves near the main trunk from two trees of each replicate, enclosing leaves in small black plastic bags covered with aluminum foil for at least 1 h before measurements in the pressure chamber (model 3005, Soil Moisture Equipment Co., Santa Barbara, CA, USA).

Oil Extraction. Olive fruits were harvested at mid-December (DOY 345). Extra virgin olive oil was extracted using an Abencor system, and the obtained oil was separated by decanting. Samples for analysis were filtered and stored at −18 °C in darkness using amber glass bottles without headspace until analysis.

Chemicals and Reagents. PhytoPs (Figure 1), including 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP, *ent*-16-*epi*-16-F_{1t}-PhytoP, 9-D_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, *ent*-16-B_1-PhytoP, 16-B_1-PhytoP, *ent*-9-L_1-PhytoP, and 9-L_1-PhytoP, were synthesized according to our published procedures.[34−36] As internal standard, 8-iso-$PGF_{2\alpha}$d4 was used (Cayman Chemicals, Ann Arbor, MI, USA). Hexane was purchased from Panreac (Castellar del Vallés, Barcelona, Spain), bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (Bis-Tris) was from Sigma-Aldrich (St. Louis, MO, USA) and LC-MS grade solvents, methanol and acetonitrile, were purchased from J. T. Baker (Phillipsburg, NJ, USA). The SPE cartridges used was Strata cartridge (Strata X-AW, 100 mg/3 mL), which was acquired from Phenomenex (Torrance, CA, USA).

Phytoprostanes Extraction. PhytoPs were extracted by using a dilution followed by a solid-phase extraction (SPE), as described previously.[21,37] Briefly, 1 mL of extra virgin olive oil samples was diluted in 10 mL of *n*-hexane, and this solution was rediluted in 2 mL of methanol, stirred, and then rediluted in 2 mL of Bis-Tris buffer (0.02 M, HCl, pH 7). Immediately after, the emulsion of each sample (oil + hexane + Bis-Tris buffer) was subjected to SPE using a Strata X-AW cartridge (100 mg/3 mL), which was, prior to the oil sample loading, conditioned and equilibrated with 2 mL of hexane, 2 mL of methanol, and 2 mL of Milli-Q water. Later, to remove undesired compounds, the cartridge was washed with a series of solvents in the following sequence: 2 mL of hexane, 2 mL of Milli-Q water, 2 mL of a solution of methanol/Milli-Q water (1:3), and 2 mL of acetonitrile. Retained compounds were eluted with 1 mL of methanol, which was evaporated by using a SpeedVac concentrator (Savant SPD121P, Thermo Scientific, Waltham, MA, USA). The residue of each sample was redissolved in 200 μL of a mixture of A/B solvents (90:10, v/v), employing a mixture of Milli-Q water/0.01% acetic acid as solvent A and a solution of methanol/0.01% acetic acid as solvent B. Reconstituted samples were sonicated for 10 min and passed through a 0.45 μm filter (Millipore, Beford, MA, USA) before 20 μL of each was analyzed by UHPLC-QqQ-MS/MS (Agilent Technologies, Waldbronn, Germany).

UHPLC-QqQ-MS/MS Analyses. Ten PhytoPs were analyzed by reversed phase using UHPLC coupled with a 6460 triple-quadrupole MS/MS (Agilent Technologies), as reported by Collado-González et al.[37] Briefly, chromatographic separation was carried out on a BEH C18 column (2.1 × 50 mm, 1.7 μm) (Waters, Milford, MA, USA), the temperature of which was 6 °C (both left and right sides of the column). The solvents used as mobile phase were water/acetic acid (99.99:0.01, v/v) (A) and methanol/acetic acid (99.99:0.01, v/v) (B). The MS analysis was applied in the multiple reaction monitoring (MRM negative) ESI mode. The injection volume was 20 μL, and elution was performed at a flow rate of 0.2 mL/min. The linear gradient started with 60% B at 0 min, rose to 62% B at 2 min and 62.5% B at 4 min to reach 65% B at 8 min and at 8.01 min returned to the initial conditions. In this way, the acquisition time was 8.01 min for each sample, with a postrun of 2 min for the column equilibration. ESI conditions and ion optics were as previously described.[37] Data acquisition and processing were performed using the MassHunter software version B.04.00 from Agilent Technologies. The eight detected PhytoPs were quantified using authentic standards: 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, 9-D_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, *ent*-16-B_1-PhytoP, 16-B_1-PhytoP, and *ent*-9-L_1-PhytoP, 9-L_1-PhytoP.

Statistical Design and Analysis. The design of the experiments was completely randomized with four replications, each replication consisting of five adjacent tree rows, each with nine trees. Measurements were taken on the inner trees of the central row of each replicate, which were very similar in appearance (leaf area, trunk cross-sectional area, height, ground shaded area, etc.), whereas the other trees served as border trees. Data were analyzed using SPSS software.[38] Analysis of variance was performed, and mean values were compared by a $Tukey_{0.05}$ test. Values for each replicate were averaged before the mean and standard error of each treatment were calculated. To check the regression model hypothesis (linearity, homoscedasticity, normality, and independency) Kolmogorov−Smirnov was used with the Liliefors correction and the Shapiro−Wilk tests for normality and the Levene test for homoscedasticity on the typified residuals. To do the Levene test, data were divided into two groups according to the median of the abscissa data.

RESULTS

Plant Water Status. During the irrigation season (DOY 134−273), average daily maximum and minimum air temperatures were 31.4 and 13.2 °C, respectively, with a mean relative humidity of 37%. VPD_m ranged from 0.09 to 3.50 kPa, and accumulated ETo was 849 mm (Figure 2). Rainfall achieved 96 mm, taking place in 10 daily episodes mainly during September (Figure 2).

Ψ_{stem} values for T0 plants were high and nearly constant, reaching mean values of −1.48 MPa during the measurement periods (−1.40 MPa during phase I and −1.50 during phases II and III) (Figure 3). During phases I and III of fruit growth Ψ_{stem} values for the three treatment plants were very similar and

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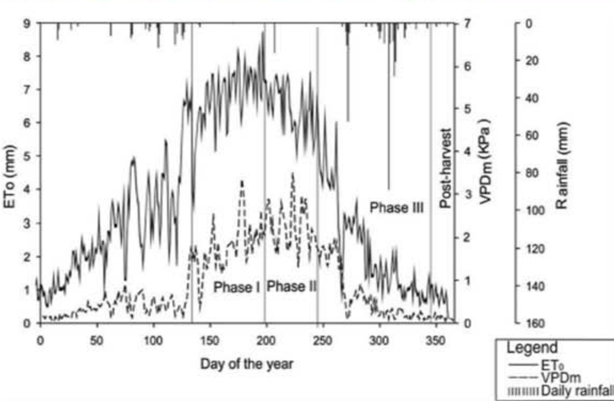

Figure 2. Daily crop reference evapotranspiration (ETo, solid line), mean daily air vapor pressure deficit (VPD_m, short-dash line), and daily rainfall (vertical bars) during year 2012.

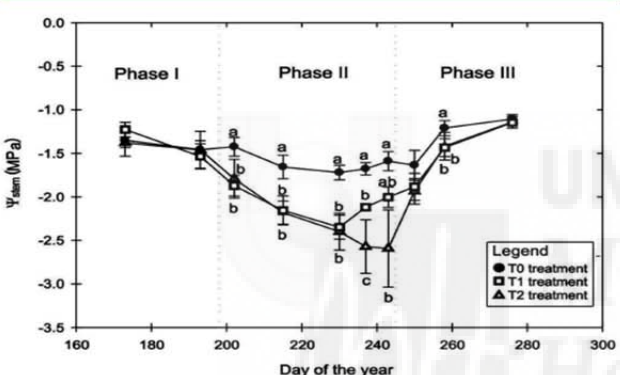

Figure 3. Midday stem water potential (Ψ_{stem}) in T0 (soild symbols), T1 (open squares), and T2 (open triangles) plants during the measurement period. Bars on data points are ±SE of the mean (not shown when smaller than symbols). Different letters on data points at each time indicate significant differences according to the Tukey test ($P \leq 0.05$). Each point is the mean of six values.

slightly increased during phase III of fruit growth (Figure 3). Ψ_{stem} values for T1 and T2 treatments significantly decreased during phase II at a similar rate of around 0.023 MPa day^{-1}, reaching minimum values of −2.35 and −2.59 MPa on DOY 230 and 243, respectively. However, differences between T1 and T2 plants in Ψ_{stem} values during this phenological period were significant only on DOY 237 (Figure 3).

Effect of Water Deficit on PhytoP Content. The PhytoPs profile of the extra virgin olive oil is shown in Figure 4 and Table 1, which indicates the presence of 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, 9-D_{1t}-PhytoP, 16-B_1-PhytoP, *ent*-16-B_1-PhytoP, 9-L_1-PhytoP, and *ent*-9-L_1-PhytoP. It is important to emphasize that two series of PhytoPs, the 16-B_1-PhytoP and the 9-L_1-PhytoP, were not separated, using achiral chromatographic conditions. Each pair of enantiomers was quantified together (sum of both for each pair of enantiomers). PhytoPs were identified on the basis of their mass spectra considering their pseudomolecular ion (m/z 327.2, 325.2, and 307.2), their most characteristic fragmentations, and their

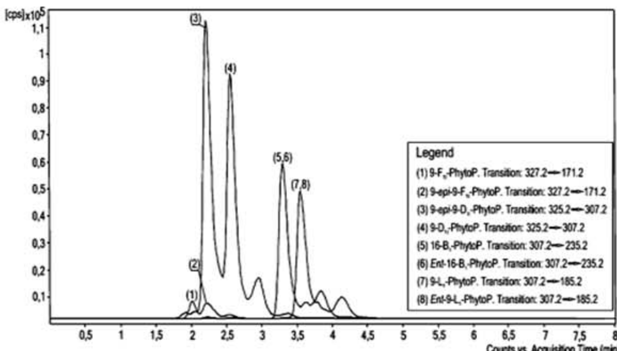

Figure 4. UHPLC-QqQ-MS/MS chromatograms of PhytoPs detected in olive oil. 9-F_{1t}-PhytoP (1), transition 327.2 → 171.2; 9-*epi*-9-F_{1t}-PhytoP (2), transition 327.2 → 171.2; 9-*epi*-9-D_{1t}-PhytoP (3), transition 325.2 → 307.2; 9-D_{1t}-PhytoP (4), transition 325.2 → 307.2; 16-B_1-PhytoP (5), transition 307.2 → 235.2; *ent*-16-B_1-PhytoP (6), transition 307.2 → 235.2; 9-L_1-PhytoP (7), transition 307.2 → 185.2; *ent*-9-L_1-PhytoP (8), transition 307.2 → 185.2.

Table 1. Multiple Reaction Monitoring (MRM) Transitions and Retention Time for Quantification of PhytoPs Using UHPLC-QqQ-MS/MS

phytoprostane	retention time (min)	precursor ion (m/z)	product ion (m/z)
9-F_{1t}-PhytoP	1.879	327.2	171.2
9-*epi*-9-F_{1t}-PhytoP	2.086	327.2	171.2
9-*epi*-9-D_{1t}-PhytoP	2.087	325.2	307.2
9-D_{1t}-PhytoP	2.380	325.2	307.2
16-B_1-PhytoP + *ent*-16-B_1-PhytoP	3.070	307.2	235.2
9-L_1-PhytoP + *ent*-9-L_1-PhytoP	3.294	307.2	185.2

elution order according to their retention times as described in Table 1.

The quantification of total PhytoPs in extra virgin olive oil oscillated from 9.18 to 19.31 ng/mL, the dominant PhytoP class being the F_{1t}-PhytoP and the D_1-PhytoPs class the minor components (Table 2). The total PhytoPs content in the extra virgin olive oil increased as a result of water deficit effect, although the differences between T1 and T2 contents were not significant (Table 2). However, the behavior observed in each PhytoP was not similar. In this sense, 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, and a racemic mixture of 16-B_1-PhytoP + *ent*-16-B_1-PhytoP showed a response to water deficit similar to that observed considering the total PhytoPs content, whereas the other PhytoPs (9-*epi*-9-D_{1t}-PhytoP, 9-D_{1t}-PhytoP, and a racemic mixture of 9-L_1-PhytoP + *ent*-9-L_1-PhytoP) showed similar levels in T0 and T1 olive oil, which were significantly lower than those observed in T2 oil (Table 2).

The observations of total PhytoPs and all individual PhytoPs in extra virgin olive oil from the three irrigation treatments showed significant first-order and second-order polynomic correlations with minimum Ψ_{stem} values in phase II of fruit growth, except when 9-F_{1t}-PhytoP was considered in the first-order correlation and a racemic mixture of 9-L_1-PhytoP + *ent*-9-L_1-PhytoP in the second-order polynomic correlation (Tables 3 and 4). Moreover, most equations in Tables 3 and 4 predict a negative PhytoP value under high Ψ_{stem} values.

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Table 2. Effect of Irrigation Treatment (T0, T1, and T2) on the PhytoP Content (ng/mL) in Cornicabra Extra Virgin Olive Oil[a]

phytoprostane	T0	T1	T2	ANOVA
9-F_{1t}-PhytoP	5.24 ± 0.21 b	10.00 ± 2.02 a	9.73 ± 2.06 a	0.022
9-*epi*-9-F_{1t}-PhytoP	2.83 ± 0.21 b	4.86 ± 0.49 a	5.26 ± 0.93 a	0.006
9-*epi*-9-D_{1t}-PhytoP	0.13 ± 7.6 × 10^{-4} b	0.13 ± 0.21 b	0.26 ± 0.08 a	0.032
9-D_{1t}-PhytoP	nd	0.19 ± 0.05 b	0.45 ± 0.11 a	0.022
16-B_1-PhytoP + *ent*-16-B_1-PhytoP	0.32 ± 0.02 b	0.72 ± 0.19 a	0.75 ± 0.09 a	0.008
9-L_1-PhytoP + *ent*-9-L_1-PhytoP	0.66 ± 0.12 b	1.28 ± 0.06 b	2.88 ± 0.88 a	0.005
total	9.18 ± 0.31 b	17.18 ± 2.70 a	19.31 ± 3.38 a	0.006

[a]Means within a row followed by different letters are significantly different by Tukey's test ($P \leq 0.05$). nd, not detected.

Table 3. Intercept (a), Slope (b), Coefficient of Determination (r^2), Number of Data Points (n), and Mean Square Error (MSE) of First-Order Linear Equations ($y = a + bx$) between PhytoP Content (ng/mL) and Minimum Mid-day Stem Water Potential (Ψ_{stem}, MPa) in Phase II of Fruit Growth, Using All Data Pooled[a]

phytoprostane	a	b	r^2	n	MSE
9-F_{1t}-PhytoP	0.393^{ns}	-3.482^{ns}	0.3476^{ns}	9	5.555
	(4.180)	(1.803)			
9-*epi*-9-F_{1t}-PhytoP	0.087^{ns}	−1.857*	0.4719*	9	0.942
	(1.721)	(0.742)			
9-*epi*-9-D_{1t}-PhytoP	-0.119^{ns}	−0.128**	0.6917**	9	0.002
	(0.075)	(0.032)			
9-D_{1t}-PhytoP	−0.748***	−0.421***	0.9132***	9	0.004
	(0.114)	(0.049)			
16-B_1-PhytoP + *ent*-16-B_1-PhytoP	-0.252^{ns}	−0.373*	0.5520*	9	0.028
	(0.294)	(0.127)			
9-L_1-PhytoP + *ent*-9-L_1-PhytoP	-2.144^{ns}	−1.647*	0.4875*	9	0.696
	(1.480)	(0.638)			
total	-2.783^{ns}	−7.907*	0.5118*	9	14.560
	(6.767)	(2.919)			

[a]*, significant at $P < 0.05$; **, significant at $P < 0.01$; ***, significant at $P < 0.001$; and ^{ns}, nonsignificant. Standard error shown in parentheses.

Table 4. Equations, Coefficients of Determination (r^2), Number of Data Points (n), and Mean Square Error (MSE) of Second-Order Polynomic Equations ($y = a + bx + cx^2$) between PhytoP Content (ng/mL) and Stem Water Potential (Ψ_{stem}, MPa) in Phase II of Fruit Growth, Using All Data Pooled[a]

phytoprostane	a	b	c	r^2	n	MSE
9-F_{1t}-PhytoP	−44.840*	−43.250**	−8.432*	0.7838*	9	2.148
	(13.260)	(11.487)	(2.424)			
9-*epi*-9-F_{1t}-PhytoP	−21.063**	−20.452**	−3.943**	0.9270***	9	0.152
	(3.526)	(3.055)	(0.645)			
9-*epi*-9-D_{1t}-PhytoP	0.688*	0.582*	0.150*	0.8974**	9	6.9 × 10^{-4}
	(0.237)	(0.206)	(0.043)			
9-D_{1t}-PhytoP	-0.777^{ns}	-0.446^{ns}	-0.005^{ns}	0.9132***	9	0.005
	(0.627)	(0.543)	(0.115)			
16-B_1-PhytoP + *ent*-16-B_1-PhytoP	-2.836^{ns}	−2.644*	-0.482^{ns}	0.7494*	9	0.018
	(1.212)	(1.050)	(0.222)			
9-L_1-PhytoP + *ent*-9-L_1-PhytoP	-11.311^{ns}	-9.706^{ns}	-1.709^{ns}	0.5998^{ns}	9	0.634
	(7.205)	(6.241)	(1.317)			
total	−80.138**	−75.917**	− 14.420**	0.8760**	9	4.314
	(18.789)	(16.276)	(3.435)			

[a]*, significant at $P < 0.05$; **, significant at $P < 0.01$; ***, significant at $P < 0.001$; ^{ns}, nonsignificant. Standard error shown in parentheses.

