DEPARTAMENTO DE
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# ASSESSMENT OF THE INFLUENCE OF PROCESSING CONDITIONS ON THE ANTIOXIDANT POTENTIAL OF EXTRACTS OBTAINED FROM OLIVE OIL INDUSTRY BYPRODUCTS

**PhD. THESIS**

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Que la memoria titulada "*ASSESSMENT OF THE INFLUENCE OF PROCESSING CONDITIONS ON THE ANTIOXIDANT POTENTIAL OF EXTRACTS OBTAINED FROM OLIVE OIL INDUSTRY BYPRODUCTS* ", presentada por Dña. Margarita Hussam Ahmad Qasem Mateo para aspirar al grado de Doctora en Ciencia, Tecnología y Gestión Alimentaria y realizada bajo nuestra dirección en el Departamento de Tecnología de Alimentos de la Universitat Politècnica de València, cumple las condiciones adecuadas para su aceptación como Tesis Doctoral, por lo que

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*A mis padres,*

*por ser el mejor ejemplo de lucha y superación,*

*por enseñarme a enfrentar la vida con una sonrisa.*

*Gracias por todo el amor y la alegría,*

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*el respeto y la comprensión*

*que llenan mi día a día.*

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# ABSTRACT / RESUMEN / RESUM

# Assessment of the influence of processing conditions on the antioxidant potential of extracts obtained from olive oil industry byproducts

## ABSTRACT

The olive oil industry generates an important number of byproducts, such as olive leaves and olive pomace. It has been demonstrated that these vegetable wastes are rich in the same phenolic compounds which are also present in the olive oil. Nevertheless, olive oil byproducts have not yet been exploited on an industrial scale, for example as sources of bioactive compounds. For this purpose, it is necessary to thoroughly study how the processing conditions (raw material pretreatment, extraction, etc.) affect the bioactive potential, as well as to explore novel applications in the food industry. Therefore, the main goal of this Thesis was to determine the influence of the main processing stages involved in the obtaining of natural extracts with high antioxidant potential from byproducts originating in the olive oil industry.

Firstly, the effect of freezing and/or the drying methods applied to olive oil byproducts on the polyphenol content and antioxidant capacity of the extracts which were subsequently obtained was addressed. For this purpose, two byproducts were considered: olive leaves and olive pomace. On the one hand, olive leaves (fresh, conventionally frozen at -28 °C or frozen in liquid $N_2$) were hot air dried at two different temperatures, 70 or 120 °C, or freeze dried. On the other hand, olive pomace drying was analyzed at different temperatures (from 50 to 150 °C) and mathematically described by means of diffusion and Weibull models.

Secondly, the feasibility of intensifying the extraction of olive leaf polyphenols by means of a new technology, such as power ultrasound, was approached taking both compositional and kinetic issues into account. For this purpose, the influence of some of the main process parameters (the electric power supplied, emitter surface and temperature) was assessed. The extraction kinetics were mathematically described by Naik's model.

Thirdly, how the processing conditions (drying and extraction) influence the extract's stability was evaluated. Thus, on the one hand, a set of experiments was carried out on extracts obtained from dried olive leaves (hot air dried at 70 and 120 °C, and freeze dried) by means of conventional or ultrasound assisted extraction. These extracts were subjected to *in vitro* digestion. On the other hand, in another set of experiments, fresh, hot air dried at 120 °C and freeze dried olive leaves were used to obtain different kinds of extracts. One part of these extracts was kept in a liquid state and another was dehydrated at 120 °C or vacuum dehydrated at 55 °C until a powder product was obtained. All the extracts (liquids and powders) were stored at 4, 25 and 35 °C for 4 weeks.

Finally, the possibility of obtaining a dried vegetable matrix (apple), rich in olive leaf phenolic compounds, was explored by addressing the influence of apple pretreatments (blanching and freezing) and drying on the final retention of infused polyphenols. Raw and blanched apple cubes were initially air dried (60 °C) or freeze dried. In the latter case, the samples were previously submitted to different freezing methods: conventional (-28 °C), blast freezing (-30 °C) and liquid $N_2$ (-196 °C). Then, the dried apples were impregnated with the phenolic extract. Once the polyphenolic infusion was completed, the samples were dried for the final stabilization by means of three different methods: hot air drying at 60 °C with and without ultrasound application and freeze drying.

The antioxidant potential of extracts and the retention of infused polyphenols in apple were evaluated through the total phenolic content and antioxidant capacity analysis, and the main olive leaf polyphenols were identified and quantified by HPLC-DAD/MS-MS. Moreover, in apple samples, the polyphenol oxidase and peroxidase activity and microstructure were also analyzed.

The experimental results highlighted that both drying and freezing methods significantly ($p<0.05$) influenced the concentration of the main polyphenols identified in the olive leaf extracts. Thus, the best processing conditions to use in order to obtain extracts of high antioxidant capacity and phenolic content were those in which the highest drying temperature tested (120 °C) was used. This effect of the

drying conditions was less relevant when olive pomace was used as the phenolic source, since the antioxidant potential of extracts was only mildly influenced by the drying temperature. However, it was not only the highest temperature tested (150 °C) but also long drying times, leading to sample overheating, increased the antioxidant potential of olive pomace extracts. Olive leaves ended up as a more promising source than olive pomace from which to obtain natural extracts rich in phenolic compounds.

Ultrasound application was found to be a relevant, non-thermal way of speeding-up the antioxidant extraction from olive leaves. Thus, by appropriately tuning-up the process variables, the ultrasonic assisted extraction shortened the extraction time from the 24 h needed in conventional extraction to 15 min, without modifying either the extract composition or the antioxidant potential. However, it is important to remark that not all the studied process variables had the same influence on the extraction process. Thus, both the electric power supplied and the emitter surface were relevant factors in the improvement of the extraction performance, whereas the influence of temperature was not clear at the tested values.

As far as extract stability is concerned, the processing conditions used to obtain the olive leaf extracts did not have a meaningful influence on bioaccessibility. In every case, the phenolic content was significantly reduced ($p<0.05$) by the digestion. Oleuropein and verbascoside practically disappeared at the end of the *in vitro* simulation. Nevertheless, luteolin-7-O-glucoside exhibited a high degree of stability to *in vitro* digestion (43 % bioaccessibility).

Unlike what was observed during the *in vitro* digestion, the processing conditions did affect the extract stability during storage. Thus, the drying of olive leaves influenced not only the initial composition of the extracts but also the evolution of their bioactive potential. Regardless of the method used, stabilizing the extracts by means of dehydration only reduced both the antioxidant capacity and the total phenolic content by around 10 %. Moreover, the storage conditions (temperature and extract form: liquid or powder) did not have a significant ($p<0.05$) effect on the antioxidant potential of the extracts for the 28 days of storage.

*Abstract*

A stable dried product (apple), rich in natural phenolic compounds (from olive leaves or tea extracts), was obtained by combining drying–impregnation–drying steps. However, it should be considered that fresh apple drying played a more significant role in the retention of infused olive leaf polyphenols than the further drying of the impregnated apple. Thus, the structure and oxidative enzymatic activity of samples obtained after fresh apple drying played a key role in the retention of phenolic compounds in the final product.