In general, correlations for the second-order polynomic equations of minimum Ψ_{stem} versus PhytoPs were tighter than those of first-order equations, except when 9-D_{1t}-PhytoP or a racemic mixture of 9-L_1-PhytoP + *ent*-9-L_1-PhytoP was considered (Tables 3 and 4). In this respect, in the case of a racemic mixture of 9-L_1-PhytoP + *ent*-9-L_1-PhytoP versus Ψ_{stem} because the coefficient of determination of the second-order polynomic equation was not significant, and in the case of 9-D_{1t}-PhytoP because both equations showed similar coefficients of determination (Tables 3 and 4).

It is important to indicate that the PhytoPs that increased their contents in T1 and T2 extra virgin olive oil (9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, and a racemic mixture of 16-B_1-PhytoP + *ent*-16-B_1-PhytoP) (Table 2) showed a second-order polynomic relationsship with respect to minimum Ψ_{stem} values characterized by two different phases (Figure 5). Above minimum Ψ_{stem} values of around −2.5 MPa, PhytoP values increased

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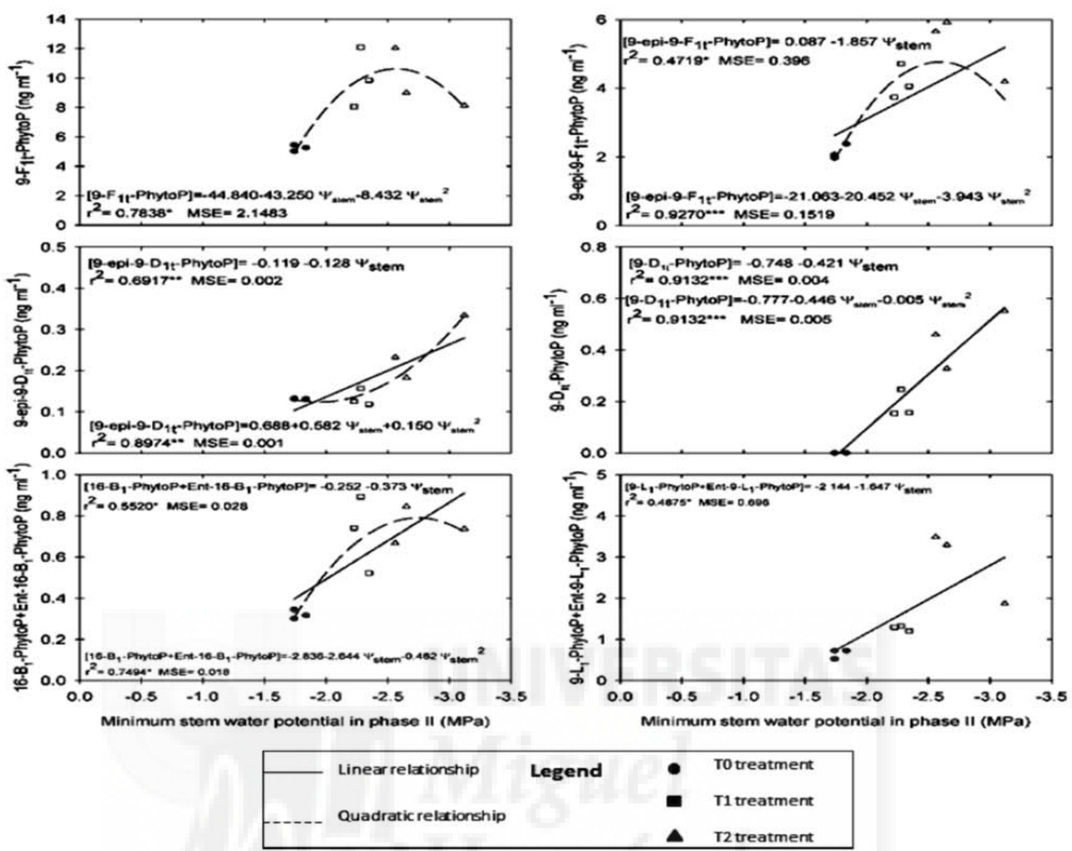

Figure 5. Relationship between PhytoPs content and minimum Ψ_{stem} values in T0 (solid circles), T1 (open squares), and T2 (open triangles) plants during phase II of fruit growth. Each value is the mean of three measurements.

sharply as minimum Ψ_{stem} decreased, and when minimum Ψ_{stem} values were below this threshold value, the relationship changed and any further reduction in minimum Ψ_{stem} was associated with a decrease in PhytoP contents. In contrast, the PhytoPs showed contents in T1 olive oil lower than in T2 (9-*epi*-9-D_{1t}-PhytoP, 9-D_{1t}-PhytoP, and a racemic mixture of 9-L_1-PhytoP + *ent*-9-L_1-PhytoP) and linear regression may be sufficient for modeling the relationship between Ψ_{stem} and PhytoP in the water stress range studied (minimum Ψ_{stem} values in phase II from −3.12 to −1.73 MPa) (Figure 5).

■ DISCUSSION

The fact that Ψ_{stem} values for T0 plants during phases I, II, and III of fruit growth were slightly below the threshold Ψ_{stem} values (Figure 3) could be because irrigation amounts were not properly managed when Ψ_{stem} values were below the threshold values. Nevertheless, Ψ_{stem} mean values in each fruit growth phase were quite high and nearly constant,[39−41] suggesting that T0 plants were not under irrigation-related stress during the experimental period.[33]

Despite Ψ_{stem} values for T1 and T2 plants during phase II decreased at a similar and slow rate,[42] minimum Ψ_{stem} values indicated that both treatment plants achieved substantial water deficit levels, but were more important in T2 plants at the end of this phenological period (Figure 3).[43,44]

In a previous paper, Collado-González et al.[37] showed in a branded extra virgin olive oil a different PhytoP profile from that shown in Table 2, because they identified 9-*epi*-9-D_{1t}-PhytoP, 9-D_{1t}-PhytoP, 16-B_1-PhytoP + *ent*-16-B_1-PhytoP, and *ent*-16-*epi*-16-F_{1t}-PhytoP + *ent*-16-F_{1t}-PhytoP. In other words, they additionally detected *ent*-16-*epi*-16-F_{1t}-PhytoP + *ent*-16-F_{1t}-PhytoP, even though did not detect 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, and 9-L_1-PhytoP + *ent*-9-L_1-PhytoP (Table 2). However, the level of total PhytoPs (14.64 ng/mL) in that branded extra virgin olive was within the range found under our experimental conditions in Cornicabra extra virgin olive oil (9.18−19.31 ng/mL). Taking into consideration that both olive oils were extra virgin and that analyses were performed with the same method, using synthetic standards, it is clear that these differences in composition can be ascribed to the decisive effect of cultivar, oil extraction technology, and/or storage conditions prone to autoxidation.[27]

To the best of our knowledge, no previous information exists on the effect of the water deficit on the PhytoPs content. In this sense, the increase in PhytoPs by water deficit effect during pit hardening (phase II of fruit growth) (Table 2) showed that this phenological period can be noncritical considering fruit yield,[18] but is clearly critical for PhytoPs content in olive oil. In this sense, Gómez del Campo and García[45] showed that virgin olive oils from deficit irrigated stress from the end of fruit drop to the

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end of July exhibited significantly higher oxidative stability, which coincided with significantly higher contents in phenol derivatives, although deficit irrigation strategies in summer did not determine significant changes in the parameters legally established for evaluating the level of commercial quality of the virgin oils. Moreover, this increase in PhytoPs content could be related to the fact that under drought stress the frail balance between reactive oxygen species (ROS) and scavenging that defines the normal steady-state level of intracellular ROS suffers an upward shift enhancing ROS production due to stomatal closure and the concomitant limitation on CO_2 fixation.[46] A consequence of this enhanced ROS formation in plants is the formation of an array of lipid peroxidation products, including structural congeners of jasmonates, the PhytoPs.[24]

Because all PhytoPs are induced by oxidative stress (OS) from common precursors, the G_1-PhytoPs,[24,47] the fact that the behavior observed in each PhytoP in response to water deficit was not similar (Table 2) indicated that those which increased their contents in the T1 extra virgin olive oil might require a lower OS level to be induced from the precursor and could be used as early markers of water deficit in extra virgin olive oil.

From an nutritional point of view, the increase in total PhytoPs in T1 and T2 olive oil, nearly 2-fold with respect to control (T0) olive oil (Table 2), could be considered a potential beneficial aspect of the extra virgin olive oils from trees cultivated under water deficit conditions during pit hardening, because even though there is no detailed knowledge of the biological effects of PhytoPs, there is some evidence of their beneficial effects on human health, such as regulation of immune function[28,48] and anti-inflammatory and apoptosis-inducing activities.[29]

The fact that the relationships between 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP or a racemic mixture of 16-B_1-PhytoP + *ent*-16-B_1-PhytoP and minimum Ψ_{stem} values in phase II were tighter using second-order polynomic equations, whereas the relations for 9-*epi*-9-D_{1t}-PhytoP or 9-D_{1t}-PhytoP using linear or quadratic equations showed similar levels of significance for their coefficients of determination and the relationship for a racemic mixture of 9-L_1-PhytyoP + *ent*-9-L_1-PhytyoP was defined only by a linear equation (Figure 5), could indicate different development levels of the same behavior. In this sense, Horner[49] indicated that the relationship between plant water status and secondary metabolite contents is defined by a quadratic relationship. Also, some authors showed that the relationship between plant water status and some metabolite contents is not defined by a linear correlation when a wide range of plant water status is considered.[50,51] Then, the first-order relationship between plant water deficit and PhytoPs content (Figure 5) could correspond to the first phase of the quadratic relationship proposed by Horner,[49] indicating that a more severe water deficit could be necessary to induce a decrease in the 9-*epi*-9-D_{1t}-PhytoP, 9-D_{1t}-PhytoP, or a racemic mixture of 9-L_1-PhytoP + *ent*-9-L_1-PhytoP content.

The fact that most equations in Tables 3 and 4 showed a negative intercept could contraindicate the use these equations under high Ψ_{stem} values because negative PhytoPs values could be predicted, which does not make sense from a metabolic or physiological point of view. Nevertheless, PhytoPs values could be predicted using these equations when Ψ_{stem} values were within the range of values used to determine these equations (minimum Ψ_{stem} values in phase II from −3.12 to −1.73 MPa). Therefore, using these Ψ_{stem} boundary values negative PhytoPs values are not predicted.

Overall, the results showed for the first time that pit hardening (phase II of olive fruit growth), a phenological period noncritical for fruit yield, is clearly critical for extra virgin olive oil composition because water deficit enhances the PhytoPs content, with the concomitant potential beneficial aspects on human health because they are bioavailable in humans according to previous results.[48] Also, qualitative and quantitative differences in PhytoPs content with respect to those reported by other authors indicate a decisive effect of cultivar, oil extraction technology, and/or storage conditions prone to autoxidation. The content of each individual PhytoP in the olive oil from T0, T1, and T2 treatments could define different phases of the same behavior. In this sense, when minimun Ψ_{stem} values in phase II were above −2.5 MPa, 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, and a racemic mixture of 16-B_1-PhytoP + *ent*-16-B_1-PhytoP values increased sharply as Ψ_{stem} decreased, and when Ψ_{stem} values were below this threshold value, the relationship changed and any further reduction in Ψ_{stem} was associated with a decrease in PhytoPs contents. In contrast, when minimun Ψ_{stem} values in phase II decreased from −1.73 to −3.12 MPa, the 9-*epi*-9-D_{1t}-PhytoP, 9-D_{1t}-PhytoP, and a racemic mixture of 9-L_1-PhytoP + *ent*-9-L_1-PhytoP increased, indicating that a more severe water deficit could be necessary to induce a decrease in their contents. Therefore, from a physiological and agronomical point of view, 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, and a racemic mixture of 16-B_1-PhytoP + *ent*-16-B_1-PhytoP could be considered as early candidate biomarkers of water stress in olive tree.

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Notes

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ABBREVIATIONS USED

ALA, α-linolenic acid; DOY, day of the year; ETo, estimated according to daily crop reference evapotranspiration; ETc, crop irrigation requirements; IsoPs, isoprostanes; MRM, multiple reaction monitoring; OS, oxidative stress; PG, prostaglandin; PhytoPs, phytoprostanes; PUFAs, polyunsaturated fatty acids; RDI, regulated deficit irrigation; ROS, reactive oxygen species; SPE, solid phase extraction; Ψ_{stem}, stem water potential; UHPLC-QqQ-MS/MS, ultrahigh-performance liquid chromatography coupled to triple-quadrupole mass spectrometry

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Research Article

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Effect of the season on the free phytoprostane content in Cornicabra extra virgin olive oil from deficit-irrigated olive trees

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Abstract

BACKGROUND: The effect of regulated deficit irrigation (RDI) on the phytoprostane (PhytoP) content in extra virgin olive (*Olea europaea* L., cv. Cornicabra) oil (EVOO) was studied. During the 2012 and 2013 seasons, T0 plants were irrigated at 100% ETc, while T1 and T2 plants were irrigated avoiding water deficit during phases I and III of fruit growth and saving water during the non-critical phenological period of pit hardening (phase II), developing a more severe water deficit in T2 plants. In 2013, a fourth treatment (T3) was also performed, which was similar to T2 except that water saving was from the beginning of phase II to 15 days after the end of phase II.

RESULTS: 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, 9-D_{1t}-PhytoP, 16-B_1-PhytoP and 9-L_1-PhytoP were present in Cornicabra EVOO, and their contents increased in the EVOO from RDI plants.

CONCLUSION: Deficit irrigation during pit hardening or for a further period of 2 weeks thereafter to increase irrigation water saving is clearly critical for EVOO composition because of the enhancement of free PhytoPs, which have potential beneficial effects on human health. The response of individual free PhytoPs to changes in plant water status was not as perceptible as expected, preventing their use as biomarkers of water stress.
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Keywords: mass spectrometry; water stress integral; UHPLC; water stress; regulated deficit irrigation; inter-seasonal behaviour in olive oil

INTRODUCTION

Olive trees are widely cultivated in Mediterranean coast countries, and olive oil consumption has increased from 1.67×10^6 t in 1990 to 3.09×10^6 t in 2011.[1] This strong increase could be due to the direct relationship between olive oil consumption and health benefits.[2,3]

According to the European regulation,[4] virgin olive oil means oil obtained solely by mechanical or other physical means under conditions that do not lead to alterations in the oil. In addition, extra virgin olive oil (EVOO) means virgin olive oil having a maximum free acidity, in terms of oleic acid, of 0.8 g per 100 g, and other characteristics detailed in the commission implementing regulation,[5] which contribute to the fact that these oils exhibit the best organoleptic features, with tastes and aromas which perfectly reproduce the fruit of origin. Moreover, EVOOs can have different proportions of oleic acid, polyphenols, squalene, triterpenes, tocopherols and other minor constituents, depending on cultivar,[2,6] season[2] and agricultural management, mainly irrigation,[6,7] fertilization[8,9] and harvesting date.[2,10]

Phytoprostanes (PhytoPs) are components of an oxidative injury-sensing, archaic signaling system which could not only be potentially used as markers of oxidative degradation of plant-derived foodstuffs but are also considered biologically

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active molecules.[11,12] Although there is little information about the biological effects of PhytoPs, it is known that free PhytoPs are the only prostanes able to be absorbed by humans.[13] They have been identified in urine and found esterified to lipids in the plasma of healthy men consuming vegetable oil,[14] with some evidence that they can modulate the function of the immune and vascular systems.[15,16] Despite the fact that most olive orchards in the Mediterranean area have been traditionally grown under rain-fed conditions, it has been demonstrated that irrigation is a vital practice in improving both olive production and productivity.[17] Nevertheless, the Mediterranean coast is characterized by the aridity of the climate and the persistent shortage of water resources. In recent years, strong competition for the water that is available has arisen with other non-agricultural users. To cope with this water scarcity situation, deficit irrigation strategies such as regulated deficit irrigation (RDI) may offer an approach for saving water in woody crops by minimizing or eliminating negative impacts on yield and crop revenue.[18]

RDI strategies are accomplished by imposing water deficits during the phenological stages relatively tolerant to water stress (non-critical periods). Studies on olive trees have reported that pit hardening is the most resistant (non-critical) phenological period to drought.[19] Traditionally, it has been assumed that oil accumulation starts towards the end of the olive pit-hardening period.[20] However, recently, Pérez-López *et al.*[21] showed that olive oil accumulation starts a few days after the onset of pit sclerification rather after the end of massive pit hardening. Thus, at the end of olive pit hardening, the olive fruit has accumulated between 25 and 40% of its final oil content.

For the above reasons, this work was focused on (1) studying the effect of different water deficit levels during the early stages of oil accumulation in olive fruit (pit-hardening period) on free PhytoP levels, and whether a longer water deficit situation during olive oil accumulation (just after pit hardening) is able to enhance free PhytoP accumulation, and (2) evaluating the effect of season on the profile and contents of free PhytoPs in Cornicabra EVOO.

MATERIALS AND METHODS

Plant material and experimental conditions

The experiment was carried out in 2012 and 2013 on a farm near the city of Ciudad Real, Spain (39° 00′ N, 3° 56′ W, altitude 640 m). The plant material consisted of self-rooted 14-year-old olive trees (*Olea europaea* L., cv. Cornicabra). Tree spacing followed a 7 m × 4.76 m (300 trees ha^{-1}) pattern.

The soil is an alkaline (pH 8.1) and shallow Alfisol Xeralf Tipic Haploxeralf with a clay loam texture, low electrical conductivity (0.2 dS m^{-1}), organic matter (10.5 g kg^{-1}), nitrogen (1.2 g kg^{-1}) and potassium (17×10^{-4} mol kg^{-1}) and high cationic exchange capacity (0.186 mol kg^{-1}). A discontinuous petrocalcic horizon is located between 0.75 and 0.85 m. The soil water content for the first 0.3 m depth is 228 g kg^{-1} at field capacity (soil matrix potential −0.03 MPa) and 121 g kg^{-1} at permanent wilting point (soil matrix potential −1.5 MPa), whereas from 0.3 to 0.75 m it was 430 and 211 g kg^{-1} respectively. The orchard was managed under no-tillage conditions; weeds were controlled with post-emergence herbicides. Pest control and fertilization practices were those usually used by local growers. Irrigation was performed daily and during the night using a drip irrigation system with four emitters (each delivering 8 L h^{-1}) per tree and irrigation water with an electrical conductivity of 2.6–2.9 dS cm^{-1}.

Treatments

From mid-May to the end of September, control plants (T0 treatment) were irrigated at 100% ETc of the previous week. When midday stem water potential (Ψ_{stem}) values of that week were below −1.2 MPa before pit hardening (phase I of fruit growth) or −1.4 MPa during and after pit hardening (phases II and III of fruit growth respectively), irrigation amounts were increased by 10% in order to ensure no irrigation-related stress.[22] In addition to T0, two RDI treatments (T1 and T2) were applied, which were based on avoiding water deficit during phases I and III of fruit growth, maintaining Ψ_{stem} values around the threshold values indicated for T0 plants, and saving irrigation water during the non-critical phenological period of pit hardening (phase II), developing different situations of water deficit (threshold Ψ_{stem} values of −2.00 and −3.00 MPa in T1 and T2 plants respectively). In 2013, a fourth treatment (T3) was also performed, which was based in an irrigation protocol similar to that used for T2, except that the Ψ_{stem} threshold value of −3.00 MPa was used from the beginning of phase II to 15 days after the end of phase II.

The irrigation protocol in T1, T2 and T3 plants was based on that proposed by Moriana *et al.*[22] Irrigation amounts were determined weekly based on the Ψ_{stem} measurements and irrigation was begun when measured Ψ_{stem} values were lower than the threshold values suggested.

During the 2012 and 2013 irrigation seasons (days of year (DOY) 134–273 and 148–273 respectively), ETc was 202 and 183 mm respectively. Cumulative amounts of applied water in T0, T1 and T2 respectively, measured by means of flow meters integrated in the irrigation system, were 407, 196 and 141 mm in the 2012 season and 338, 164 and 112 mm in the 2013 season, whereas the irrigation water amount in T3 (2013) was 88 mm.

Extra virgin olive oil extraction

Olive fruits were harvested in mid-December (DOY 345 in 2012 and DOY 346 in 2013). EVOO was extracted using an Abencor system and the oil obtained was separated by decanting. Oil samples were filtered and stored in amber glass bottles without headspace at −18 °C in darkness until analysis.

Measurements

Climate, plant water status and yield

Meteorological data, namely air temperature, solar radiation, air relative humidity, rainfall and wind speed 2 m above the soil surface, were collected in a nearby automatic weather station. Mean daily air vapour pressure deficit (VPD_m) was calculated according to Allen *et al.*[23] Crop irrigation requirements (ETc) were estimated according to daily crop reference evapotranspiration (ETo), calculated using the Penman–Monteith equation,[23] and a crop factor based on the time of year[24] and taking into consideration canopy size.[25]

Midday (12:00 solar time) stem water potential (Ψ_{stem}) was measured on the middle third of the trees, in fully developed leaves from two trees of each replicate, enclosing leaves in small black plastic bags covered with aluminium foil for at least 2 h before measurements in the pressure chamber (Model 3005, Soil Moisture Equipment Co., Santa Barbara, CA, USA). Minimum daily leaf conductance values were measured with a steady state porometer (LICOR 1600, Lincoln, NE, USA) at midday on the abaxial surface of the leaves and in a similar number and type of leaves as used for the Ψ_{stem} measurements.

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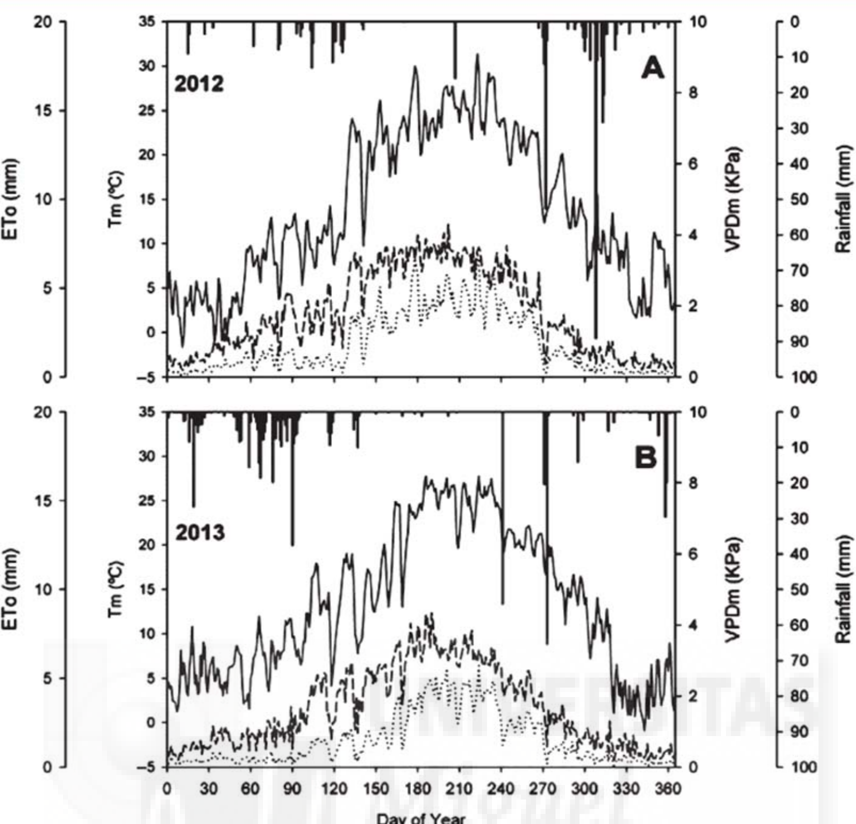

Figure 1. Daily mean temperature (Tm, –), crop reference evapotranspiration (ETo, – –), mean daily air vapour pressure deficit (VPDm, ·····) and daily rainfall (vertical lines) during years (A) 2012 and (B) 2013.

In order to describe the cumulative effect of the water deficit, the water stress integral ($S_{\Psi_{stem}}$) was calculated from the Ψ_{stem} data using the expression proposed by Myers.[26] Moriana *et al.*[22] suggested the use of stem potential threshold values of −1.2 MPa before the beginning of pit hardening and −1.4 MPa from that moment to harvesting. This change improves the capacity of comparison between different experiments and/or locations. The expression used was

$$S_{\Psi_{stem}} = |\sum (\overline{\Psi}_{stem} - b) \times n|$$

where $\overline{\Psi}_{stem}$ is the average Ψ_{stem} value for any interval, b is the stem potential threshold value and n is the number of the days in the interval.

Marketable olive fruits were harvested and the mean fruit weight was determined according to the weight and number of fruits per box in randomly selected boxes per replicate.

Chemicals and reagents

PhytoPs (9-F$_{1t}$-PhytoP, 9-*epi*-9-F$_{1t}$-PhytoP, *ent*-16-F$_{1t}$-PhytoP, *ent*-16-*epi*-16-F$_{1t}$-PhytoP, 9-D$_{1t}$-PhytoP, 9-*epi*-9-D$_{1t}$-PhytoP, 16-B$_1$-PhytoP and 9-L$_1$-PhytoP, named according to the Taber/Roberts nomenclature, which was approved by IUPAC for identification of structures unequivocally[27,28]) were synthesized according to the published procedures.[27,29,30] Solid phase extraction (SPE) cartridges (Strata X-AW, 100 mg per 3 mL) were acquired from Phenomenex (Torrance, CA, USA). Bis-Tris (bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane) was obtained from Sigma-Aldrich (St Louis, MO, USA) and hexane from Panreac (Castellar del Vallés, Barcelona, Spain). Liquid chromatography/mass spectrometry (LC/MS)-grade solvents methanol and acetonitrile were purchased from J.T. Baker (Phillipsburg, NJ, USA).

Free phytoprostane extraction and UHPLC/QqQ-MS/MS analyses

EVOO samples (1 mL) were subjected to dilution and SPE using Strata X-AW cartridges, following the procedure described by Collado-González *et al.*[31] Briefly, samples diluted in 10 mL of *n*-hexane and rediluted in 2 mL of methanol were further diluted in 2 mL of Bis-Tris buffer and applied to previously conditioned and equilibrated cartridges, which were subsequently washed to remove unwanted compounds. Target compounds were eluted with methanol and dried using a SpeedVac concentrator (Savant SPD121P, Thermo Scientific, Waltham, MA, USA). Dry extracts were reconstituted with 200 μL of a mixture of A/B solvents (90:10, v/v), sonicated and filtered through a 0.45 μm filter (Millipore, Bedford, MA, USA). Samples (20 μL) were analysed in a UHPLC/QqQ-MS/MS system (Agilent Technologies, Waldbronn, Germany).

Free PhytoP separations were performed using a BEH C18 column (2.1 mm × 50 mm, 1.7 μm; Waters, Milford, MA, USA). The mobile phase used was a mixture of (A) water/acetic acid

(99.99:0.01, v/v) and (B) methanol/acetic acid (99.99:0.01, v/v). The flow rate (0.2 mL min^{-1} using a linear gradient), electrospray ionization (ESI) conditions and ion optics were as described previously.[31] The MS analysis was applied in multiple reaction monitoring (MRM) negative ESI mode. Data acquisition and processing were performed using MassHunter Version B.04.00 software (Agilent Technologies). For quantification of free PhytoPs, all PhytoPs synthesized were used.

Statistical design and analysis

The design of the experiments was completely randomized with four replications, each replication consisting of five adjacent tree rows, each with nine trees. Measurements were taken on the inner trees of the central row of each replicate, which were very similar in appearance (leaf area, trunk cross-sectional area, height, ground shaded area, etc.). Data were analysed using SPSS software.[32] Analysis of variance was performed and mean values were compared by Tukey's test ($P < 0.05$). Values for each replicate were averaged before the mean and standard error (SE) of each treatment were calculated. To check the regression model hypothesis (linearity, homoscedasticity, normality and independency), the Kolmogorov–Smirnov test was used with the Liliefors correction and the Shapiro–Wilk test for normality and the Levene test for homoscedasticity on the typified residuals. To do the Levene test, data were divided into two groups according to the median of abscissa data.

RESULTS

Climate, plant water status and fruit yield

During the irrigation season, average daily maximum and minimum air temperatures were 31.4 and 13.1 °C in 2012 and 31.3 and 12.7 °C in 2013 (Fig. 1). VPD_m ranged from 0.09 to 3.50 kPa in 2012 and from 0.09 to 2.85 kPa in 2013 (Fig. 1). Accumulated ETo was 856 and 721 mm in 2012 and 2013 respectively (Fig. 1). There were important differences in rainfall in 2012 and 2013, with levels of 90 and 326 mm respectively before the beginning of the irrigation seasons and 96 and 159 mm respectively during the irrigation season (Fig. 1).

In both seasons, $S_{\Psi_{stem}}$ values in all irrigation treatment plants increased progressively during the measurement period, with the characteristic that the highest increases were observed mainly in T1, T2 and T3 plants during pit hardening, coinciding with deficit irrigation (Fig. 2). $S_{\Psi_{stem}}$ values in T0, T1 and T2 were higher in 2012 than in 2013 (Fig. 2). In 2012, $S_{\Psi_{stem}}$ values in T1 and T2 plants were similar and, from the final stage of pit hardening to the end of the measurement period, higher than those in T0. In 2013, from the beginning of pit hardening, significant differences between treatments were found, with T3 plants showing the highest and T0 plants the lowest $S_{\Psi_{stem}}$ values, while T1 and T2 plants showed $S_{\Psi_{stem}}$ values similar to those in T0 and T3 plants (Fig. 2).

No differences between treatments were observed in leaf conductance values except during the pit-hardening period, when on some dates significant differences between treatments were observed (Fig. 3). On those dates, T0 plants reached leaf conductance values higher than those of RDI (T1, T2 and T3) plants, which showed similar leaf conductance values (Fig. 3).

No differences between treatments were found in fruit yield, mean crop load and average fruit weight in both seasons (Table 1). However, significant differences between seasons were found in fruit yield and mean crop load because of the very low yield in 2012 (Table 1).

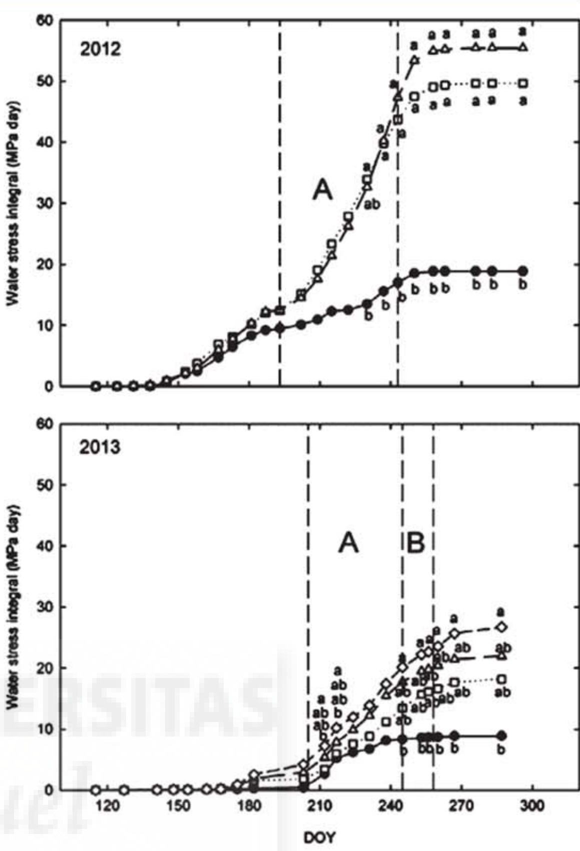

Figure 2. Water stress integral in T0 (●, –), T1 (□, ····), T2 (△, – –) and T3 (◇, - - -) during 2012 and 2013 irrigation seasons. Different letters on data points at each date indicate significant differences according to Tukey's test ($P \leq 0.05$). Broken vertical lines indicate pit hardening (A) and 15 days after pit hardening (B).

Free phytoprostane profile

Identification of free PhytoPs was performed on the basis of their pseudomolecular ion (*m/z* 327.2, 325.2 and 307.2) and their elution order according to their retention times as described in Fig. 4. The PhytoP profile of the Cornicabra EVOO is shown in Fig. 4, which indicates the presence of 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, 9-D_{1t}-PhytoP, 16-B_1-PhytoP and 9-L_1-PhytoP.

Water deficit and season effects on free PhytoP content

The total content of free PhytoPs in the Cornicabra EVOO from the different irrigation treatments ranged from 9.18 to 19.31 ng mL^{-1} in 2012 and from 31.92 to 67.87 ng mL^{-1} in 2013, with F_{1t}-PhytoP being the dominant PhytoP class and the D_1-PhytoP class the minor component (Table 2). The total free PhytoP content in the oil increased as a result of the water deficit effect, although no significant differences between T1 and T2 contents in 2012 and T1, T2 and T3 contents in 2013 were found (Table 2). Also, the results indicated that the highest total free PhytoP contents in olive oil from the different irrigation treatments were obtained in the season of maximum yield (Tables 1 and 2).

The response of each free PhytoP to water stress was not the same and some inter-seasonal differences were observed (Table 2).