In overall terms, olive leaves can be considered a potential source of natural phenolic compounds. Notwithstanding this, the previous drying and freezing steps applied in the raw material processing are decisive factors when obtaining natural extracts with high antioxidant potential. Moreover, enhancing the extraction by applying power ultrasound was stated as a non-thermal means of shortening processing times. The stability of olive polyphenols during storage and *in vitro* digestion was closely related with the individual component considered. Finally, the exploitation of olive leaf extracts as a means of enriching solid foodstuffs requires the use of porous solid matrices free of oxidative enzymes.

# Estudio de la influencia de las condiciones de procesado en el potencial antioxidante de extractos obtenidos a partir de subproductos de la industria del aceite de oliva

## RESUMEN

La industria del aceite de oliva genera una cantidad importante de subproductos, tales como las hojas de olivo y el orujo. Se ha demostrado que estos residuos vegetales son ricos en los mismos compuestos fenólicos que se encuentran presentes en el aceite de oliva. Sin embargo, estos subproductos todavía no han sido explotados a nivel industrial como fuentes de compuestos bioactivos. Para ello, es necesario estudiar exhaustivamente cómo las condiciones procesado (pretratamiento de la materia prima, extracción, etc.) afectan al potencial bioactivo, así como explorar las posibles aplicaciones de estos compuestos en la industria alimentaria. Por lo tanto, el objetivo principal de esta Tesis fue determinar la influencia de las principales etapas de procesado implicadas en la obtención de extractos naturales con alto potencial antioxidante a partir de los subproductos originados en la industria del aceite de oliva.

En primer lugar, se evaluó el efecto de los métodos de congelación y/o secado, empleados en el procesado de la materia prima, sobre el contenido polifénolico y la capacidad antioxidante de los extractos obtenidos posteriormente. Para ello, se seleccionaron dos subproductos de la elaboración de aceite de oliva: hojas y orujo. Por una parte, las hojas de olivo (frescas, congeladas convencionalmente a -28 °C o congeladas en $N_2$ líquido) fueron secadas por aire caliente a dos temperaturas diferentes, 70 ó 120 °C, o liofilizadas. Por otra parte, el secado del orujo de oliva se llevó a cabo a diferentes temperaturas (de 50 a 150 °C), describiendo matemáticamente el proceso mediante modelos difusivos y de Weibull.

En segundo lugar, se abordó la viabilidad de intensificar la extracción de polifenoles de hoja de olivo a través una nueva tecnología, los ultrasonidos de potencia, teniendo en cuenta tanto la composición final de los extractos como la

cinética del proceso. Con este fin, se evaluó la influencia de algunos de los principales parámetros del proceso (la potencia eléctrica suministrada, la superficie del emisor y la temperatura). Las cinéticas de extracción fueron matemáticamente descritas con el modelo de Naik.

A continuación, se estudió cómo las condiciones de procesado (secado y extracción) podían influir en la estabilidad de los extractos. Así, por una parte, se obtuvieron extractos a partir de hojas de olivo secadas (aire caliente a 70 y 120 °C y liofilización) y mediante extracción convencional o asistida por ultrasonidos. Estos extractos fueron, posteriormente, sometidos a una digestión *in vitro*. Por otra parte, se obtuvieron diferentes extractos a partir de hojas frescas, secadas por caliente a 120 °C y liofilizadas. Estos extractos fueron conservados en estado líquido, o bien deshidratados (a 120 °C o aplicando vacío a 55 °C) hasta poder obtener un producto en polvo. Todos los extractos (líquidos y polvos) fueron almacenados a 4, 25 y 35 °C durante 4 semanas.

Por último, se exploró la posibilidad de obtener una matriz vegetal deshidratada (manzana) y rica en compuestos fenólicos de hoja de olivo. Para ello, se evaluó la influencia de los pretratamientos de la manzana (escaldado y congelación) y del secado en la retención final de los polifenoles impregnados. Así, cubos de manzana fresca o escaldada fueron inicialmente secados por aire (60 °C) o liofilizados. En este último caso, las muestras fueron previamente congeladas empleando distintos métodos: congelación convencional (-28 °C), congelación rápida o "blast freezing" (-30 °C) y congelación en $N_2$ líquido (-196 °C). Posteriormente, las manzanas secas fueron impregnadas con el extracto fenólico. Una vez completada la infusión fenólica, las muestras se secaron de nuevo para su estabilización mediante tres métodos diferentes: aire caliente a 60 °C con y sin aplicación de ultrasonidos y liofilización.

El potencial antioxidante de los extractos y la retención de los polifenoles incorporados a la manzana se determinaron a través de la medida del contenido total en compuestos fenólicos y de la capacidad antioxidante, así como de la identificación y cuantificación mediante HPLC-DAD/MS-MS de los principales polifenoles presentes

en la hoja de olivo. Además, en las muestras de manzana, se midió la actividad enzimática de la polifenol oxidasa y peroxidasa y se analizó la microestructura.

Los resultados experimentales pusieron de manifiesto que tanto el método de secado como el de congelación influyeron significativamente ($p<0.05$) en la concentración de los principales polifenoles identificados en los extractos de hoja de olivo. Así, el secado a la mayor temperatura ensayada (120 °C) resultó ser el mejor tratamiento para obtener extractos con alta capacidad antioxidante y alto contenido fenólico. La influencia de las condiciones de secado fue menos relevante cuando se empleó como fuente de compuestos fenólicos el orujo de oliva. En este caso, el potencial antioxidante de los extractos tan sólo se vio ligeramente afectado por la temperatura de secado. Sin embargo, no sólo la temperatura más alta evaluada (150 °C) sino también tiempos largos de secado, que supusieron un sobrecalentamiento de la muestra, dieron lugar a un aumento del potencial antioxidante de los extractos de orujo de oliva. En comparación, las hojas de olivo pueden considerarse una fuente más prometedora que el orujo de oliva para la obtención de extractos naturales ricos en compuestos fenólicos.

La aplicación de ultrasonidos resultó ser una alternativa no térmica muy interesante para acelerar la extracción de antioxidantes de hojas de olivo. Con la combinación adecuada de las variables del proceso, la aplicación de ultrasonidos redujo el tiempo de extracción de 24 h necesarias en extracción convencional a 15 min, sin modificar la composición de los extractos y su potencial antioxidante. Aun así, es importante remarcar que no todas las variables estudiadas tuvieron la misma influencia en el proceso de extracción. La potencia eléctrica suministrada y la superficie del emisor fueron factores clave en la mejora del rendimiento de la extracción, mientras que la influencia de la temperatura no fue clara en el rango de temperaturas evaluado.

En cuanto a la estabilidad del extracto, las condiciones de procesado empleadas para la obtención de extractos de hoja de olivo no tuvieron una influencia significativa en su bioaccesibilidad. En todos los casos, la digestión redujo significativamente ($p<0.05$) el contenido fenólico. La oleuropeína y el verbascósido

prácticamente desaparecieron al final de la digestión *in vitro*. Por el contrario, la luteolina-7-O-glucósidó mostró una buena estabilidad (bioaccesibilidad del 43 %).

A diferencia de lo observado durante la digestión *in vitro*, las condiciones de procesado sí afectaron a la estabilidad del extracto durante su almacenamiento. Así, el secado de las hojas de olivo no sólo determinó la composición inicial de los extractos sino también la evolución del potencial bioactivo. Independientemente del método utilizado, la estabilización de los extractos por deshidratación sólo redujo la capacidad antioxidante y el contenido total en compuestos fenólicos en torno a un 10 %. Además, las condiciones de almacenamiento (temperatura y forma del extracto: líquido o polvo) no mostraron ningún efecto significativo ($p<0.05$) sobre el potencial antioxidante durante los 28 días de almacenamiento.

Combinando las etapas de secado-impregnación-secado, fue posible desarrollar un producto deshidratado (manzana), estable y rico en compuestos fenólicos naturales (de hojas de olivo o extractos de té). No obstante, cabe destacar que el secado de la manzana fresca jugó un papel más importante en la retención de los polifenoles de hoja de olivo infundidos que el secado final de la manzana impregnada. En consecuencia, la estructura y la actividad enzimática oxidativa de las muestras obtenidas tras el secado de la manzana fresca fueron clave para la retención de compuestos fenólicos en el producto final.

En términos generales, las hojas de olivo pueden considerarse como una fuente potencial de compuestos fenólicos naturales. No obstante, el secado y la congelación durante el procesado de la materia prima son factores decisivos para la obtención de extractos naturales con alto potencial antioxidante. Además, la aplicación de ultrasonidos de potencia durante la extracción puede resultar una alternativa no térmica muy interesante de cara a acortar el tiempo de procesado. La estabilidad de los polifenoles de la hoja de olivo, durante el almacenamiento y la digestión *in vitro*, dependió claramente del compuesto individual considerado. Finalmente, el empleo del extracto de hoja de olivo como medio para enriquecer alimentos sólidos requiere del uso de matrices sólidas porosas y libres de enzimas oxidativas.

# Valoració de la influència de les condicions de processament en el potencial antioxidant d'extractes obtinguts de subproductes de la indústria de l'oli d'oliva

## RESUM

La indústria de l'oli d'oliva genera un nombre important de subproductes, com ara les fulles d'olivera i la pinyolada. S'ha demostrat que aquests residus vegetals són rics en els mateixos compostos fenòlics que l'oli d'oliva. No obstant això, els subproductes de l'oli d'oliva no s'han explotat encara a escala industrial, per exemple com a fonts de compostos bioactius. Per aquest motiu, cal estudiar exhaustivament com les condicions de processament (pretractament de la matèria primera, extracció, etc.) afecten el potencial bioactiu d'aquests subproductes i, al mateix temps, explorar-ne noves aplicacions en la indústria alimentària. Per tant, l'objectiu principal d'aquesta tesi va ser determinar la influència de les principals etapes de processament implicades en l'obtenció d'extractes naturals amb alt potencial antioxidant procedents de subproductes de la indústria de l'oli d'oliva.

En primer lloc, es va estudiar l'efecte de la congelació i/o els mètodes d'assecatge aplicats a subproductes de l'oli d'oliva sobre el contingut fenòlic i la capacitat antioxidant dels extractes obtinguts subsegüentment. Amb aquesta finalitat, es van prendre en consideració dos subproductes: les fulles d'olivera i la pinyolada. D'una banda, unes fulles d'olivera (fresques, congelades convencionalment a -28 °C o congelades en $N_2$ líquid) van ser assecades amb aire calent a dues temperatures diferents, 70 o 120 °C, o liofilitzades. D'altra banda, l'assecatge de la pinyolada va ser analitzat a diferents temperatures (de 50 a 150 °C) i descrit matemàticament amb els models de difusió i Weibull.

En segon lloc, es va avaluar, tenint en compte la composició dels extractes i la cinètica del procés d'extracció, la viabilitat d'intensificar l'extracció de polifenols de fulla d'olivera utilitzant una tecnologia nova, ultrasons de potència. Amb aquest objectiu es va avaluar també la influència d'alguns dels principals paràmetres del

procés (l'energia elèctrica subministrada, la superfície de l'emissor i la temperatura). La cinètica d'extracció va ser descrita matemàticament pel model de Naik.

En tercer lloc, es va avaluar com les condicions de processament (assecatge i extracció) poden influir en l'estabilitat dels extractes. Així, d'una banda, extractes obtinguts de fulles d'olivera assecades (aire calent a 70 i 120 °C i liofilització) i amb extracció convencional o assistida per ultrasons van ser sotmesos a una digestió *in vitro*. D'altra banda, fulles d'olivera fresques, assecades a 120 °C i liofilitzades, van ser usades per a obtenir extractes diferents. Una part d'aquests extractes va ser mantinguda en estat líquid i l'altra va ser deshidratada a 120 °C o aplicant-hi buit a 55 °C fins a aconseguir un producte en pols. Tots els extractes (líquids i en pols) van ser emmagatzemats a 4, 25 i 35 °C durant 4 setmanes.

Finalment, es va explorar la possibilitat d'obtenir una matriu vegetal deshidratada (poma) i rica en compostos fenòlics de fulla d'olivera considerant la influència del pretractament de la poma (escaldament i congelació) i de l'assecatge sobre la retenció final dels fenòlics introduïts en la poma. Daus escaldats i frescos de poma van ser primerament assecats amb aire (60 °C) o liofilitzats. En el segon cas, les mostres van ser congelades prèviament utilitzant diferents mètodes de congelació: convencional (-28 °C), congelació ràpida o *blast freezer* (-30 °C) i $N_2$ líquid (-196 °C). A continuació, les pomes assecades van ser impregnades amb extracte fenòlic. Una vegada es va completar la infusió polifenòlica, les mostres van ser assecades perquè s'estabilitzaren, emprant tres mètodes diferents: liofilització i assecatge amb aire calent a 60 °C, amb i sense aplicació d'ultrasons.

El potencial antioxidant dels extractes i la retenció dels polifenols impregnats dins la poma es van avaluar determinant el contingut fenòlic total i la capacitat antioxidant, així com identificant i quantificant els principals polifenols de la fulla d'olivera per HPLC-DAD/MS-MS. A més, es va analitzar a les mostres de poma l'activitat enzimàtica de la polifenoloxidasa i la peroxidasa i la microestructura.

Els resultats experimentals van destacar que tant el mètode d'assecatge com el de congelació van influir significativament ($p<0,05$) en la concentració dels principals polifenols identificats en els extractes de fulla d'olivera. L'assecatge a la temperatura

més alta que es va provar (120 °C) va resultar la millor condició de processament per a obtenir extractes amb una alta capacitat antioxidant i un alt contingut fenòlic. Aquest efecte de les condicions d'assecatge va ser menys rellevant quan s'usà la pinyolada com a font fenòlica. El potencial antioxidant dels extractes tan sols es va veure lleugerament influït per la temperatura d'assecatge. No obstant això, no sols la temperatura més alta provada (150 °C) va augmentar significativament ($p<0,05$) el potencial antioxidant del extractes de pinyolada; els temps llargs d'assecatge, els quals van significar un sobreescalfament de la mostra, també van contribuir positivament a incrementar-ne el potencial antioxidant. En comparació, les fulles d'olivera van resultar ser una font més prometedora que la pinyolada per a obtenir-ne extractes naturals rics en compostos fenòlics.