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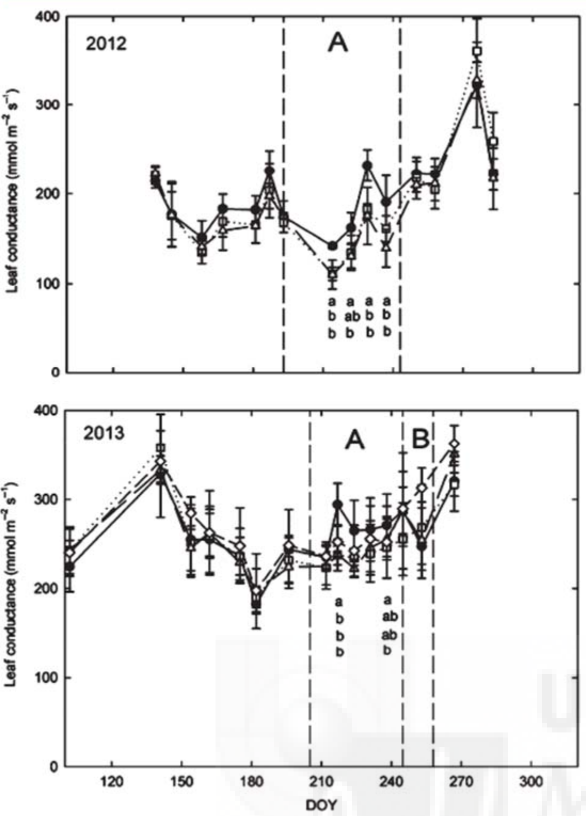

Figure 3. Midday leaf conductance values (mean ± SE, not shown when smaller than symbols) in T0, T1, T2 and T3 during 2012 and 2013 irrigation seasons. Symbols as in Fig. 2.

For example, 9-*epi*-9-F_{1t}-PhytoP content showed a similar response to water deficit to that observed in total free PhytoP content in both seasons, 9-F_{1t}-PhytoP showed similar content in T1 and T2 in 2012, whereas a gradual increase from T0 to T2 was observed in 2013, and 9-D_{1t}-PhytoP showed different content in T1 and T2 in 2012 and a content in T1 similar to that observed in T0 and T2 in 2013 (Table 2).

Similarly, the inter-seasonal behaviour of some free PhytoPs was different from that observed for total free PhytoPs (Table 2). In this sense, the dominant free PhytoPs (9-F_{1t}-PhytoP and 9-*epi*-9-F_{1t}-PhytoP) showed a behaviour similar to that observed for total free PhytoP content. In contrast, 9-*epi*-9-D_{1t}-PhytoP and 16-B_1-PhytoP contents in the different irrigation treatments were similar in both seasons. Furthermore, 9-L_1-PhytyoP content in T1 and T2 and 9-D_{1t}-PhytoP content in T2 were higher in 2012 (Table 2).

Results showed that total and individual contents of free PhytoPs in EVOO from all irrigation treatments applied in 2012 and 2013 presented significant first-order correlations with respect to achieved $S_{\Psi_{stem}}$ values, except the correlations obtained in 2012 for 9-F_{1t}-PhytoP and 9-D_{1t}-PhytoP (Table 3). These equations were characterized by (1) very low values of slope in most of them, except in the equations of the dominant free PhytoP class (F_{1t}-PhytoP) in 2013, which showed the highest slope values, (2) the highest significant correlations in 2012 and 2013 being found

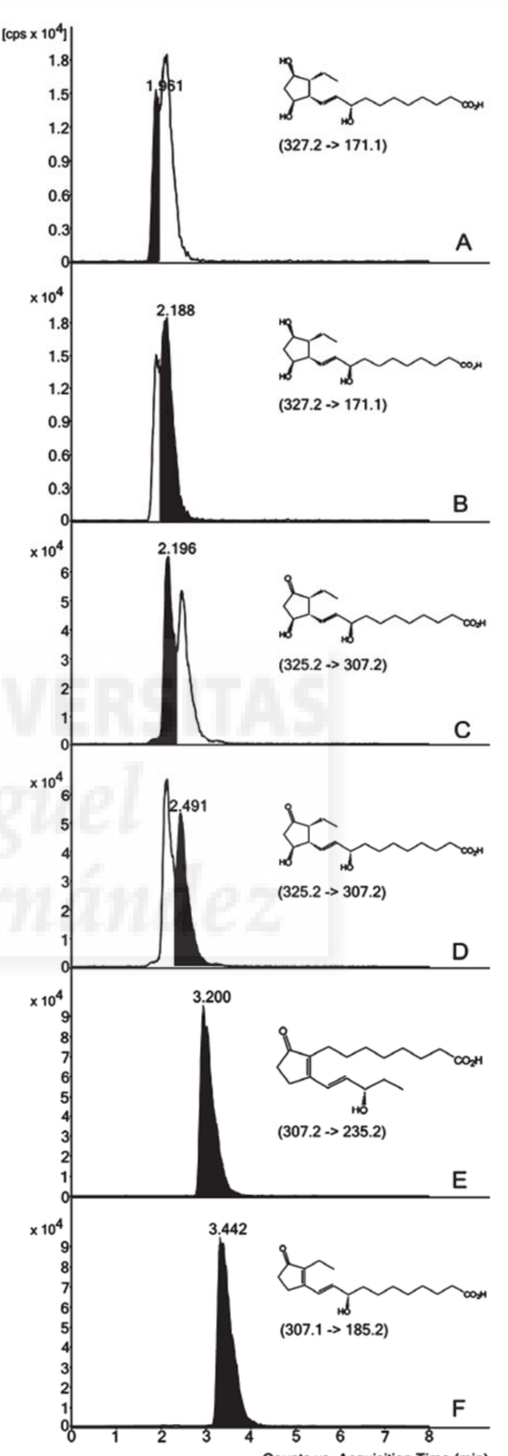

Figure 4. UPHLC/QqQ-MS/MS chromatograms of PhytoPs detected in olive oil, namely 9-F_{1t}-PhytoP (A), 9-*epi*-9-F_{1t}-PhytoP (B), 9-*epi*-9-D_{1t}-PhytoP (C), 9-D_{1t}-PhytoP (D), 16-B_1-PhytoP (E) and 9-L_1-PhytoP (F), and their preferential MRM transitions.

Table 1. Effect of irrigation treatments (T0, T1, T2 and T3) on Cornicabra olive fruit yield, mean crop load and average fruit weight in 2012 and 2013 seasons

Season	Treatment	Yield (kg per tree)	Crop load (number of fruits per tree)	Fruit weight (g)
2012	T0	1.95aB	780aB	2.50aA
	T1	4.88aB	2228aB	2.19aA
	T2	3.02aB	1135aB	2.66aA
2013	T0	31.92aA	16583aA	1.93aA
	T1	28.74aA	14468aA	1.99aA
	T2	31.01aA	16204aA	1.91aA
	T3	31.02a	16684a	1.86a

Means within a column followed by different capital letters and within a column for each season followed by different lowercase letters are significantly different at $P = 0.05$ by Tukey's test.

for 16-B_1-PhytoP and 9-F_{1t}-PhytoP respectively and (3) all significant first-order equations predicting a positive free PhytoP value in the range of $S_{\Psi stem}$ studied (Table 3).

DISCUSSION

Taking into consideration ETc and irrigation water levels applied in the 2012 and 2013 seasons, T0 plants were irrigated above crop water requirements (202 and 185% ETc respectively). Moreover, deficit-irrigated plants reduced the seasonal water applied with respect to estimated ETc by 3 and 10% in T1 plants and 30 and 39% in T2 plants in 2012 and 2013 respectively and by 52 % in T3 plants in 2013.

To explain why $S_{\Psi stem}$ and leaf conductance values in all treatments in 2012 were significantly higher and lower respectively than those in 2013 in spite of the fact that irrigation water amounts were similar in the two seasons (Fig. 2), it is key to take into account the scarcity of rainfall in 2012 and that the drip irrigation system in the olive orchard was installed in March 2012. Thus in most of the 2012 season the root system dissemination could have been that typical of olive trees under rain-fed conditions, whereas in 2013 a very important proportion of the root system could be confined to the volume of soil wetted by drip irrigation.[33] Consequently, the irrigation efficiency in most of 2012 could be lower than that in 2013, leading to $S_{\Psi stem}$ and leaf conductance values in T0, T1 and T2 clearly higher and lower respectively in 2012 than in 2013 (Fig. 2). Additionally, the very low yield and crop load in 2012 (Table 1) could contribute to the fact that $S_{\Psi stem}$ and leaf conductance values were higher and lower respectively than those in 2013.[34,35] The fact that rainfall reached 485 mm during the 2013 irrigation season can explain why the differences in $S_{\Psi stem}$ and leaf conductance values between treatments were not as important as expected (Fig. 2).

The very important difference in the yield obtained in the two seasons (Table 1) matches with the better adaptation of the trees to the drip irrigation system in 2013 and with the characteristic alternate bearing pattern of olive trees, which exhibits a frequency and intensity regulated by the genotype and growing conditions.[36] The increase in 2013 fruit yield seemed to be due to the higher number of fruits, because the average fruit weight was similar in the two seasons (Table 1). The fact that in both seasons no effect of T1 and T2 on fruit yield was observed confirms that the pit-hardening phenological period is not a critical period from the yield point of view.[19] However, the increase in total free PhytoP content in Cornicabra EVOO due to T1 and T2 clearly indicated that the pit-hardening period is critical for the PhytoP content in olive oil. Moreover, the fact that the yield was not affected and the free PhytoP content in EVOO increased in response to T3 indicated that it is possible to extend the water deficit period by 2 weeks after pit hardening, leading to a similar effect to that of T1 and T2 but increasing irrigation water saving (Tables 1 and 2).

The increase in free PhytoP content in EVOO from deficit-irrigated trees (T1, T2 and T3) could be related to stomatal regulation (Fig. 3) and the concomitant limitation on CO_2 fixation under water stress, which enhances reactive oxygen species formation[37] and promotes the formation of various lipid peroxidation products, including PhytoPs.[38]

It is difficult to understand why the highest total free PhytoP content in the EVOO from the different irrigation treatments was obtained in the season of better plant water status (Fig. 2, Table 2). In this sense, Berenguer *et al.*[39] showed that the level of linolenic acid, the precursor of PhytoPs, increased significantly with irrigation in one year out of two. However, other authors have found inconsistent fluctuations in some fatty acids from year to year.[39–41] In any case, it can be considered that the composition of EVOO results from a very complex multivariate interaction between the genotypic potential and the environmental, agronomic and technological factors that characterize fruit growth and ripening as well as oil extraction and storage.[20,42] Thus the minor components in EVOO may vary independently, depending on factors that are not always interrelated.

Thoma *et al.*[38] and Loeffler *et al.*[12] indicated that free PhytoPs are excellent biomarkers of oxidative degradation of plant-derived foodstuffs. In this sense, total and individual free PhytoP contents in Cornicabra oil increased as a result of water stress (Table 2). However, this response was not as perceptible as expected, because most correlations in Table 3 present very low values of slope, indicating that a large change in $S_{\Psi stem}$ is needed to get any change in individual free PhytoP contents. Also, it is important to consider that most correlations for individual free PhytoPs changed from one season to another (Table 3). In the light of these results, it is difficult to conclude that these individual free PhytoPs can be used as biomarkers of water stress, probably owing to the fact that the only requirement for forming PhytoPs is the presence of linolenic acid and molecular oxygen. Nevertheless, considering that there is some evidence that PhytoPs are biologically active lipids,[11,16,43] it is clear that the overall significant increase in free PhytoPs constitutes a potential nutritionally beneficial aspect of Cornicabra EVOO from trees cultivated under water deficit conditions during summer.

CONCLUSION

The results showed for the first time that water deficit during pit hardening or for a further period of 2 weeks thereafter to increase irrigation water saving is clearly critical for EVOO composition because of the enhancement of free PhytoPs. Moreover, an important inter-seasonal change in PhytoP content was observed, probably due to a very complex multivariate interaction between factors that are not always interrelated. Both circumstances are crucial for potential beneficial effects of Cornicabra EVOO on human health. The response of individual free PhytoPs to changes in plant water status was not as perceptible as expected, preventing their use as biomarkers of water stress when it is evaluated using $S_{\Psi stem}$ values.

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Table 2. Effect of irrigation treatments (T0, T1, T2 and T3) on PhytoP contents (ng mL^{-1}) in Cornicabra EVOO in 2012 and 2013 seasons

		Treatment			
Phytoprostane	Year	T0	T1	T2	T3
9-F_{1t}-PhytoP	2012	5.24bB	10.00aB	9.73aB	-
	2013	19.31cA	32.61bA	39.19aA	39.26a
9-*epi*-9-F_{1t}-PhytoP	2012	2.83bB	4.86aB	5.26aB	–
	2013	11.68bA	23.01aA	27.28aA	25.81a
9-*epi*-9-D_{1t}-PhytoP	2012	0.13bA	0.13bA	0.26aA	–
	2013	0.16bA	0.22aA	0.23aA	0.23a
9-D_{1t}-PhytoP	2012	ND	0.19bA	0.45aA	–
	2013	0.12b	0.15abA	0.21aB	0.22a
16-B_1-PhytoP	2012	0.32bA	0.72aA	0.75aA	–
	2013	0.41bA	0.52 abA	0.63aA	0.60a
9-L_1-PhytyoP	2012	0.66bA	1.28bA	2.88aA	–
	2013	0.25bA	0.25bB	0.34aB	0.34a
Total	2012	9.18bB	17.18aB	19.31aB	–
	2013	31.92bA	56.76aA	67.87aA	66.45a

Means within a row for each PhytoP and season followed by different lowercase letters and within a column for each PhytoP and treatment followed by different capital letters are significantly different at $P = 0.05$ by Tukey's test. ND, not detected.

Table 3. Intercept (*a*), slope (*b*), coefficient of determination (r^2), number of data points (*n*) and mean square error (MSE) of first-order linear equations ($y = a + bx$) between each PhytoP content (ng mL^{-1}) and water stress integral (MPa day) in phases II and II, using all data pooled

Phytoprostane	Year	*a*	*b*	r^2	*n*	MSE
9-F_{1t}-PhytoP	2012	5.4214^{*} (1.6274)	0.0965^{NS} (0.0477)	0.3694^{NS}	9	5.3702
	2013	17.1334^{***} (3.3534)	0.8919^{***} (0.1771)	0.6444^{***}	16	29.2136
9-*epi*-9-F_{1t}-PhytoP	2012	2.6388^{**} (0.6010)	0.0558^{*} (0.0176)	0.5895^{*}	9	0.7324
	2013	9.8121^{**} (2.7128)	0.6999^{***} (0.1432)	0.6304^{***}	16	19.1181
9-*epi*-9-D_{1t}-PhytoP	2012	0.0881^{NS} (0.0394)	0.0028^{*} (0.0012)	0.4535^{*}	9	0.0031
	2013	0.1597^{****} (0.2207)	0.0028^{*} (0.0012)	0.2960^{*}	16	0.00013
9-D_{1t}-PhytoP	2012	-0.075^{NS} (0.2529)	0.0098^{NS} (0.0061)	0.3868^{NS}	9	0.0202
	2013	0.1022^{***} (0.0235)	0.0042^{**} (0.0012)	0.4542^{**}	16	0.0014
16-B_1-PhytoP	2012	0.2439^{*} (0.0858)	0.0117^{**} (0.0025)	0.7571^{**}	9	0.0149
	2013	0.3609^{****} (0.0477)	0.0102^{**} (0.0025)	0.5391^{**}	16	0.0059
9-L_1-PhytyoP	2012	0.3320^{NS} (0.0609)	0.0424^{*} (0.0178)	0.4471^{*}	9	0.7511
	2013	0.2191^{****} (0.0293)	0.0042^{*} (0.0016)	0.3413^{*}	16	0.0022
Total	2012	8.6264^{*} (2.5854)	0.2194^{*} (0.0757)	0.5456^{*}	9	13.5541
	2013	27.7895^{***} (5.9164)	1.6130^{***} (0.3124)	0.6557^{***}	16	90.9377

Values are mean ± SE (in parentheses). Significance:
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; NS, non-significant.

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The phytoprostane content in green table olives is influenced by Spanish-style processing and regulated deficit irrigation

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ABSTRACT

No previous information exists on the effect of deficit irrigation during pit hardening and the processing of Manzanilla de Sevilla Spanish style green table olives on the phytoprostanes (PhytoPs) contents in the fruit flesh. In this paper, the influence of different irrigation treatments during pit hardening on the PhytoPs content of raw and processed olive flesh was studied. PhytoPs profile in the raw olive flesh was characterized by the presence of 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, 16-B_1-PhytoP and 9-L_1-PhytyoP.

Fruit yield and fruit size was not affected by deficit irrigation, but PhytoPs content in the raw and processed olive flesh was enhanced. After olive fruit processing only 9-F_{1t}-PhytoP and 9-*epi*-9-F_{1t}-PhytoP were detected, which enhanced their contents. Consequently, table olive tree culture under deficit irrigation conditions during pit hardening and the processing of its fruits to obtain Spanish-style olives can be considered as complementary actions to enhancing the PhytoP content and hence their potential beneficial effects on human health.

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1. Introduction

Olive fruit production in the Mediterranean basin, which was traditionally a rainfed crop, has increased, reaching around 700,000 tons and is now frequently irrigated (Eris & Barut, 1995; IOOC, 2013) because olive yield is highly correlated with applied irrigation water amounts (Moriana et al., 2013), being possible to raise olive crop productivity with very low volumes of applied water (Orgaz & Fereres, 2004).

In recent years, in addition to the aridity of the climate and the persistent scarcity of water resources in the Mediterranean agrosystems other non-agricultural users has arisen increasing pressure to reduce agricultural water consumption. An option for olive tree culture could pay special attention towards the use of deficit irrigation in phenological periods in which adverse effects on productivity are minimized (regulated deficit irrigation, RDI), allowing significant water savings with a minimum impact on yield and fruit quality (Naor, 2006). So, previously Goldhamer (1999) reported that pit hardening is a non-critical phenological period.