L'aplicació d'ultrasons va ser una manera rellevant i no tèrmica d'accelerar l'extracció d'antioxidants de les fulles d'olivera. Així, amb la combinació adequada de les variables del procés, l'extracció assistida per ultrasons va escurçar el temps d'extracció, de les 24 h requerides en l'extracció convencional a 15 min, sense modificar la composició de l'extracte ni el potencial antioxidant. No obstant això, és important remarcar que no totes les variables van tenir la mateixa influència en el procés d'extracció. Tant l'energia elèctrica subministrada com la superfície de l'emissor van ser factors determinants en la millora de l'extracció, mentre que la influència de la temperatura no va ser clara en el rang de valors estudiat.

Quant a l'estabilitat de l'extracte, les condicions de processament utilitzades per a l'obtenció dels extractes de fulla d'olivera no van tenir una influència significativa en la bioaccessibilitat. En tots els casos, la digestió va reduir significativament ($p<0,05$) el contingut fenòlic. L'oleuropeïna i el verbascòsid pràcticament van desaparèixer al final de la simulació *in vitro*. Per contra, la luteolina-7-O-glucòsid va mostrar una bona estabilitat davant les condicions de la digestió *in vitro* (43 % de bioaccessibilitat).

A diferència del que s'ha observat durant la digestió *in vitro*, les condicions de processament van afectar l'estabilitat de l'extracte durant l'emmagatzematge. Així, l'assecatge de fulles d'olivera va influir no sols en la composició inicial dels extractes,

sinó també en l'evolució del potencial bioactiu d'aquests. Independentment del mètode utilitzat, l'estabilització dels extractes per mitjà de la deshidratació només va reduir la capacitat antioxidant i el contingut fenòlic total al voltant d'un 10 %. A més, les condicions d'emmagatzematge (temperatura i forma de l'extracte: líquid o pols) no van mostrar cap efecte significatiu ($p<0,05$) en el potencial antioxidant dels extractes durant els 28 dies d'emmagatzematge.

Combinant etapes d'assecatge-impregnació-assecatge fou possible obtenir un producte assecat estable (poma) i ric en compostos fenòlics naturals (de fulles d'olivera o te). No obstant això, cal destacar que l'assecatge de la poma fresca va ser més important i determinant en la retenció dels polifenols de fulla d'olivera que no l'assecatge de la poma impregnada. Així, l'estructura i l'activitat enzimàtica oxidativa de mostres obtingudes després de l'assecatge de la poma fresca van tenir un paper clau en la retenció dels compostos fenòlics en el producte final.

En termes generals, les fulles d'olivera es poden considerar com una font potencial de compostos fenòlics naturals. No obstant això, l'aplicació d'assecatge i congelació durant el processament de la matèria primera són factors decisius per a l'obtenció d'extractes naturals amb un alt potencial antioxidant. A més, l'aplicació d'ultrasons de potència durant l'extracció resultà ser una forma no tèrmica de millorar el procés, tot reduint-ne el temps d'extracció. L'estabilitat dels polifenols d'olivera durant l'emmagatzematge i la digestió *in vitro* va dependre del compost individual considerat. Finalment, la utilització d'extractes de fulla d'olivera per a desenvolupar aliments sòlids enriquits requereix l'ús de matrius sòlides poroses i lliures d'enzims oxidatius.

# 1. INTRODUCTION

## 1.1. Food industry byproducts as sources of phenolic compounds

Nowadays, the processing of plant foods in the food industry generates a large number of byproducts. Although in the past these byproducts were considered only a waste without any value, constituting an important environmental problem in some cases, now they represent an opportunity to improve the profitability of food factories. In fact, byproducts have started to be considered as possible sources of natural additives and ingredients for the food, chemical and pharmaceutical industries.

The number of scientific papers concerning phenolic compounds, especially those present in foodstuffs, has been increasing recently (Fig. 1). In the last five years, a rise of 63.9 % has been observed, which demonstrates the interest shown in these compounds and the potential of foods as natural sources able to provide them.

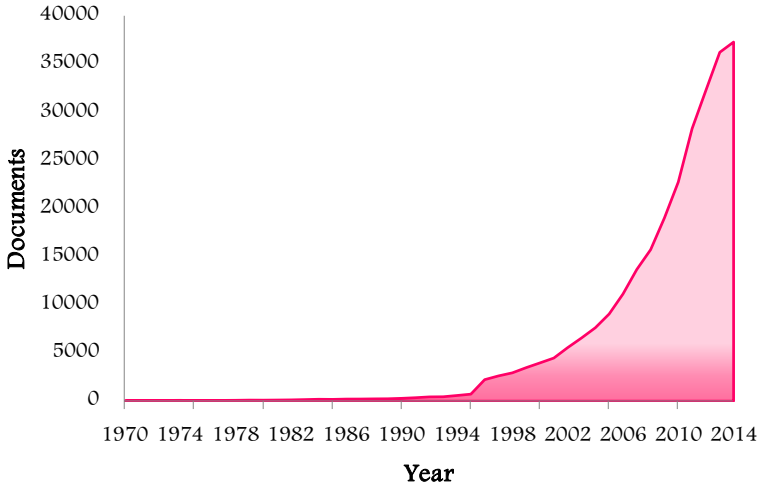


**Fig. 1.** Evolution in the number of published research papers concerning antioxidants in food over the last few years (Search: (TITLE-ABS-KEY (antioxidant OR polyphenol) AND TITLE-ABS-KEY (food)), Scopus, 2015).

Fruits, vegetables and their derived beverages are the major sources of phenolic compounds in the human diet (Hertog et al., 1993). These compounds are believed to contribute to the protective effect that many food commodities have on the health. Thus, they have even been named the "vitamins of the 21$^{st}$ century" (Stich, 2000). Notwithstanding, it has been demonstrated that byproducts originating from the

processing of fruits, vegetables and even other plant foods, such as cereals and oilseeds, are also rich sources of bioactive compounds, including phenolic compounds. The supplementation of foods with polyphenols recovered from waste materials could be a valuable strategy with which to increase the dietary ingestion of these compounds. Moreover, they could constitute a source of natural additives for food preservation and cosmetic and pharmaceutical formulations, implying in every case an increase in the value of food industry byproducts.

There is extensive literature on the extraction of phenolic compounds from food industry wastes. Grapes are one of the most important crops, with an annual production of almost 60 million tons in 2012 (FAOSTAT, 2015). One of the main grape byproducts, solid pomace, has been used for the recovery of different compounds, such as dietary fiber (Zhu et al., 2014) and anthocyanins (Mazza, 1995; Monrad et al., 2014). Moreover, grape seeds have been exploited in order to obtain seed oil and phenolic antioxidants (Duba & Fiori, 2015; Maier et al., 2009) as well as sugars and lignin (Yedro et al., 2014). As far as grape stalks are concerned, they have not only been studied as a source of antioxidants (García-Pérez et al., 2010), but they are also employed in the preparation of activated carbon (Ozdemir et al., 2014) and in the recovery of fermentable sugars for use in bioethanol production (Egües et al., 2013).

Apple pomace, what is leftover after juice processing, consists mainly of skin and flesh (95 %), seeds (2-4 %) and stems (1 %) (Bhushan et al., 2008). It has been shown to be a good source of polyphenols with strong antioxidant activity, the main ones being quercetin derivatives and anthocyanins (Grigoras et al., 2013; Lu & Foo, 2000; Sadilová et al., 2006). Despite the interest in phenolic compounds, pectin extraction is still considered the most efficient way of apple pomace valorization (Endress, 2000; Wang et al., 2014). Thus, a method for the combined recovery of pectin and polyphenols from apple pomace has been developed (Schieber et al., 2003).

There are also some cases in which the antioxidant potential of byproducts is much greater than in the food product. Thereby, the quantity of total phenolics in peaches, pears and apples peels was twice that in the edible fleshy parts (Gorinstein et

al., 2002). In the citrus industry, Gorinstein et al. (2001) found that the total phenolic content in the peels of lemons and oranges was 15 % higher than that found in the peeled fruits.

Apple, grape and citrus fruit are some of the most widely consumed fruits. Nevertheless, there are others, such as the tropical fruits, whose manufacturing can also generate byproducts rich in phenolic compounds. For example, the main wastes of mango processing are peels and seed kernels, representing between 35–60 % of the total fruit weight. Peels are a good source not only of extractable polyphenols but also dietary fiber (Vergara-Valencia et al., 2007) and pectin (Berardini et al., 2005; Maran et al., 2014). As regards the seed kernels, they are considered a promising source of edible oil and characterized by their natural antioxidant content, mainly gallic and ellagic acids (Soong & Barlow, 2006).

As previously mentioned, it is not only fruits which have large amounts of phenolic compounds but also vegetables. Potatoes are one of the most important staple crops for human consumption. Of the processing waste, peels constitute the fraction containing most of the valuable compounds, such as phenolics and glycoalkaloids (Mäder et al., 2009). Therefore, a method for the simultaneous recovery and subsequent separation of phenolic acids and glycoalkaloids for food and pharmaceutical purposes has recently been developed (Sánchez-Maldonado et al., 2014).

Onions are one of the major vegetable crops grown in Europe. Byproducts derived from the manipulation and preparation for their commercialization have been considered a suitable source for the development of natural food ingredients with antioxidant and antibrowning properties (Roldán et al., 2008). The relevance of these byproducts resides in the fact that they contain the largest amount of quercetin (Hertog et al., 1992), one of the most abundant phenolic compounds in vegetables and fruits (Moon et al., 2000). Moreover, onion wastes are a source of flavor and fiber compounds (Benítez et al., 2011; Waldron, 2001).

There are other well known vegetables, such as tomato, in which it has been found that peels and seeds are richer sources of phenolic compounds than the fleshy

pulp (Toor & Savage, 2005). Nevertheless, less consumed vegetables can also provide wastes to be exploited. Thus, garlic husks contain phenolic compounds, such as caffeic, *p*-coumaric ferulic and di-ferulic acids, which make this byproduct an easily accessible source of natural bioactive compounds (Kallel et al., 2014). Artichoke residues (leaves, external bracts and stems) represent a huge amount of discarded material in industrial canning processing (Lattanzio et al., 2009) but recently the possibility of recovering these wastes for subsequent use has been assessed. Hence, it has been proved that artichoke byproducts represent an interesting material to be used in the functional food industry, with caffeoylquinic acids, such as chlorogenic acid, luteolin-7-glucoside and apigenin-7-glucoside being the main identified phenolic compounds (Abu-Reidah et al., 2013; Ruiz-Cano et al., 2014).

Apart from fruits and vegetables, there are other agro-industrial wastes with a high content of phenolics. For example, byproducts from nuts, such as pistachio (Goli et al., 2005) and almond hulls (Takeoka & Dao, 2002) as well as oil extraction wastes. Of the latter byproducts, the pressing residues originating from sunflower oil extraction can be highlighted. They are still rich in phenolic compounds, the main ones being monocaffeoyl quinic acids, which amount to 84-92 % of the total phenolics in kernels (Weisz et al., 2009). The olive oil industry has also attracted considerable interest because of the phenolic potential of its byproducts. Consequently, its importance as a natural source of bioactive compounds will be explained in more detail in the following sections.

## 1.2. The olive oil industry

Nowadays, olive trees (*Olea europaea* L.) are cultivated in several countries around the world. Notwithstanding this, 97 % of the total world production of olive oil is concentrated in the countries of the Mediterranean basin (López-Villalta, 1998). Thereby, only three countries (Spain, Greece and Italy) encompass almost 80 % of the production, with more than 30 % produced in Spain (Bas et al., 2001).

Olive oil is a high-value edible oil, which is prized for its flavor as well as its health-related benefits (Stefanoudaki et al., 2011). The health benefits are mainly

ascribed to the presence of a high content of monounsaturated fatty acid and functional bioactive compounds, including tocopherols, carotenoids, phospholipids and phenolics, with multiple biological activities (Covas et al., 2006; Covas, 2008). However, despite its nutritional and economic importance, the olive oil industry is characterized by the serious impact it has on the environment due to the production of highly polluted wastewater and/or solid residue (olive pulp, skin and stone), the amounts of which depend on the oil extraction process (Azbar, 2004).

### 1.2.1. Oil extraction systems and byproducts

There are three main methods of extracting the olive oil: a traditional pressing method and two centrifugation techniques called three-phase and two-phase systems (Roig et al., 2006). Even though traditional pressing is an obsolete technology, some olive oil producers still use it as a sign of quality. After the extraction by pressing, both a solid byproduct (pomace or "orujo") and an emulsion containing oil and water are obtained. Olive oil is separated by decantation giving rise to a liquid byproduct, the olive mill wastewater ("alpechín").

The three-phase system substitutes the slow decantation operation for centrifugation, generating the olive oil and the same byproducts as those in the conventional system, the pomace and the wastewater, at the end of the process. This system makes process automation easier and reduces the necessary processing area. However, it also presents some issues, such as greater water and energy consumption, costly facilities and a more significant production of wastewater as the result of water addition during the oil extraction. For these reasons, at the beginning of the 1990s, a new centrifugation system was developed in order to reduce olive mill wastes. This new centrifugation system, called two-phase, drastically reduces the water consumption during the process and produces only two fractions: the olive oil and a semi-solid byproduct ("alperujo").

The different olive oil byproducts may present slightly different composition and characteristics depending on the production techniques. Olive mill wastewater or "alpechín", is a mixture of water and oil from the olive fruit, with additional water added from processing. Thus, it contains the remains of the pulp, mucilage, pectins,

oil, etc., suspended in a relatively stable emulsion (Paredes et al., 1999). This byproduct can be used as an organic fertilizer (Kotsou et al., 2004; Rinaldi et al., 2003) or as resource for the production of biohydrogen and biopolymers (Ntaikou et al., 2009).

Olive pomace, also known as "orujo", olive cake or olive husk, is composed of pulp, peels and stones. It has been proposed as a potentially renewable energy source being studied, for example, in the production of biodiesel and biogas (López et al., 2014; Tekin & Dalgic, 2000). However, it is often consumed as fertilizer and animal feed (Manios, 2004).

"Alperujo", also called olive wet husk, wet pomace or olive mill waste, consists of a thick sludge with pieces of stone and the pulp of the olive fruit. It can be considered as a suspension mixture of "alpechín" (liquid phase) and "orujo" (solid phase). This semi-solid effluent has a water content of about 65 % and a very high content of organic matter, mainly composed of lignin, hemicellulose and cellulose. It also contains a considerable proportion of fats, proteins, water-soluble carbohydrates and a small, but active, fraction of hydrosoluble phenolic compounds (Alburquerque et al., 2004). Due to its high water content, it is dried and then re-used by performing a second extraction with solvents of the remaining oil. Afterwards, the exhausted olive cake is usually used as fuel to obtain energy through combustion (Caputo et al., 2003).