Table olives are prepared from the fruit of the olive tree (*Olea europaea* L.) because fresh olives are not edible (Boskou, Camposeo, & Clodoveo, 2015). Their consumption is increasing worldwide because table olives are a highly functional food with a high amounts of phenolic compounds and a balanced content of fats made up mainly of monounsaturated oleic acid (Bautista-Gallego et al., 2015; Boskou et al., 2015; Ramírez, Gandul-Rojas, Romero,

Abbreviations: ETo, annual reference evapotranspiration; ETc, crop evapotranspiration; PhytoP, phytoprostane; RDI, regulated deficit irrigation; SI, water stress integral; TGR, trunk growth rate; Ψ_{stem}, midday stem water potential.

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Brenes, & Gallardo-Guerrero, 2015).

The bioactivity and subsequently the quality of olive fruits are affected by several factors such as plant cultivar, olive fruit maturation and the technological process applied to turn olives edible. Among the different types of commercial table olives, Spanish-style green olives are the most popular. Their processing consists in lye treatment followed by typical lactic acid fermentation after brining (Bautista–Gallego et al., 2015; Boskou et al., 2015; Malheiro et al., 2014; Ramírez et al., 2015). Recent studies reported that fermented table olives with high phenolic content and high antioxidant activity displayed higher microbial growth inhibition (Malheiro et al., 2014). In addition, the application of methods that promote the growth of the desirable microbial strains like the pied-de-cuve technology or the use of a mixture of diverse chloride salts in the initial brine in the Spanish-style green Manzanilla olive fermentation can avoid the generation of undesired off-odours and off-flavours, favouring the quality of the final products (Bautista-Gallego et al., 2015; Martorana et al., 2015).

Concerning plant oxidative stress, phytoprostanes (PhytoPs) are prostaglandin analogs formed from α-linolenic acid via a nonenzymatic free radical-catalysed pathway (Jahn, Galano, & Durand, 2010). These bioactive compounds act as endogenous mediators capable of protecting cells from damage under various conditions related to oxidative stress (Loeffler et al., 2005), they play a role in regulation of immune function (Barden, Croft, Durand, Guy, & Mueller, 2009; Traidl-Hoffmann et al., 2005) and display potent anti-inflammatory and apoptosis-inducing activities similar to other prostanoids (Durand et al., 2011).

There are some qualitative and quantitative studies on the PhytoPs profile in different vegetable oils (Collado-González, Medina, et al., 2015; Collado-González, Perez-López, et al., 2015; Karg et al., 2007). However, no previous information has been provided concerning PhytoPs occurrence in olive fruit flesh, and the effect of the deficit irrigation during early fruit growth (phase I) or pit hardening (phase II) on these compounds. For these reasons, the present study aimed to look at the effect of different RDI (regulated deficit irrigation) treatments on the PhytoPs qualitative and quantitative profile in the olive flesh and if the elaboration process of Manzanilla de Sevilla Spanish style green table olives modify the PhytoPs content.

2. Materials and methods

2.1. Experimental conditions and treatments

The experiment was performed in a 0.5 ha olive (*Olea europaea* L., cv Manzanilla de Sevilla) adult orchard at the experimental farm of the IRNAS-CSIC in southwest of Spain (37° 17′ N, 6° 3′ W, 30 m a.s.l). The soil is a sandy loam (Xerochrept) of a depth varying between 0.9 and 2 m with a quite homogeneous root zone with average values of 735 g/Kg coarse sand, 47 g/Kg fine sand, 70 g/Kg silt and 148 g/Kg clay. The content in organic matter is between 6 and 10 g/kg. The values of soil water content at field capacity (−0.01 MPa) and wilting point (−1.5 MPa) were 0.21 and 0.10, respectively. Crop water requirements (ETc) were estimated taking into account crop reference evapotranspiration (ETo), a crop coefficient and the percentage of ground covered by the crop (Fernández et al., 2006).

The experiment was performed in the 2012 and 2013 growing seasons, in which pit hardening period took place from around early July to end of August. Plants were irrigated daily and during the night from mid-April (day of the year 105 to the mid-September (day of the year 258) using a drip irrigation system with 5 emitters (each delivering 8 L/h) per tree and irrigation water with an electrical conductivity of 2.0 dS/m.

Control treatment trees (T0) were over-irrigated (125% ETc). T1 and T2 treatment trees were submitted to RDI according to trunk diameter fluctuations data (Moriana et al., 2013). Both seasons, before and after pit hardening, T1 plants were irrigated in order to maintain trunk growth rate (TGR) near to that of T0 trees. During 2012 pit hardening T1 plants were irrigated for obtaining maximum daily trunk shrinkage values around the 90% of the maximum daily trunk shrinkage values observed in T0 plants. During 2013 pit hardening, T1 plants were irrigated only when TGR was lower than −10 μm day^{-1}. Both seasons, before pit hardening period, T2 plants were irrigated in order to maintain TGR values around the 50% of those observed in T0 plants. During pit hardening, T2 plants were irrigated in order to obtain maximum daily trunk shrinkage values around the 75% of those observed in T0 plants. After pit hardening, T2 plants were irrigated in order to obtain TGR values similar to those observed in T0 plants.

2.2. Processing of green olives

At mid-September, when the olive fruits skin from each irrigation treatment had reached a yellowish green colour (Hermoso, Uceda, Frías, & Beltrán, 2006), they were harvested and processed to obtain so called ‘Spanish-style’ or ‘Seville-style’ olives (Sánchez, García, & Rejano, 2006). Fruit samples were dressed in a table olive processing company (Cooperativa Las Virtudes, La Puebla de Cazalla, Sevilla, Spain) with the usual processing steps for these fruits. Fruit processing began with a lye treatment (NaOH, 20–40 g/L) for 6–12 h until 75% of the fruit fleshes were dyed (IOOC, 1990; Tarrado-Castellarnau, Domínguez, Tarrado-Castellarnau, & Pleite-Gutiérrez, 2013). The degree of lye penetration in the fruit was checked dying with a solution of phenolphthalein longitudinal fruits cuts. Then the fruits were washed during 15 h to eliminate the lye and placed in brine (NaCl 100–120 g/L) where natural lactic fermentation took place for around 7 months. This fermentation was controlled with periodically measurements of pH, free acidity and salt content and addition of acid (HCl) or lye (NaOH). The concentration of salt was increased when the lactic fermentation was completed ($pH < 4$) to improve the preserving process (IOOC, 1990). Then, the fruits were packed with covering liquid (IOOC, 2004) in 2 kg plastic containers and stored at 4 °C in darkness until analysis. PhytoPs analyses were performed in the flesh of raw and treated olives and in the treated olives covering liquid from 2012 and 2013 harvests.

2.3. Measurements

2.3.1. Climate, plant water status and fruit yield

Meteorological data, namely air temperature, solar radiation, air relative humidity, rainfall, and wind speed 2 m above the soil surface, were collected in 50 m away from the orchard automatic weather station. Daily ETo was estimated using the Penman–Monteith equation (Allen, Pereira, Raes, & Smith, 1998).

Micrometric trunk diameter fluctuations were measured throughout the experimental period in four trees per treatment, using a set of linear variable displacement transducers (model DF ± 3 mm, accuracy ± 10 μm, Solartron Metrology, Bognor Regis, UK) attached to the main trunk of 6 trees per treatment (Moriana et al., 2013) with a special bracket made of invar and aluminium. Sensors were placed on the north side and measurements were taken every 2 s and the datalogger (model CR10X with AM25T multiplexer, Campbell Scientific Ltd, Logan, USA) was programmed to report 15 min means. Maximum daily trunk shrinkage was calculated as the difference between maximum and minimum daily trunk diameter and TGR was calculated as the difference of two consecutive maximum daily trunk diameter values.

Midday (12 h solar time) stem water potential (Ψ_{stem}) was

measured on the middle third of the trees, in fully developed leaves near to the main trunk from two trees of each replicate, enclosing leaves in small black plastic bags covered with aluminium foil for at least 2 h before measurements in the pressure chamber (PMS 1000, PMS Instruments Company, Albany, USA). In order to describe the cumulative effect of the water deficit, the water stress integral (SI) was calculated from the Ψ_{stem} data. The expression used was:

$$SI = \sum(\overline{\Psi}_{stem} - (-1.4)) \times n$$

where $\overline{\Psi}_{sem}$ is the average Ψ_{stem} values for any interval and n is the number of the days in the interval (Moriana, Pérez-López, Prieto, Ramírez-Santa-Pau, & Pérez-Rodriguez, 2012).

Olive fruits were harvested at mid-September, controlling the fruit yield per tree. The mean weight of olive fruit was determined according to the weight and number of fruits per box in randomly selected boxes per replicate.

2.3.2. Chemicals

PhytoPs, including 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP, *ent*-16-*epi*-16-F_{1t}-PhytoP, 9-D_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, *ent*-16-B_1-PhytoP, 16-B_1-PhytoP, *ent*-9-L_1-PhytoP, and 9-L_1-PhytoP, were synthesised according to our published procedures (El Fangour, Guy, Vidal, Rossi, & Durand, 2005; El Fangour et al., 2004). As internal standard, d4-15-F2t-IsoP (8-iso-ProstaglandinF$_{2\alpha}$-d$_4$; molecular weight: 358.2; $C_{20}H_{30}D_4O_5$) was used (Cayman Chemicals, Ann Arbor, MI, USA). This internal standard was prepared at a concentration of 1000 nmol/L. Hexane was purchased from Panreac (Castellar del Vallés, Barcelona, Spain), Bis-Tris (bis (2-hydroxyethyl)amino-tris(hydroxymethyl)methane) and butylhydroxyanisole was purchased from Sigma–Aldrich (St. LOUIS, MO USA) and all LC-MS grade solvents, methanol and acetonitrile, were purchased from J.T. Baker (Phillipsburg, New Jersey, USA). The SPE cartridges used was Strata cartridge (Strata X-AW, 100 mg/3 mL), which were acquired from Phenomenex, (Torrance, CA, USA).

2.3.3. Phytoprostanes extraction and UHPLC-QqQ-MS/MS analyses

Olive flesh from 10 treated or raw olives were milled during 30 s and 2 g of this material was crushed in a mortar containing 10 mL of a solution of MeOH and BHA (1 g butylhydroxyanisole/1 L MeOH) and transferred to a polypropylene tube. Then the sample was vortexed during 5 min and centrifuged during 10 min at 2000g and 4 °C. Supernatant was collected and filtered through a Sep-pak. Filtrate (1 mL) was subjected to a dilution and a solid-phase extraction (SPE) using a Strata X-AW cartridge, following the procedure described by Collado-González, Medina, et al. (2015). PhytoPs in the brine of the treated olive fruits were also extracted following the same procedure described above (Collado-González, Medina, et al., 2015).

Free PhytoPs separations were performed using a BEH C18 column (2.1 × 50 mm, 1.7 μm) (Waters, Milford, M. A.), and the used mobile phase was a mixture of two solvents: acetic acid prepared in water (0.105 g/L) (A) and acetic acid prepared in methanol (0.105 g/L) (B). The flow rate (0.2 mL/min using a linear gradient), ESI conditions, and ion optics were as previously described (Collado-González, Medina, et al., 2015). The MS analysis was applied in the multiple reaction monitoring negative ESI mode (Fig. 1).

2.4. Statistical design and analysis

The design of the experiments was completely randomised with four replications, each replication consisting of five adjacent tree rows, each one with nine trees. Measurements were taken on the inner trees of the central row of each replicate while the other trees served as border trees. Data were analysed using SPSS software (SPSS, 2002). Analysis of variance was performed and mean values were compared by Tukey$_{0.05}$ test. Values for each replicate were averaged before the mean and the standard error of each treatment was calculated.

3. Results

3.1. Plant water status, yield and fruit features

During the experimental period, climate conditions presented only slight differences between seasons (Table 1). In this sense, the 2012 season showed an accumulated ETo of 886 mm, whereas in 2013 were achieved 847 mm. Rainfall was higher in 2012 (73 mm) than in 2013 (50 mm), but they occured mainly in spring (April and May) not affecting the pit hardening period. Total amount of water received by each treatment, without considering precipitation, was 412, 130 and 111 mm in 2012 season and 369, 207 and 106 mm in 2013 season for T0, T1 and T2 treatments, respectively (Table 1).

Minimum Ψ_{stem} (min Ψ_{stem}) values in each treatment were very similar in both seasons (Table 1). Moreover, both season min Ψ_{stem} values in T0 plants were high but differences between treatments were significant only in the 2012 season due to a decrease in T2 plants values respect to T0 plants. In contrast, both years SI values showed a significant tendency to increase as a result of decreasing irrigation volumes. SI vales in T1 and T2 plants were clearly higher in the 2012 season than in the 2013 season (Table 1).

Both seasons, olive fruit yield and crop load were within the historical average of the orchard (data not shown). In spite that total olive yield showed a tendency to decrease by water deficit effect, no significant differences between treatments were found (Table 1). Moreover, fruit weight ranged from 4 to 5 g and did not show significant differences between treatments and seasons (Table 1).

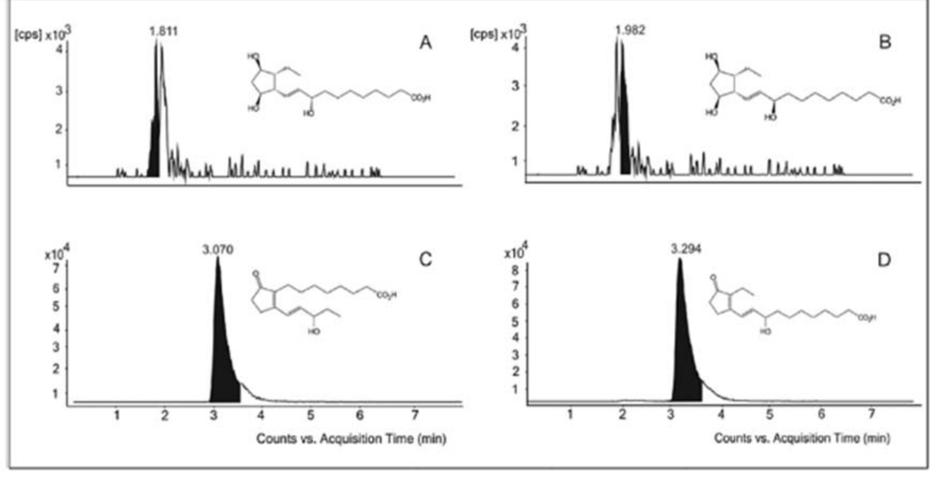
3.2. Qualitative and quantitative PhytoPs profile

PhytoPs were identified on the basis of their mass spectra considering their pseudomolecular ion (*m/z* 327.2, 325.2 and 307.2), their most characteristic fragmentations, and their elution order according to their retention times as described in Fig. 1. The PhytoP profile of the raw fruit fleshes is shown in Fig. 1, which indicate the occurrence of 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, 16-B_1-PhytoP and 9-L_1-PhytyoP, but the absence of regioisomers *ent*-16-F_{1t}-PhytoP + *ent*-16-*epi*-16-F_{1t}-PhytoP. It is important to underline that 16-B_1-PhytoP and 9-L_1-PhytoP presented enantiomeric forms due to their chiral nature.

The quantification of total PhytoPs in raw olive flesh oscillated from 581 to 1000 ng/100 g FW, being 9 series of F_{1t}-PhytoPs the dominant PhytoPs class and the 9 series of L_1-PhytoPs class the minor components (Table 2). The PhytoPs profile in the flesh of treated table olive fruits changed respect to that observed in raw olive fruits, because the L_1-and B_1-PhytoPs were not detected, being only identified 9-F_{1t}-PhytoP and 9-*epi*-9-F_{1t}-PhytoP (Table 2). Total PhytoPs in the flesh of treated fruits ranged from 5888 to 8786 ng/100 g FW (Table 2). The PhytoPs profile in the brine was similar to that observed in the flesh of treated olive fruits (Table 2), with the characteristic that total PhytoPs were very low, ranging only from 11 to 97 ng/mL, respectively (Table 2).

3.3. Effect of water deficit on the PhytoPs content

The total PhytoPs content in raw T0 fruits increased as a result of water deficit effect, being the total PhytoPs content in T1 fruits



PhytoPs	Retention time (min)	Precursor ion (*m/z*)	Product ion (*m/z*)
9-F1t-PhytoP (A)	1.811	327.2	171.2
9-*epi*-9-F1t-PhytoP (B)	1.982	327.2	171.2
16-B1-PhytoP (C)	3.070	307.2	235.2
9-L1-PhytoP (D)	3.294	307.2	185.2

Fig. 1. Representative UHPLC-MS/MS chromatograms of the PhytoPs found in the flesh of raw Manzanilla de Sevilla table olive fruits with their chemical structures and their MRM (multiple reaction monitoring) transitions for quantification. 9-F_{1t}-PhytoP (A); 9-*epi*-9-F_{1t}-PhytoP (B); 16-B_1-PhytoP (C); 9-L_1-PhytoP (D).

Table 1
Accumulated daily crop reference evapotranspiration (ETo, mm), total rainfall (mm) and irrigation water amount applied (WA, mm), without considering precipitation, total fruit yield (TY, kg tree^{-1}), average fruit weight (FW, g), minimum Ψ_{stem} (min Ψ_{stem}, MPa) and water stress integral (SI, MPa x day) in each irrigation treatment (T0, T1 and T2) during each season.