Olive leaves and branches are agricultural residues stemming not only from pruning but also from the harvesting operations. They can also be considered as industrial byproducts, representing 10 % of the total weight of the fruit reaching oil factories (Espínola, 1997). In spite of being used in traditional medicine to reduce the symptoms of fever or malaria, the leaves of olive trees have not been exploited industrially. At the present time, most of these wastes are simply disposed of by burning (Rada et al., 2007). Only a small part is employed as animal feed (Delgado-Pertíñez et al., 2000; Martín-García & Molina-Alcaide, 2008) or consumed as a dietary component in the form of an extract (Sedef & Karakaya, 2009).

In addition to the abovementioned olive oil byproducts, there is another important waste which originates in table olive industries, olive stones. This lignocellulosic residue is mainly used to produce electric energy or heat by combustion. There are other uses of this biomass, such as the production of activated carbon, applied in the removal of unwanted colors and dyes (Najar-Souissi et al., 2005), odors, tastes or contaminants, such as arsenic (Budinova et al., 2006). Furfural, xylitol and ethanol production (Montane et al., 2002; Saleh et al., 2014) and plastic filling (Siracusa et al., 2001) have also been cited. Furthermore, it has been reported that olive stones are used as metal biosorbent (Rodríguez et al., 2008), animal feed (Carraro et al., 2005), and in resin formation (Tejeda-Ricardez et al., 2003).

### 1.2.2. Olive phenolic compounds

Table olives and extra virgin olive oil, both typical products of the Mediterranean food culture, are certainly the principal food sources of olive biophenols (Uccella, 2001). Nevertheless, the increasing worldwide demand for olive biomolecules has led to the search for alternative phenolic sources. It has been proven that phenolic compounds are present in almost every part of both the olive tree and olive oil industry byproducts (Table 1). Nevertheless, their nature and concentration vary greatly from one tissue to another, in some cases, the content being higher in wastes than in oil. The phenolic composition is very complex and, in this sense, the average concentration of these compounds depends on several factors, including the maturation stage, the part of the fruit, the variety, the age, the season, the packaging, the storage, the climatological conditions and how they are processed (Bengana et al., 2013; Boskou et al., 2005; Ranalli et al., 2006).

The major phenolic compounds of olive fruit and its byproducts are secoiridoids and flavonoids (Makris et al., 2007). Nevertheless, there are other compounds which belong to other groups, such as phenolic acids, phenolic alcohols and hydroxycinnamic acid derivatives.

**Table 1.** Concentration of the main olive phenols found in extracts from some olive oil and olive tree materials (Japón-Luján et al., 2008).

| Phenol (mg/kg) | Olive oil and byproducts | | | | |
|---|---|---|---|---|---|
| | Oil | Alperujo | Leaves | Small branches | Stones |
| Hydroxytyrosol | 3.0 ± 0.2 | 831 ± 22 | n.d. | 22 ± 2 | 18.1 ± 1.9 |
| Luteolin-7-glucoside | n.d. | 14 ± 2 | 155 ± 10 | 175 ± 8 | 6.2 ± 0.8 |
| Apigenin-7-glucoside | n.d. | 6.2 ± 0.9 | 207 ± 10 | 10.9 ± 0.8 | 0.09 ± 0.01 |
| Verbascoside | 0.08 ± 0.02 | 20 ± 3 | 1428 ± 46 | 1560 ± 50 | 0.15 ± 0.03 |
| Oleuropein | n.d. | 37 ± 4 | 19050 ± 880 | 673 ± 34 | 0.06 ± 0.02 |
| Apigenin | 0.65 ± 0.04 | 23 ± 3 | n.d. | n.d. | n.d. |
| Luteolin | 8.6 ± 0.9 | 22 ± 3 | n.d. | n.d. | 1.2 ± 0.2 |
| Diosmetin | 0.60 ± 0.08 | n.d. | n.d. | n.d. | n.d. |

Oleuropein is the major constituent of the secoiridoid family in unripe olive fruit and, as a consequence of its metabolization to hydroxytyrosol, it becomes less concentrated as the fruit matures (Fernández-Bolaños et al., 2006). It has been found to be present in proportions of 0.005-0.12 % in olive oil, up to 0.87 % in alperujo and 1-14 % in olive leaves (Luque de Castro & Japón-Luján, 2006). One of the most prominent properties of oleuropein is its strong antioxidant activity, particularly as a free radical scavenger (Cicerale et al., 2012). Many studies have also documented its antimicrobial (Furneri et al., 2002) and antiviral (Lee-Huang et al., 2003) activities. Moreover, this compound has exhibited other effects; for example it is cardioprotective (Andreadou et al., 2009) and preventive against age-related bone loss and osteoporosis (Santiago-Mora et al., 2011). As for the role of oleuropein in obesity, the addition of oleuropein to high-fat diet results in a decrease in body weight gain and liver weight and improves the lipid profiles in both the plasma and the liver (Park et al., 2011). Recently, it has also been proven that oleuropein has potent anti-breast cancer properties (Elamin et al., 2013).

Luteolin and apigenin are the flavonoid compounds of olive fruit (Bendini et al., 2007), flavonol glycosides, such as luteolin-7-O-glucoside and apigenin-7-O-glucoside, being their main derivatives (Romani et al., 1999). Luteolins have been reported to possess strong antioxidative, antiinflammatory and antiallergic activities (Chen et al., 2007; Seelinger et al., 2008), as well as an antitumor effect (Hwang et al.,

2011). Most recently, the ability of luteolin to protect the brain from traumatic injury has also been investigated (Xu et al., 2014) and its potential use in the treatment against arthritis (Shi et al., 2014). As far as apigenins are concerned, as in the case of luteolin and other flavonoids, they can be considered antiallergic substances (Kawai et al., 2007). They also inhibit platelet adhesion and thrombus formation (Navarro-Núñez et al., 2008) and exhibit antioxidant activity (Benavente-García et al., 2000). In addition, apigenin is one of the most interesting of the flavonoids used in the fight against cancer. Its anticarcinogenic effects have been demonstrated in several studies related to thyroid (Yin et al., 1999), colon (Van Dross et al., 2003), prostate (Shukla & Gupta, 2007) and breast (Long et al., 2008) cancer, among other forms.

Verbascoside has been detected in a wide range of plant species, even in olive trees (*Olea europaea*). In this case, and despite not belonging to the two biggest phenolic families present in olive fruit, it is the main hydroxycinnamic acid derivative. In leaves and branches, the presence of verbascoside cannot be underestimated when it is considered that the ratio of the verbascoside to oleuropein content examined in 13 Spanish cultivars varied from 0.05 to 0.63 (Japón-Luján & Luque de Castro, 2007). Their biological properties have been extensively reported and include antioxidant (Frum et al., 2007), anti-inflammatory and wound healing activities (Akdemir et al., 2011), as well as antifungal (Oyourou et al., 2013), antimicrobial (Shikanga et al., 2010) sedative (Daels-Rakotoarison et al., 2000) and antiatherogenic (Funes et al., 2009; Liu et al., 2003) properties. Furthermore, it highlights both the antiinflammatory effect of verbascoside on the skin (Lee et al., 2006) and its use in the prevention of some kinds of cancer. Of the other compounds, verbascoside has recently been considered the best candidate for topical photoprotection and, consequently, for the chemoprevention of UV-induced non-melanoma skin cancers (Kostyuk et al., 2013).