Season	ETo	Rainfall	Treatment	WA	TY	FW	min Ψ_{stem}	SI
2012	886	73	T0	412	23	4	−2a	8b
			T1	130	18	4	−22ab	32a
			T2	111	21	4	−3b	48a
2013	847	50	T0	369	32	4	−2	8b
			T1	207	29	5	−2	15ab
			T2	106	23	5	−3	34a

Means within a column for each season that do not have a common letter are significantly different by Tukey$_{0.05}$ test.

significantly lower than in T2 and higher than in T0 fruits (Table 2). This behaviour was similar in each PhytoP, except in 9-*epi*-9-F_{1t}-PhytoP which in 2012 showed similar levels in T1 and T2 fruits, and in 2013 the levels in T1 fruits were similar to those observed in T0 and T2 fruits (Table 2). The behaviour observed in total PhytoPs content in the flesh of fruits from T0 plants did not changed with season, whereas the season effect was significant in fruits from deficit irrigated plants (T1 and T2), which presented lower total PhytoPs content in the flesh of 2013 fruits (Table 2).

During the experimental period, the observations of min Ψ_{stem} showed significant first-order correlation with the total PhytoPs content and each PhytoPs content, except 9-*epi*-9-F_{1t}-PhytoP (Table 3). On the contrary, SI showed significant correlations in all cases and the highest coefficients of determination (Table 3), suggesting that SI is a better predictor for PhytoPs content. Moreover, it is important to underline that in the flesh of treated fruits only was significant the regression between 9-F_{1t}-PhytoP and SI (Fig. 2) and

Table 2
Effect of irrigation treatments (T0, T1 and T2) on phytoprostanes (PhytoPs) content in the raw and treated fleshes and in the covering liquid of Spanish-style treated Manzanilla de Sevilla table olive fruits from 2012 and 2013 harvests.

PhytoPs	Season	Treatment			LSD
		T0	T1	T2	
Raw flesh (ng/100 g FW)					
9-F1t-PhytoP	2012	364 cA	539 bA	628 aA	28
	2013	383 cA	432 bB	469 aB	13
9-epi-9-F1t-PhytoP	2012	194 bA	232 aA	254 aA	11
	2013	155 bB	184 abB	201 aB	12
16-B1-PhytoP	2012	32 cA	48 bA	83 aA	5
	2013	30 cA	41 bA	59 aB	3
9-L1-PhytyoP	2012	12 cA	21 bA	35 aA	3
	2013	13 cA	16 bA	23 aB	1
Total content	2012	602 cA	841 bA	1000 aA	35
	2013	581 cA	673 bB	751 aB	20
Treated flesh (ng/100 g FW)					
9-F1t-PhytoP	2012	2504 bA	3588 aA	3730 aA	236
	2013	2628 bA	3522 aA	3625 aA	127
9-epi-9-F1t-PhytoP	2012	3904 bA	5000 aA	4542 aA	202
	2013	3260 bB	5264 aA	4990 aA	136
Total content	2012	6408 bA	8588 aA	8273 aA	370
	2013	5888 bA	8786 aA	8615 aA	236
Covering liquid (ng/mL)					
9-F1t-PhytoP	2012	26 cA	59 aA	39 bA	2
	2013	5 cB	15bB	26 aB	1
9-epi-9-F1t-PhytoP	2012	25 cA	38 aA	31 bA	1
	2013	6 cB	22 bB	25 aB	1
Total content	2012	51 cA	97 aA	7 bA	2
	2013	11 cB	37 bB	51 aB	1

Means within a row for each PhytoP and season followed by different small letter, and within a column for each PhytoP and treatment followed by different capital letter are significantly different at P = 0.05 by Tukey's test. Four replicates were carried out for each sample. LSD: least significative difference.

Table 3
Intercept (a), slope (b), coefficient of determination (r^2), number of data points (n) and standard error (SE) of first-order linear equations ($y = a + bx$) between each PhytoP (ng/100 g FW) in the flesh of raw Manzanilla de Sevilla table olive fruits and min Ψ_{stem} (MPa) and SI (MPa x day) using all data pooled.

PhytoP	a	b	r^2	n	SE
PhytoP vs. min Ψ_{stem}					
9-F_{1t}-PhytoP	9	−210	0.7593*	6	49
9-*epi*-9-F_{1t}-PhytoP	58	−66	0.5556^{ns}	6	24
16-B_1-PhytoP	−46	−44	0.8633**	6	7
9-L_1-PhytyoP	−22	−19	0.8981**	6	3
Total content	−1	−339	0.7861*	6	74
PhytoP vs. SI					
9-F_{1t}-PhytoP	330	6	0.8742**	6	35
9-*epi*-9-F_{1t}-PhytoP	158	2	0.6975*	6	19
16-B_1-PhytoP	21	1	0.8977**	6	6
9-L_1-PhytyoP	8	1	0.9314**	6	2
Total content	517	9	0.9003**	6	50

**, * Significant at $P < 0.01$ and $P < 0.05$. ns = not significant.

the data showed higher coefficient of determination for the logistic than for the lineal relationship.

Total PhytoPs, 9-F_{1t}-PhytoP and 9-*epi*-9-F_{1t}-PhytoP content was similar in the treated flesh of T1 and T2 fruits, even though these values were higher than those observed in T0 fruits (Table 2). Moreover, no significant effect of the season was observed (Table 2). PhytoPs content in the covering liquid of deficit irrigated olives did not show a clear behaviour because in 2013 the T2 covering liquid showed a 9-F_{1t}-PhytoP and 9-*epi*-9-F_{1t}-PhytoP content higher than that observed in T1 covering liquid, which was also higher than that of T0 covering liquid (Table 2). However, in 2012 PhytoPs content in T2 covering liquid was lower than in T1 and higher than in T0 fruits (Table 2). Significant differences between seasons were showed in 9-F_{1t}-PhytoP and 9-*epi*-9-F_{1t}-PhytoP contents in the covering liquid, with higher values in the 2012 covering liquid and lower values in 2013 covering liquid (Table 2). The addition of total PhytoPs content in the flesh of treated olives and its covering liquid showed values from 13 to 23 folds higher than that in raw olives flesh (Table 4). This ratio in 9-*epi*-9-F_{1t}-PhytoP was higher than that in 9-F_{1t}-PhytoP, ranging from 27 to 43 folds and from 9 to 18 folds, respectively (Table 4).

4. Discussion

In spite that in RDI treatments (T1 and T2) irrigation water

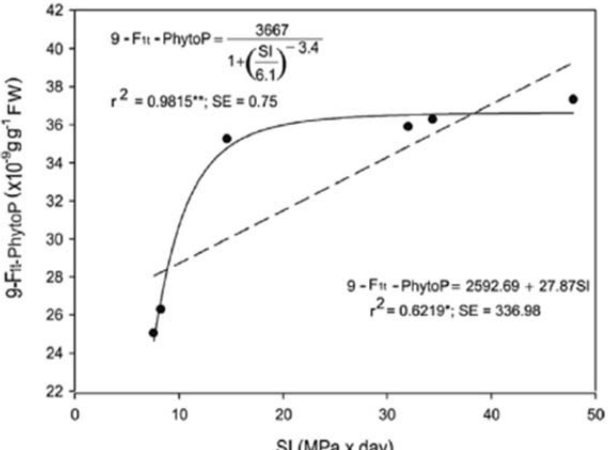


Fig. 2. Relationship between 9-F_{1t}-PhytoP content in the flesh of treated olive and stress integral (SI) using all data pooled. Each value is the mean of four measurements.

Table 4
Ratio between the addition of the content of PhytoPs in the flesh of treated olives and its covering liquid and the content in raw olives flesh in 2012 and 2013 seasons.

PhytoPs	Season	Treatment			LSD
		T0	T1	T2	
9-F_{1t}-PhytoP	2012	14 bA	18 aA	13 bA	1
	2013	9 bB	12 aB	14 aA	1
9-*epi*-9-F_{1t}-PhytoP	2012	34 abA	40 aA	33 bA	2
	2013	27 bA	43 aA	38 aA	3
16-B_1-PhytoP	2012	0	0	0	0
	2013	0	0	0	0
9-L_1-PhytyoP	2012	0	0	0	0
	2013	0	0	0	0
Total	2012	20 abA	23 aA	17 bA	1
	2013	13 bB	20 aB	19 aA	1

Means within a row for each PhytoP and season followed by different small letter, and within a column for each PhytoP and treatment followed by different capital letter are significantly different at P = 0.05 by Tukey's test. Four replicates were carried out for each sample. LSD: least significative difference.

amounts were drastically reduced leading to a clear plant water deficit the effects on yield and fruit weight were not significant (Table 1). In this sense, Goldhamer (1999) previously reported than a moderate water restriction did not affect olive yield and maximize grower profit. Moreover, the fact that average olive fruit weight were not affected by RDI treatments, corroborating previous finding of Moriana et al. (2013), is of top importance because olive fruit size is a key feature for table olives.

To the best of our knowledge, no previous information on the occurrence of PhytoPs in raw and Spanish-style processed olive fruit flesh and on the effect of RDI on the content of these compounds. Nevertheless, there are some papers on the concentration of PhytoPs in olive oil. In this sense, Karg et al. (2007) in olive oil identified PhytoPs from A_1-, B_1-, E_1-and F_1- classes, each class represented a complex isomeric mixture that consisted of two series (9 and 16) (Imbusch & Mueller, 2000). In addition, each series is theoretically comprised of 16 stereisomers (Imbusch & Mueller, 2000). However, in a more specific manner, in extra virgin olive oil (0.8°), Collado-González, Medina, et al. (2015) identified the following PhytoPs: *ent*-16-*epi*-16-F_{1t}-PhytoP + *ent*-16-F_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, 9-D_{1t}-PhytoP and 16-B_1-PhytoP. However, the PhytoPs profile in the raw olive fruit flesh (Table 2) was very different, with only 16-B_1-PhytoP as common PhytoPs. This fact induced to think that the cultivar and/or the oil extraction process can significantly affect to the PhytoPs profile and content.

Loeffler et al. (2005) indicated that the only requirements for PhytoPs formation are the presence of α-linolenic acid and molecular oxygen, suggesting that PhytoPs formation not necessarily requires metabolic activity of living cells. Considering this conclusion, the increase of 9-F_{1t}-PhytoP and 9-*epi*-9-F_{1t}-PhytoP contents in the flesh of treated olive fruits respect to that observed in raw olive fruits (Table 2) could be due to the olive processing conditions and/or storage, which could favoured the formation of these PhytoPs. At first sight, the no detection of 16-B_1-PhytoP and 9-L_1-PhytyoP in the flesh of treated olives (Table 2) could be ascribed to their diffusion from flesh into covering liquid during processing and/or storage. However, the absence of these PhytoPs in the covering liquid (Table 2) and the fact that these PhytoPs were terminal compounds (end products) in the non-enzymatic lipid peroxidation from α-linolenic acid (Thoma et al., 2003), induce to think that those PhytoPs were exposed to degradation reactions during the olive processing and/or storage (Table 2).

The fact that PhytoPs content increased as a result of water deficit (Tables 2 and 3) could be related to the results showed by Cruz de Carvalho (2008) who suggested that one of the inevitable consequences of drought stress is enhanced reactive oxygen

species production in the different cellular compartments. A consequence of this enhanced reactive oxygen species formation in plants is the formation of an array of lipid peroxidation products, including the PhytoPs (Thoma et al., 2003). Moreover, the increase of PhytoPs content in treated and raw olive fleshes by deficit irrigation effect (Table 2) was in agreement with previous results in which water deficit during pit hardening (phase II of fruit growth) enhanced PhytoPs content in extra virgin olive oil (Collado-González, Perez-López, et al., 2015), and confirmed that phase II of fruit growth phenological period can be non-critical considering fruit yield or fruit size (Moriana et al., 2013) but is clearly critical not only for PhytoPs content in olive oil (Collado-González, Perez-López, et al., 2015) but also for PhytoPs content in treated and raw olive fleshes (Table 2).

In raw olive flesh, the fact that the first-order linear relations between total PhytoPs and each PhytoP content and SI values in all cases showed significant coefficients of determination and higher than those obtained with min Ψ_{stem} (Table 3) showed SI as a better predictor for PhytoPs content and that the length of water stress instead of maximum stress is a key factor in the content of these compounds. In this sense, the fact that in treated olive flesh the only significant relation was between SI and 9-F_{1t}-PhytoP content confirm the validity of this predictor to estimate PhytoPs content.

In spite that deficit irrigation conditions during pit hardening and Spanish-style green olive processing enhanced PhytoPs content in the olive flesh, the order of magnitude was very different because of deficit irrigation increased total PhytoPs content less than two folds and the olive processing increased total PhytoPs content between 13 and 23 folds (Tables 2 and 4). As a consequence, from a human health point of view, the Spanish-style olive processing is more beneficial, since there is evidences of the PhytoPs effect on regulation of immune function (Barden et al., 2009; Traidl-Hoffmann et al., 2005) and anti-inflammatory and apoptosis-inducing activities (Durand et al., 2011).

5. Conclusions

Overall, deficit irrigation during olive pit hardening affects neither table olive yield nor fruit size, maximizing farmer incomes. However, this phenological period was clearly critical for table olive composition because water deficit enhanced the PhytoPs content in the flesh of raw olives and Spanish style processed olives. SI was a better predictor than min Ψ_{stem} for predicting the PhytoPs content in the olive flesh, pointing to the idea that the increase in PhytoPs depends in a greater extension of the length of water stress instead of maximum stress achieved. PhytoPs profile in raw olive flesh changed by Spanish style olive processing effect. In this way, the absence of 16-B_1-PhytoP and 9-L_1-PhytyoP in the flesh of treated olives and the important increase in the 9-F_{1t}-PhytoP and 9-*epi*-9-F_{1t}-PhytoP content indicated that the olive processing conditions favour both the formation of these terminal PhytoPs and the degradation of 16-B_1-PhytoP and 9-L_1-PhytoP. Consequently, table olive tree culture under RDI conditions and the processing of its fruits to obtain Spanish-style olives can be considered as complementary actions to enhancing the PhytoPs content and hence their potential beneficial effects on human health, since there are evidences of the PhytoPs effect on regulation of immune function and anti-inflammatory and apoptosis-inducing activities.

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9. Results and Discussion

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Analysis of phytoprostanes

To study the PhytoP content of olive fruits and extra virgin olive oil it was necessary to develop a suitable extraction and purification processes. So, cartridges (Strata X-AW cartridge) were assayed to perform a solid phase extraction (SPE), since in previous studies carried out in biological samples with compounds structurally similar to PhytoPs the Strata X-AW cartridge provided the best results due to its higher recovery rates.

The analysis of PhytoPs of plant-derived foodstuffs is challenging due to the presence of several structural classes and isomers and to the low abundance of these compounds compared to the high levels of primary and secondary metabolites in plant materials (Thoma, et al., 2004). Therefore, for their characterization, analysis and quantification in plants, several techniques such as 1H or 13C nuclear magnetic resonance, gas chromatography coupled to mass spectrometry (GC-MS), high performance liquid chromatography (HPLC) coupled to fluorescence detector, and ESI-MS have been developed. Although the HPLC-UV analysis method is rapid, its sensitivity and specificity are not sufficient to analyze these compounds in plant-derived materials. So far, the most sensitive method developed for quantifying these compounds is a NICI GC-MS method (Parchmann and Mueller, 1998; Imbusch and Mueller, 2000a; Imbusch and Mueller, 2000b; Thoma, et al., 2003; Thoma, et al., 2004; Karg et al., 2007; Göbel and Feussner, 2009). The drawback of this method is, however, that PhytoPs must be subjected to derivatization to their corresponding pentafluorobenzyl ester and trimethylsilyl ether so that the different PhytoPs and their regioisomers can be quantified. This handicap can be solved by employing LC/MS because the derivatization is not required. LC/MS would be more specific and sensitive because it would allow the separation of these compounds and identification of their regioisiomers. However, LC/MS has limitations such as the time required for the analysis and the restricted number of transitions that can be monitored.

In accordance to the first objective of this work, a rapid, reliable and efficient analytical method was developed that allows identification and quantification in the same chromatogram of F_1-, D_1-, B_1-, L_1-PhytoPs and their regioisomers in olive oil, using ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry (UHPLC/MS-MS) (see section 8.1).

The separation of the target compounds and the internal standard, using the chromatographic conditions, was satisfactory except for three pairs of PhytoPs, since they were not completely differentiated by their retention times as a pair of them co-elute and the others two pairs correspond to two pairs of enantiomers, which present identical physical and chemical properties, yielding the same fragmentation efficiency. Hence, they were quantified in samples as pairs of PhytoPs. Moreover, chiral compounds can only be separated by using chiral additives and/or columns.

For the unequivocal identification and quantification of the target compounds the most intense multiple reaction monitoring (MRM) transition was selected. None of the MRM transitions corresponded solely to a single PhytoP, but rather to a pair of diastereoisomers of these compounds. For example, the transition of 327.2→251.2 and 327.2→171.2 corresponded to two pairs of diastereoisomers of F_{1t}-PhytoP (*ent*-16-*epi*-16-F_{1t}-PhytoP and *ent*-16-F_{1t}-PhytoP and 9-F_{1t}-PhytoP and 9-*epi*-9-F_{1t}-PhytoP, respectively). In the same way, the transition 325.2→307.2 matched to a pair of diastereoisomers of D_{1t}-PhytoP, which were 9-*epi*-9-D_{1t}-PhytoP and 9-D_{1t}-PhytoP. The transition of 307.2→235.2 and 307.2→185.2 is characteristic of the B_1-PhytoPs enantiomers (16-B_1-PhytoP and *ent*-16-B_1-PhytoP) and L_1-PhytoPs enantiomers (9-L_1-PhytoP and *ent*-9-L_1-PhytoP), respectively.

In accordance with the ‘Guidance for Industry-Bioanalytical Method Validation’ of the US Food and Drug Administration (FDA) (FDA, 2001) and the International Conference on Harmonization (ICH) (ICH, 1994), for

carrying out the validation of this method, it was necessary to determine linearity, sensitivity, precision, accuracy and recovery.

The linearity of the method was assessed for each metabolite by evaluating the correlation coefficient, which, if higher than 0.995, indicated that the analytical method presented an adequate linearity. The limit of detection (LOD) and the limit of quantification (LOQ) of our method were evaluated, both of these values being metabolite-dependent. The LOD values of our method ranged from 0.29 to 2.56 ng mL^{-1} and the LOQs from 0.59 to 5.12 ng mL^{-1}. The linearity range established for the PhytoPs measured by this method was from 5.12 to 328.44 ng mL^{-1} for *ent*-16-*epi*-16-F_{1t}-PhytoP, 9-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP and 9-*epi*-9-F_{1t}-PhytoP, from 0.62 to 326.43 ng mL^{-1} for 9-*epi*-9-D_{1t}-PhytoP and 9-D_{1t}-PhytoP, and finally from 0.59 to 308.41 ng mL^{-1} for 16-B_1-PhytoP, 9-L_1-PhytoP, *ent*-16-B_1-PhytoP and *ent*-9-L_1-PhytoP the linearity range reached (Table 1).

The intra-day variations for all target compounds ranged from 2.24 to 13.64 % and the accuracies varied from 80.33 to 119.64 %. The inter-day variations for the same compounds ranged from 0.01 to 13.69 % and the accuracies from 80.34 to 119.90 %. The relative standard deviation (RSD) values for three injections were lower than 15 %, indicating that this method is very reproducible for the PhytoPs analyzed. Results of the absolute recovery provided high extraction efficiencies, ranging from 102.90 to 140.64 %, which reflected the adequate and good efficiency of the solid-phase extraction (SPE). In addition, the low standard deviations obtained highlighted the good repeatability of the process. Therefore, we have created a model by a robust analytical platform able to unravel the behaviour and generation of these compounds from linolenic acid linked to direct or undirect oxygen-mediated processes towards free radical attacks and oxidative pathways.

The ‘Oxygen Paradox’ outlines that plants and all living organisms cannot exist without oxygen, which, nevertheless is inherently dangerous to their existence. Under normal conditions, due to their reductive environment, cell plants continuously promote the reduction of oxygen,

fostering the endogenous production of ROS (Mueller, 2004). The production of these ROS increases as a consequence of almost any stress (abiotic or biotic) to which the plant is subjected. These ROS readily attack ALA in membrane lipids that are non-enzymatically oxidized to a PhytoP family (Mueller, 2004; Cruz de Carvalho, 2008). The importance of PhytoPs is not only based on their biological activity, but also on their use as biomarkers of oxidative degradation of plant-derived foodstuffs (Barden, et al. 2009). Further, previous studies have suggested that the intake of these PhytoPs appears to be related to the regulation of the immune function in humans (Gutermuth, et al., 2007; Barden, et al. 2009). Despite the negative connotation of PhytoP production linked to co-lateral oxidation processes of ALA, Barden, et al. (2009) used by GC-MS to measure the total content of F_1-PhytoPs in plasma samples and 24 h urine samples of humans consuming olive oil or flaxseed oil during a four week intervention, finding that the flaxseed oil supplementation significantly increased the concentration of plasma F_1-PhytoPs relative to olive oil. Therefore, these compounds are absorbed at physiological level in humans. However, the pharmacokinetic curve and the physiological effects in the human body are still unknown.

Previous studies have measured the concentration of PhytoPs by GC-MS in tissues of several plants (Imbusch and Mueller, 2000a; Imbusch and Mueller, 2000b) and other studies have also detected them in cell cultures of different types of plants such as *Nicotiana tabacum* (Solanaceae), *Glycine max* (Fabaceae), *Rauvolfia serpentina* (Apocynaceae), *Agrostis tenuis* (Poaceae), *Slix alba*, *Arabidopsis thaliana*, *Tilia cordata* and *Betula pendula* (Parchmann and Mueller, 1998; Thoma, et al., 2003). Furthermore, GC-MS has been used to measure several PhytoPs in edible oils and in biological samples after the administration to human volunteers (Karg, et al., 2007; Barden, et al., 2009). Sensitivity and selectivity of our method was reflected by LOQ values, which ranged from 0.0019 μM (0.59 ng mL^{-1}) to 0.0156 μM (5.12 ng mL^{-1}), depending on the compound analyzed. These values were better than those found by Karg

et al (2007) for the determination of different PhytoPs in vegetable oils by GC-MS, whose LOQ values varied from 0.01 µM (3.08 ng mL^{-1}) to 0.56 µM (182.80 ng mL^{-1}) depending on the type of edible oil and the compound tested. This fact underlines the higher sensitivity shown by our method using this technique (UHPLC-QqQ-MS/MS) compared to previous methods using GC-MS.

As regards with the total content of PhytoPs in different oils, it should be noted that in commercial olive oils only four PhytoPs were obtained, the dominant PhytoP class being D_1-PhytoP, which agrees with the results of Karg et al (2007), who reported that, in all the oils studied, either D_1-PhytoP or F_1-PhytoP was the dominant PhytoP class, while B_1-PhytoPs (both enantiomers) were in a minority. However, in refined sunflower oil the major PhytoP class comprised both B_1-PhytoPs. Besides, the results for this oil showed a higher amount and a larger number of PhytoPs than those obtained for olive oil. This could be due to the milder extraction treatment used to obtain olive oil compared to the harsh treatment used refining sunflower oil. The radical temperature treatment for sunflower oil could induce higher cross oxidation reactions of ALA at 100 °C or 240 °C.

The sum of the PhytoP content for 0.8° extra virgin olive oil and olive oil (extra virgin olive oil/refined olive oil (50/50, *v/v*) was 14.97 and 39.35 ng mL^{-1}, respectively, whereas 0.2º refined sunflower oil contained 297.45 ng mL^{-1}. In other words, the refined sunflower oil had a 20 fold higher concentration of PhytoPs than extra virgin olive oil and 8 fold higher concentration than olive oil. Karg et al. (2007) studied the total PhytoP contents of olive oil (*vs* Brändle vita) and reported that it was approximately 1057 ng mL^{-1}. It is important to note that, in their assay, they obtained the total amount of each compound from the different classes of PhytoP starting from ALA, whose *in vitro* and *in vivo* autoxidation may yield not only a large number, but also high amounts of PhytoPs (Parchmann and Mueller, 1998; Imbusch and Mueller, 2000a; Imbusch and Mueller, 2000b; Thoma, et al., 2003; Thoma, et al., 2004;

Sattler et al., 2006; Karg, et al., 2007). In contrast, in our study we worked with individual synthetic standards (ten in total). This explains why our results are lower than those found by other authors. In addition, in their study, Karg et al (2007) did not study the total content of PhytoPs in sunflower oil, while our results show that sunflower oil displays higher qualitative profile and content than olive oils (20 fold higher than extra virgin olive oil and 8 fold higher than olive oil). Nevertheless, they studied them in other edible oils, reporting that these compounds not only vary depending on the type of oil, but also the cultivar of the fruit from which the oil is obtained. The absolute PhytoP content of the oil diminished in the following order: Linseed oil ≈ soybean oil > olive oil > walnut oil > rapeseed oil >> grape seed oil. Although it is of note that our results obtained for the refined sunflower oil are not comparable with the results of Karg et al. (2007), for the reasons described above, in the above classification the refined sunflower oil would be ahead of the olive oil. However, it is not possible to say whether the total content of PhytoPs present in refined sunflower oil would be around the PhytoP content present in soybean oil or linseed oil. The fact that refined sunflower oil possessed a higher concentration of PhytoPs than olive oil could be related to the higher initial concentration of ALA in the olive oil, although Karg et al. (2007) reported that the total amount of PhytoPs did not correlate well with the ALA content. Regardless of the initial ALA content of the oils, the manufacturing process could be the key to explaining the different PhytoP levels since most plant oils are subjected to a refining treatment which includes dramatic temperature procedures for whitening (100 °C/ 15 min) and removing the flavours (240 °C at 2-3 mm pressure), that could increase the oxidation of this fatty acid. However, extra virgin olive oil only requires a soft mechanical extraction (De la Lastra et al., 2001). The PhytoP value for olive oil was 2.6 fold higher than in extra virgin olive oil since it is half virgin extra olive oil and half refined olive which has been exposed to the same dramatic oxidative temperature

procedures as those described for sunflower oil (FAO, 1997; Melgarejo, 2003).

Effect of water deficit during pit hardening on PhytoP content of EVOO

In line with the second and the third objectives of this work, the effects of water deficit on the PhytoP content of EVOO made from fruits of mature Cornicabra olive trees during pit hardening were studied.

The fact that Ψ_{stem} values for control (T0) plants during phases I, II and III of fruit growth were slightly below the threshold Ψ_{stem} values could have been because irrigation amounts were not properly managed when Ψ_{stem} values were below the threshold values. Nevertheless, mean Ψ_{stem} values in each fruit growth phase were quite high and near constant (Fernandez et al., 2006; Tognetti et al., 2007; Dell'Amico et al., 2012) suggesting that T0 plants were not under irrigation-related stress during the experimental period (Moriana et al., 2012).

Although Ψ_{stem} values for T1 and T2 plants during phase II decreased at a similar and slow rate (Hale and Orcutt, 1987), minimum Ψ_{stem} values indicated that both treatments achieved substantial water deficit levels especially in T2 plants at the end of this phenological period (Alegre et al., 2002; Iniesta et al., 2009).

In the previous section of this chapter, it was shown that a branded EVOO had a different PhytoP profile to that found in Cornicabra EVOO, because in the branded EVOO *ent*-16-*epi*-16-F_{1t}-PhytoP + *ent*-16-F_{1t}-PhytoP was also detected and 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP and 9-L_1-PhytoP + *ent*-9-L_1-PhytoP were not detected. However, the level of total PhytoPs (14.64 ng mL^{-1}) in the branded EVOO was within the range found in our experimental conditions in Cornicabra EVOO (9.18 to 19.31 ng mL^{-1}). Taking into consideration that both olive oils were extra virgin and that analyses were performed with the same method, using synthetic standards, it is clear that these differences in composition must be

attributed to the effect of cultivar, oil extraction technology and/or the storage conditions which made the oil on autoxidation (Karg et al., 2007).

To the best of our knowledge, no previous information exists on the effect of water deficit on the PhytoP content. In this sense, the increase in PhytoPs by water deficit effect during pit hardening (phase II of fruit growth), showed that this phenological period can be non-critical as regards fruit yield (Inglese et al., 1996), but is clearly critical for the PhytoPs content of olive oil. Gómez del Campo and García (2013) showed that virgin olive oils from deficit irrigated trees, from the end of fruit drop to the end of July, exhibited significantly higher oxidative stability, which coincided with a significantly higher content of phenol derivatives. However, deficit irrigation strategies in summer did not determine significant changes in the parameters legally established for evaluating the commercial quality of virgin oils. Moreover, this increase in the PhytoP content could be related with the fact that under drought stress the frail balance between reactive oxygen species (ROS) and scavenging that defines the normal steady-state level of intracellular ROS suffers an upwards shift, enhancing ROS production due to stomatal closure and the concomitant limitation in CO_2 fixation (Cruz de Carvalho, 2008). A consequence of this enhanced ROS formation in plants is the formation of an array of lipid peroxidation products, including structural congeners of jasmonates, the PhytoPs (Thoma et al., 2003).

Since all PhytoPs are induced by oxidative stress (OS) from common precursors, the G_1-PhytoPs (Thoma et al., 2004; Sattler et al., 2006), the fact that the behaviour observed in each PhytoP in response to water deficit differed induced us to think that those in which increased their contents in the T1 extra virgin olive oil required a lower OS level to be induced from the precursor and could be used as early markers of water deficit in extra virgin olive oil.

From a nutritional point of view, the increase in total PhytoPs in T1 and T2 olive oil, near 2-fold higher with respect to the control (T0) olive oil, could be considered a potential beneficial aspect of the extra virgin olive

oils from trees cultivated under water deficit conditions during pit hardening, because even though there is no detailed knowledge of the biological effects of PhytoPs, there is some evidence of their beneficial effects on human health, such as the regulation of immune function (Traidl Hoffmann, et al., 2005; Gutermuth et al., 2007; Barden et al., 2009) and anti-inflammatory and apoptosis-inducing activities (Durand et al., 2011).

The fact that the relationships between 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP or 16-B_1-PhytoP + *ent*-16-B_1-PhytoP and minimum Ψ_{stem} values in phase II were tighter using second-order polynomic equations, whereas the relations for 9-*epi*-9-D_{1t}-PhytoP or 9-D_{1t}-PhytoP using linear or quadratic equations showed similar levels of significance for their coefficients of determination, while the relation for 9-L_1-PhytyoP + *ent*-9-L_1-PhytyoP was defined only by a linear equation could indicate different development levels of the same behaviour. In this sense, Horner (1990) indicated that the relation between plant water status and secondary metabolite contents is defined by a quadratic relationship. Also, some authors showed that the relation between plant water status and some metabolite contents is not defined by a linear correlation when a wide-range of plant water status is considered (Mattson and Haack, 1987; Gobbo Neto and Lopes, 2007; Mellisho et al., 2012; Mena et al., 2013). So, the first-order relation between plant water deficit and PhytoP contents could correspond to the first phase of the quadratic relationship proposed by Horner (1990), indicating that a more severe water deficit could be necessary to induce a decrease in the 9-*epi*-9-D_{1t}-PhytoP, 9-D_{1t}-PhytoP or 9-L_1-PhytyoP + *ent*-9-L_1-PhytyoP contents.

The fact that most equations showed a negative intercept might suggest that these equations should not be used under high Ψ_{stem} values because negative PhytoPs values could be predicted, which does not make sense from a metabolic or physiological point of view. Nevertheless, PhytoP values could be predicted using these equations when stem values were within the range of values used to determine these equations

(minimum Ψ_{stem} values in phase II from -3.12 to -1.73 MPa). So, negative PhytoPs values were not predicted if these boundary values are used.

Overall, the results showed for first time that pit hardening (phase II of olive fruit growth), a phenological non-critical period for fruit yield, is clearly critical for extra virgin olive oil composition because water deficit enhances the PhytoP content, with the concomitant potential benefits for human health since they are bioavailable in humans according to previous results (Barden et al., 2009). Also, qualitative and quantitative differences in the PhytoP content with respect to those reported by other authors indicates a decisive effect of cultivar, oil extraction technology and/or the storage conditions on autoxidation. The content of each individual PhytoP in the olive oil from T0, T1 and T2 treatments could define different phases of the same behaviour. In this sense, when minimun Ψ_{stem} values in phase II were above -2.5 MPa, 9-F_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP and 16-B_1-PhytoP + *ent*-16-B_1-PhytoP values increased sharply as Ψ_{stem} decreased, and when Ψ_{stem} values were below this threshold value the relationship changed and any further reduction in Ψ_{stem} was associated with a decrease in PhytoP contents. In contrast, when minimum Ψ_{stem} values in phase II decreased from -1.73 to -3.12 MPa the 9-*epi*-9-D_{1t}-PhytoP, 9-D1t-PhytoP and 9-L_1-PhytyoP + *ent*-9-L_1-PhytyoP increased indicating that a more severe water deficit could be necessary to induce a decrease in their contents. Therefore, from a physiological and agronomical point of view, 9-F_{1t}-PhytoP, 9-*epi*-9-F *1t*-PhytoP and 16-B_1-PhytoP + *ent*-16-B_1-PhytoP could be considered as candidates for acting as early biomarkers of water stress in olive tree.

Effect of the season on free PhytoP content in EVOO made from deficit irrigated olive trees

Taking into consideration the fourth objective of this work we studied the effect of different water deficit levels applied at the beginning of oil accumulation in olive fruits (pit hardening period) on free PhytoPs

levels and whether a longer water deficit situation during the olive oil accumulation period (just after pit hardening) is able to enhance free PhytoP accumulation. The effect of season on the free PhytoP profile and content in Cornicabra EVOO was also evaluated.

In the 2012 and 2013 irrigation seasons, control (T0) plants were irrigated above crop water requirements (202 % ETc and 185 % ETc, respectively). Two RDI treatments (T1 and T2) were applied, which were intended to avoid water deficit during phase I and III of fruit growth and save irrigation water during the non-critical phenological period of pit hardening (phase II) by developing different situations of water deficit. In 2013, a fourth treatment (T3 treatment) was also performed, which was based on an irrigation protocol similar to that used for T2, except that the water deficit was applied from the beginning of phase II to 15 days after the end of this phenological phase.