It is claimed that hydroxytyrosol is the principal product of oleuropein hydrolysis during olive fruit ripening and oil extraction, brought about by the action of esterases (Capasso et al., 1994). Moreover, the amount of hydroxytyrosol can be increased by the acid hydrolysis of secoiridoid derivatives and verbascoside. The main feature of hydroxytyrosol is its strong antioxidant capacity, greater than oleuropein

(Benavente-García et al., 2000). However, it is difficult to extract pure hydroxytyrosol from a natural source due to the fact that it usually forms part of other molecules, such as oleuropein, demethyloleuropein, verbascoside and hydroxytyrosol glucosides. It has also been demonstrated that hydroxytyrosol acts against several types of cancerous tumors. One of the last studies focusing on liver cancer suggested that this phenol may be a promising candidate as an agent for the prevention and therapy of hepatocellular carcinoma (Zhao et al., 2014). There are studies in which the neuroprotective effect of hydroxytyrosol and its capacity to reduce oxidative stress have also been proven (Cabrerizo et al., 2013). Moreover, it has exhibited antimicrobial (Medina et al., 2007) and antithrombotic (González-Santiago et al., 2006) activities, among others.

In addition to the main phenolics mentioned above, there are other minor compounds which can also be found in olive products. Phenolic acids, such as *p*-hydroxybenzoic, *p*-coumaric, vanillic, caffeic, ferulic, sinapic or chlorogenic, and other flavonoids, such as quercetin and rutin or secoiridois, such as ligstroside (Ryan et al., 1999).

As can be appreciated, the phenolic compounds present in both the olive fruit and byproducts possess a great amount of diverse bioactive properties which support the importance of these materials. Nowadays, the exploitation of olive byproducts as sources of phenolic compounds is in its early stages, being advisable to take into account all the processing stages involved (Fig. 2). In order to achieve an efficient and effective recovery of the target compounds, the key stage is that of extraction, with the technology and the extraction conditions chosen playing a vital role. Nevertheless, there are also other processing operations which can modify, reduce or even enhance, the antioxidant potential yield achieved. Thus, pretreatments of raw material, such as drying, blanching and freezing, as well as extract stabilization (dehydration, freezing, encapsulation, etc.) so as to extend the shelf-life can be considered essential in the obtaining of antioxidant extracts. Moreover, it is necessary to explore the possible applications of extracts by evaluating not only the potential health benefits but also their capacity of assimilation by the human body.

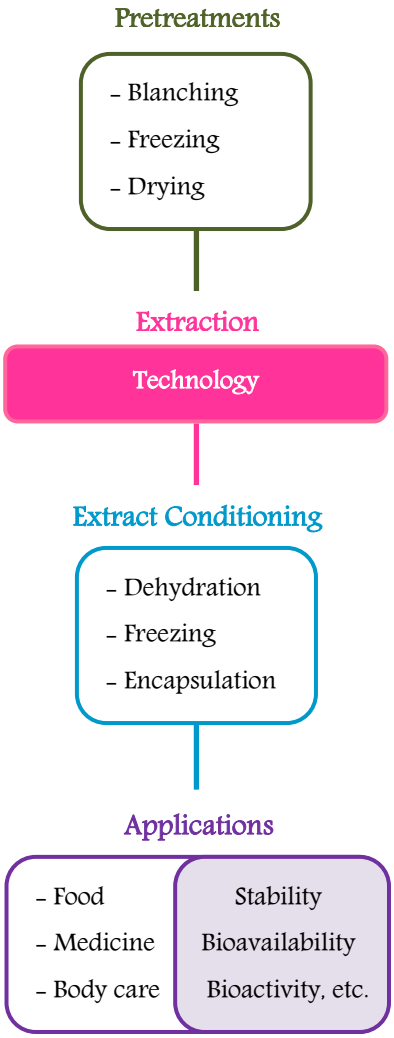


**Fig. 2.** Main stages for the exploitation of natural sources rich in bioactive compounds.

## 1.3. Drying

### 1.3.1. Fundamentals

Dehydration is defined as the process of reducing the moisture content of a product, while drying is considered the dehydration operation in which water is removed to a gas phase, which involves water evaporation (or sublimation).

The most common food dehydration technique is hot air drying. Generally, hot air drying occurs in two stages, these being differentiated by the drying rate. The first stage is characterized by a constant rate; the drying is controlled by the water evaporation and its transfer from the product surface to the air. During this period, the moisture movement inside the product is fast enough to keep the product surface saturated. Thus, the mechanism of water removal does not depend on the nature of the product. The first stage is extended until the moisture content is lower than a critical moisture content value. The second stage involves a slow drying rate since the total product surface is no longer saturated and dried areas appear. As a consequence, the movement of water inside the product, acting as a limiting factor, increases in importance. Therefore, this stage is related to the properties of the material to be dried (Ekechukwu, 1999; El-Sebaii & Shalaby, 2012; Mujumdar & Devahastin, 2000).

Drying operations are applied in a large number of industrial sectors, such as the food industry, the chemical industry and agricultural and mineral production, and so on. In particular, the removal of moisture from solids represents an important part of food engineering and, with few exceptions, most food products undergo drying at some stages of their processing (Mujumdar & Menon, 1995).

The reduction in water activity by means of moisture removal also implies a significant reduction in the weight and volume of the products, minimizing packaging, transportation and storage costs (Okos et al., 1992) and facilitating the handling. However, the main purpose of drying is the preservation of products. Thus, drying increases the shelf life of foodstuffs by inhibiting the microbial growth and forestalling certain biochemical reactions that may alter the organoleptic characteristics (Díaz-Maroto et al., 2003; Hossain et al., 2010). In addition, drying is also used as a pretreatment for reducing the interference of water in the extraction of compounds, such as antioxidants, which are retained in solid matrices (Chism & Haard, 1996; Górnaś et al., 2014).

Notwithstanding this, during the drying operation, physical, structural, chemical or nutritional changes may occur and affect quality attributes like texture, color, flavor and/or nutritional value (Di Scala & Crapiste, 2008). These alterations in food

properties do not always take place with the same intensity, since the quality of the final products is heavily dependent on the drying technique and the process variables used (Doymaz, 2005). This fact, linked to an increasing consumer demand to keep the original characteristics of raw matter in processed foods, has led the food industry to carry out continuous research into drying techniques that better preserve the material, without introducing undesirable changes. Attempts to obtain high quality dried products have mainly focused on reducing the drying temperature or time. Thus, drying technology has evolved from simple processes like those based on the use of solar energy to current technology that includes, among other things, freeze drying or spray drying. Nowadays, research is also being carried out into the introduction of novel technologies, such as microwaves, infrared, ohmic heating, radio frequency radiation, supercritical fluids or high power ultrasound. In this sense, different combinations of these technologies are being explored in order to reduce the disadvantages of a single drying method, such as long drying times, high energy consumption and the loss of nutritive compounds.