To explain why in 2012 Ψ_{stem} values in all treatments were significantly higher than those in 2013 even though the amount of irrigation water applied was similar both seasons, two factors should be taken into account: the scarcity of rainfall in 2012 and the fact that the drip irrigation system in the olive orchard was installed in March 2012. So, during most of the 2012 season the root system dissemination could have been typical of olive trees under rainfed conditions, whereas in 2013 a very important proportion of the root system would have itself within the volume of soil wetted by drip irrigation (Inglese et al., 2011). Consequently, the irrigation efficiency during most of 2012 would have been lower than that in 2013, leading to clearly higher Ψ_{stem} values in T0, T1 and T2 in 2012 than in 2013. Moreover, the fact that during the 2013 irrigation season rainfall reached 485 mm would explain why differences in Ψ_{stem} values between treatments were not as pronounced as expected.

The sustantial difference in the yield obtained both seasons would reflect the adaptation of the trees to the drip irrigation system in 2013 and the characteristic alternate bearing pattern of olive trees, which exhibit a

frequency and intensity regulated by the genotype and the growing conditions (Cruz de Carvalho, 2008). The increase in 2013 fruit yield seemed to be due to the higher number of fruits, because the average fruit weight was similar in both seasons. The fact that in neither season was any effect of T1 and T2 treatments on fruit yield observed confirms that the pit hardening phenological period is not a critical period from the yield point of view (Goldhamer, 1999). However, the increase in total free PhytoP content in Cornicabra EVOO by the effects of T1 and T2 clearly indicated that the pit hardening period is critical for PhytoP content in olive oil. Moreover, the fact that yield was not affected and that the free PhytoP content in EVOO increased in response to T3 indicated that it is possible to extend the water deficit after pit hardening by two weeks leading to a similar effect as T1 and T2, but increasing irrigation water saving.

The increase in the free PhytoP content in EVOO obtained from deficit irrigated trees (T1, T2 and T3) could be related with stomatal regulation and the concomitant limitation of CO_2 fixation under water stress, which enhances the reactive oxygen species (ROS) formation (Thoma et al., 2004), which, in turn, promotes the formation of an array of lipid peroxidation products, including PhytoPs (Berenguer et al., 2006). It is difficult to understand why the highest total free PhytoP content in the EVOOs from the different irrigation treatments were obtained in the season when the plant water status was better. In this sense, Berenguer et al. (2006) showed that linolenic acid, a precursor of PhytoPs, significantly increased with irrigation in one year out of two. However, some authors have found inconsistent fluctuations in some fatty acids between years (Montedoro and Garofolo, 1984; Inglese et al., 1996; Servilli et al., 2007). Whatever the case, it can be considered that EVOO composition results from a very complex multivariate interaction between the genotypic potential and the environmental, agronomic, and technological factors that characterize fruit growth and ripening as well as oil extraction and storage (Lavee and Wodner, 2004; Gutermuth et al.,

2007). So, the minor components in EVOO may range independently and depending on factors that are not always interrelated.

Thoma et al. (2004) and Loeffler et al. (2005) indicated that free PhytoPs are excellent biomarkers of oxidative degradation of plant-derived foodstuff. In this sense, the total and individual free PhytoPs content in Cornicabra oil increased through a water stress effect. However, this response was not as perceptible as expected because most of the correlations obtained presented very low slope values, indicating that a great change in $S_{\Psi_{stem}}$ is needed to obtain any change in the content of individual free PhytoPs. Also, it is important to consider that most correlations for individual free PhytoPs changed from one season to another. In light of these results, it is difficult to conclude that these individual free PhytoPs can be used as biomarkers of water stress, or that, rather than $S_{\Psi_{stem}}$, other plant water status indicators should have been measured and compared with total and individual free PhytoP content. It is possible that the only requirement for forming PhytoPs is ALA and molecular oxygen. Nevertheless, considering that there is some evidence that PhytoPs are biologically active lipids (Durand et al., 2011; Minghetti et al., 2014) it is clear that an overall significant increase in free PhytoPs constitutes a nutritional potential benefit of Cornicabra EVOO obtained from trees cultivated under water deficit conditions during summer.

Effect of water deficit and Spanish-style processing on PhytoP content of green table olives

In accordance with the fifth and sixth objectives of this thesis, it was decided to look at the effect of different RDI treatments on the qualitative and quantitative profile of PhytoPs in olive flesh and if the elaboration process of Manzanilla de Sevilla Spanish style green table olives modifies the PhytoPs content.

Despite the fact that in the RDI treatments irrigation water amounts were drastically reduced, leading to a clear plant water deficit, the effects on yield and fruit weight were not significant. In this sense, Goldhamer (1999) reported than a moderate water restriction did not affect olive yield and maximizes grower profit. Moreover, the fact that our results indicated that the average olive fruit weight was not affected by RDI, corroborating previous finding of Moriana et al. (2013), is of great importance because olive fruit size is a key feature for table olives.

To the best of our knowledge, no previous information exists on the occurrence of PhytoPs in raw and Spanish-style processed olive fruit flesh and on the effect of RDI on the content of these compounds. Nevertheless, there are some papers on the concentration of PhytoPs in olive oil. For example, Karg et al. (2007) in olive oil identified PhytoPs from A_1-, B_1-, E_1- and F_1- classes, each class representing a complex isomeric mixture that consisted of two series (9 and 16) (Imbusch and Mueller, 2000). In addition, each series is theoretically comprised of 16 stereisomers (Imbusch and Mueller, 2000). However, in a more specific manner, in extra virgin olive oil (0.8º), the following PhytoPs have been identified: *ent*-16-*epi*-16-F_{1t}-PhytoP + *ent*-16-F_{1t}-PhytoP, 9-*epi*-9-D_{1t}-PhytoP, 9-D_{1t}-PhytoP and 16-B_1-PhytoP (see section 8.1). Our results indicate that the PhytoPs profile in the raw olive fruit flesh was very different, 16-B_1-PhytoP being the only common PhytoP. This induced us to think that the cultivar and/or the oil extraction process can significantly affect the PhytoP profile and content.

Loeffler et al. (2005) indicated that the only requirements for PhytoPs formation are the presence of α-linolenic acid and molecular oxygen, suggesting that PhytoPs formation does not necessarily require the metabolic activity of living cells. Based on this conclusion, the increase of 9-F_{1t}-PhytoP and 9-*epi*-9-F_{1t}-PhytoP in the flesh of treated olive fruits compared with that observed in raw olive fruits could be due to the olive processing conditions and/or storage, which might have favour the formation of these PhytoPs. At first sight, the fact that 16-B_1-PhytoP and 9-

L_1-PhytyoP were not detected in the flesh of treated olives could be attributed to their diffusion from flesh into covering liquid during processing and/or storage. However, the absence of these PhytoPs in the covering liquid and the fact that these PhytoPs were seen to be terminal compounds (end products) in the non-enzymatic lipid peroxidation from α-linolenic acid (Thoma et al., 2003), leds us to think that these PhytoPs were exposed to degradation reactions during the olive processing and/or storage.

The fact that the PhytoP content increased as a result of water deficit could be related to the results of Cruz de Carvalho (2008), who suggested that one of the inevitable consequences of drought stress is enhanced reactive oxygen species (ROS) production in the different cellular compartments. A consequence of this enhanced ROS formation in plants is the formation of an array of lipid peroxidation products, including PhytoPs (Thoma et al., 2003). Moreover, the increase in PhytoP content in treated and raw olive flesh as a result of deficit irrigation agrees with previous results in which water deficit during pit hardening (phase II of fruit growth) enhanced PhytoPs content in extra virgin olive oil (see section 8.2), and confirmed that phase II of the fruit growth phenological period can be non-critical for fruit yield and fruit size (Moriana et al., 2013) but clearly critical for both the PhytoP content of olive oil and the PhytoP content of treated and raw olive fleshes.

In raw olive flesh, the fact that the first-order linear relations between total PhytoPs and each PhytoP and SI values in all cases showed significant coefficients of determination that were higher than those obtained with min Ψ_{stem} indicated that SI is a better predictor of PhytoP content and that the length of water stress, rather than maximum stress, is a key factor in the content of these compounds. In this sense, the fact that in treated olive flesh the only significant relation was between SI and the 9-F_{1t}-PhytoP content confirmed the validity of this predictor for estimating the PhytoPs content.

It is important to note that although deficit irrigation during pit hardening and Spanish-style green olive processing enhanced the PhytoPs content of the olive flesh, the order of magnitude was very different because the deficit irrigation increased the total PhytoP content less than two folds and the olive processing increased total PhytoPs content between 13 and 23 fold. As a consequence, from a human health point of view, the Spanish-style olive processing is more beneficial, since there is evidence that PhytoPs help regulate the immune function (Traidl-Hoffmann, 2005; Barden et al., 2009) and anti-inflammatory and apoptosis-inducing activities (Durand et al., 2011).

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10. Conclusions and Conclusiones

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Conclusions

1. A new and robust UHPLC–QqQ-MS/MS method for quantitative and qualitative determination of free phytoprostanes has been developed by first time.

2. Additionally, this new methodology has the advantage of being able to perform each analysis in a single assay per sample, requiring only 8 min and reducing the cost of solvents.

3. Water deficit during pit hardening (phase II of olive fruit growth), a phenological period that is not critical for fruit yield, or a longer period of two weeks just after pit hardening, is clearly critical for olive table and extra virgin olive oil composition because the water deficit involved enhances the phytoprostanes content, with the concomitant potential benefits for human health due to their bioavailability in humans.

4. The increase in phytoprostanes seems to depend on the length of water stress rather than the degree of stress achieved

5. The qualitative and quantitative differences in the phytoprostane content in extra virgin olive oil with respect to that reported by other authors indicated a decisive effect of cultivar, oil extraction technology, and/or storage conditions on autoxidation.

6. The phytoprostanes content of extra virgin olive oil is significantly affected by the season, probably due to a very complex multivariate interaction among factors that are not always interrelated.

7. The response of individual free phytoprostanes in the extra virgin olive oil to changes in plant water status was not as perceptible as expected, probably due to the plant water status parameter considered. Thus, stem water potential values showed a direct relation with phytoprostane contents, whereas this relation using the water stress integral did not clearly support the idea that these compounds could be used as biomarkers of water stress.

8. The content of each individual phytoprostane in the extra virgin olive oil obtained from trees with different water status levels could define different phases of the same behaviour. In this sense, when minimum stem water potential values in phase II were above a threshold value some phytoprostanes increased sharply as stem water potential decreased, and when stem water potential values were below this threshold value the relationship changed and any further reduction in stem water potential was associated with a decrease in phytoprostanes contents.

9. Spanish style olive processing conditions favour both the formation of two end point phytoprostanes (9-F_{1t}-PhytoP and 9-*epi*-9-F_{1t}-PhytoP) and the degradation of 16-B_1-PhytoP and 9-L_1-PhytoP.

10. Spanish style olive processing enhances the effect of water deficit during pit hardening for enhancing the phytoprostane content of olive flesh.

Conclusiones

1. Se ha desarrollado por primera vez un nuevo y robusto método con equipos UHPLC–QqQ-MS/MS para la determinación cualitativa y cuantitativa del contenido de fitoprostanos en muestras de origen vegetal.

2. Esta nueva metodología presenta la ventaja adicional de ser capaz de realizar cada análisis en sólo 8 min y con considerable ahorro de disolventes.

3. El déficit hídrico durante el endurecimiento del hueso (fase II del crecimiento de la aceituna), periodo no crítico para la producción, o un periodo más largo incluyendo dos semanas tras el endurecimiento del hueso, resultó claramente crítico para la composición de la aceituna de mesa y el aceite virgen extra ya que el contenido de fitoprostanos aumenta, con el consiguiente beneficio potencial sobre la salud humana dada su biodisponibilidad.

4. El aumento en el contenido de fitoprostanos depende en mayor medida de la duración que de la intensidad del déficit hídrico alcanzado por el cultivo.

5. Las diferencias cualitativas y cuantitativas en el contenido de fitoprostanos en aceites virgen extra de distinto origen parecen deberse a claros efectos del cultivar, tecnología de extracción del aceite y/o condiciones de almacenamiento

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6. Los niveles de fitoprostanos en aceite virgen extra resulta claramente afectado por la estación probablemente debido a la interacción de un complejo abanico de factores no siempre interrelacionados.

7. La relación entre los niveles de cada fitoprostano en el aceite virgen extra y el estado hídrico de los olivos de procedencia no resultó tan evidente como era de esperar, probablemente por la influencia del parámetro considerado para definir el estado hídrico del cultivo. De este modo, el potencial hídrico del tallo mostró una relación más sólida que la integral de estrés, la cual cuestionó el uso de los fitoprostanos como biomarcadores del estrés hídrico.

8. La relación entre cada fitoprostano en el aceite virgen extra y el estado hídrico de los olivos de procedencia presentaron distintas etapas dentro de un mismo comportamiento. Concretamente, cuando el potencial hídrico de tallo adquirió valores por encima de un valor umbral el contenido de fitoprostanos aumentó al disminuir los niveles de potencial hídrico del tallo. Por el contrario, cuando los valores de potencial hídrico del tallo fueron inferiores al umbral las reducciones de dichos valores se asociaron con reducciones en el contenido en fitoprostanos.

9. Las condiciones que acontecen durante el procesado “estilo español” de la aceituna de mesa favorecieron la formación de dos fitoprostanos terminales (9-F_{1t}-PhytoP y 9-*epi*-9-F_{1t}-PhytoP) y la degradación de otros dos (16-B_1-PhytoP y 9-L_1-PhytoP).

10. El procesado “estilo español” de la aceituna de mesa puede considerarse como una acción complementaria al déficit hídrico durante el endurecimiento del hueso para aumentar el contenido de fitoprostanos en la pulpa de las aceitunas.

11. References

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12. Appendix

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Quality of each publication of the compendium

Peer-reviewed papers from journals in the 2014 edition of Journal Citation Reports®

Collado-González, J., Medina, S., Durand, T., Guy, A., Galano, J. M., Torrecillas, A., Ferreres, F., Gil-Izquierdo, A. 2015. New UHPLC–QqQ-MS/MS method for quantitative and qualitative determination of free phytoprostanes in foodstuffs of commercial olive and sunflower oils. *Food Chemistry* 178: 212-220.

Publisher: Elsevier Science Ltd, The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, England

ISSN: 0308-8146

Research Domain: Chemistry, Applied; Food Science & Technology; Nutrition & Dietetics

JCR® Category	Quartile in Category	Rank	Impact factor	5-year impact factor
Food Science & Technology	**Q1**	8/123	3.391	3.901

Collado-González, J., Pérez-López, D., Memmi, H., Gijón, M. C., Medina, S., Durand, T., Guy, A., Galano, J.-M., Ferreres, F., Torrecillas, A., Gil-Izquierdo, A. 2015. Water deficit during pit hardening enhances phytoprostanes content, a plant biomarker of oxidative stress, in extra virgin olive oil. *Journal of Agricultural and Food Chemistry* 63: 3784-3792.

Publisher: American Chemical Society, 1155 16TH ST, NW, Washington, DC 20036

ISSN: 0021-8561

Research Domain: Agriculture, Multidisciplinary; Chemistry, Applied; Food Science & Technology

JCR® Category	Quartile in Category	Rank	Impact factor	5-year impact factor
Chemistry, Applied	**Q1**	8/123	2.912	3.269

Collado-González, J., Pérez-López, D., Memmi, H., Gijón, M. C., Medina, S., Durand, T., Guy, A., Galano, J.-M., Fernández, D. J., Carro, F., Ferreres, F., Torrecillas, A., Gil-Izquierdo, A. (2015). Effect of the season on the free phytoprostanes content in Cornicabra extra virgin olive oil from deficit irrigated olive trees. *Journal of the Science of Food and Agriculture* DOI: 10.1002/jsfa.7259

Publisher: Wiley-Blackwell. 111 River St, Hoboken 07030-5774, NJ, UK

ISSN: 0022-5142

Research Domain: Agriculture, Multidisciplinary; Chemistry, Applied; Food Science & Technology

JCR® Category	Quartile in Category	Rank	Impact factor	5-year impact factor
Agriculture, Multidisciplinary	**Q1**	7/56	1.714	1.994

Collado-González, J., Moriana, A., Girón, I. F., Corell, M., Medina, S., Durand, T., Guy, A., Galano, J.-M., Valero, E., Garrigues, T., Ferreres, F., Moreno, F., Torrecillas, A., Gil-Izquierdo, A. 2015. The phytoprostane content in green table olives is influenced by Spanish-style processing and regulated deficit irrigation. *LWT-Food Science and Technology* 64 : 997-1003.

Publisher: Elsevier Science BV, PO BOX 211, 1000 AE Amsterdam, Netherland

ISSN: 0023-6438

Research Domain: Food Science & Technology

JCR® Category	Quartile in Category	Rank	Impact factor	5-year impact factor
Food Science & Technology	**Q1**	24/123	2.416	3.095

Other peer-reviewed papers

Collado-González, J., Durand, T., Ferreres, F., Medina, S., Torrecillas, A., Gil-Izquierdo, A. 2015. Phytoprostanes. *Lipid Technology* 27: 127-130.

Publisher: Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

ISSN: 1863-5377