Natural convective drying (drying in the shade) is used because of its low cost (Soysal, 2004). However, natural drying has many disadvantages, such as an inability both to handle large quantities of the product and to achieve consistent quality standards (Soysal & Öztekin, 2001). These drawbacks have been overcome by conventional air drying, nowadays the most widely used industrial drying technique (Mujumdar & Devahastin, 2000). It usually provides dehydrated products characterized by low porosity and high apparent density (Krokida & Maroulis, 1997) with an extended shelf life. However, the quality of a conventionally dried product is, in most cases, drastically reduced when compared to the original foodstuff (Ratti, 2001) due to heat damage produced by the use of high temperatures. In addition, hot air drying presents other important drawbacks, such as low energy efficiency and lengthy drying times during the last drying stage (Soysal, 2004).

Freeze drying, also known as lyophilization, consists of the nucleation and propagation of ice crystals (freezing) followed by a sublimation process. This technique has been widely used to obtain high quality and high added value dehydrated fruits and vegetables (Huang et al., 2009a). It enables the production of

foods with minimum shrinkage, color changes, and loss of thermosensitive compounds (Pisano et al., 2011). Freeze drying can also help to maintain natural nutrients, flavor and texture with negligible changes from the original raw matter (Deng et al., 2014). Nevertheless, this technique is very expensive because of the low drying rates, which lead to relatively small throughputs, and the high capital and energy costs due to the refrigeration and vacuum needs (Zhang et al., 2006). Hence, its application depends on the uses of the final product being appropriate for products with high added value. An alternative technique to conventional freeze drying, which enables the continuous operation, is the atmospheric freeze drying systems. It has been demonstrated that freeze drying is possible at atmospheric pressure if the partial pressure of water in the drying chamber is held sufficiently low. This method avoids the requirement of a vacuum and makes continuous freeze drying operations much easier as interlock systems are not required. However, the main drawback of this technology is the long drying times needed (Santacatalina et al., 2014).

Spray drying is, together with hot air drying and freeze drying, one of the most commonly used drying techniques. Mainly applied in the drying of liquid products, it consists of the atomization of the liquid in a hot gas stream in order to obtain a powder instantaneously. Although most often considered just a simple dehydration process, it can also be used to encapsulate an active material within a protective matrix formed from a polymer or melt (Carneiro et al., 2013; Dziezak, 1988). Thus, spray drying has been successfully used in the food industry for several decades for the purposes of microencapsulation (Gouin, 2004), due to its operational flexibility, applicability to heat sensitive materials and affordability (Filková et al., 2007). Conversely, this technique requires the exhaustive optimization of all the process variables, the main ones being the product feed temperature, the air inlet temperature and the air outlet temperature (Liu et al., 2004). Moreover, the difficulty of applying it to high viscosity products, and the production, in some cases, of fine powders which need further processing are some of the disadvantages of this method.

Vacuum drying is a process in which the wet material is dried under subatmospheric pressure (Arévalo-Pinedo & Murr, 2006). The impact of the

temperature is reduced because applying vacuum allows the use of lower drying temperatures (Lewicki, 2006). Compared with conventional atmospheric hot air drying, the oxidation of products is prevented because the sample does not come into contact with air during the process. Moreover, the sensory and nutritive qualities of foodstuffs are better preserved as a result of both low drying temperatures and shorter drying times (Wu et al., 2007). However, batch processing limits its use on an industrial scale where huge volumes are processed.

The use of microwave and infrared radiations in drying operations has also been studied. On the one hand, microwave assisted drying has ended up being 1 to 2 orders of magnitude faster than conventional drying (Zhao et al., 1993) with no significant quality degradation (Clary et al., 2007; Nindo et al., 2003; Popovich et al., 2005). As a result, this technology has gained popularity as an alternative or complementary drying method for a variety of food products, such as fruits, vegetables, snack foods and dairy products (Wang & Sheng, 2006). Moreover, the combination of vacuum and microwave drying has led to an improvement in the drying rate and a reduction in the drying temperature (Clary et al., 2007; Dak & Pareek, 2014; Giri & Prasad, 2007). On the other hand, the use of infrared radiation technology as a means of dehydrating foods has also shown several advantages. These may include shortened drying times, great energy efficiency, high quality finished products, uniform temperature in the product while drying, and a reduced need for air flow around the product (Mongpreneet et al., 2002).

Recently, drying techniques using supercritical $CO_2$ have attracted interest in the field of food processing (Brown et al., 2008; Khalloufi et al., 2010; Nuchuchua et al., 2014). Their main advantages include the application of temperature near to ambient and the avoidance of vapor–liquid interfaces and capillary stress (which usually arises during air drying), which potentially results in less shrinkage and a better preservation of structure. However, the application of this technology is still under research and the available data is very limited.

Another novel and promising drying technique with many advantages is the use of high power ultrasound. Compared to other emerging technologies applied in

drying, such as infrared radiation or microwave, ultrasound has a weak thermal effect. Thus, the main ultrasound mechanisms which accelerate the drying process are those related with mechanical effects (Gallego-Juárez & Graff, 2015; García-Pérez et al., 2015). On the one hand, ultrasound affects the solid-liquid interface by generating microturbulences and microcurrents due to oscillating pressures. On the other hand, applying ultrasound interrupts the continuity of cellular membranes since the mechanical stress generated inside the product gives rise to microchannels. Thus, the mass transfer rate between the cell and its extracellular surroundings increases (Nowacka et al., 2012). Moreover, it has been found that ultrasound application during air drying increases both the effective diffusion and the mass transfer coefficients more markedly when low temperatures (below 20 °C) are used (Rodríguez et al., 2014a). This fact highlights the interest in the use of ultrasound technology in atmospheric freeze drying as a means of accelerating the process and solving the problem of long drying times (Santacatalina et al., 2014). As far as the effect of ultrasound application on product properties is concerned, it depends on the temperature, the product and the property studied. For instance, the rehydration capacity may be unaffected (Schössler et al., 2012), or even improved, whereas the color and texture might be slightly influenced (Ozuna et al., 2014).

The choice of drying method not only depends on the final quality of the dried product but also on factors such as the type of product and energy consumption (Sagar & Suresh-Kumar, 2010). Therefore, it is necessary to carry out a detailed study for each particular industrial application and commodity.

### 1.3.2. Impact on phenolic content

The impact of drying on the phenolic content of plant materials has been widely studied over the last few decades. However, despite testing different drying methods and conditions on a large number of different raw materials, it is not possible to generalize about there being an overall trend in the effect of drying on vegetable matrices rich in phenolic compounds.

Drying produces changes in the structure and composition of raw materials which can affect the further extraction of compounds of interest. It makes vegetable

tissues more brittle, which in turn results in rapid cell wall breakdown during the milling and homogenization steps. In consequence, these broken cells release more phenolic compounds into the solvents. However, as previously mentioned, drying sometimes has a negative impact on product quality traits, such as a reduction in the phenolic content. For this reason, the results of carrying out a drying step prior to extraction are contradictory in nature. On the one hand, it has been proven that, regardless of the drying method, the total phenolic content and antioxidant capacity of extracts obtained from *Lamiaceae* herbs (Hossain et al., 2010) and tomatoes (Chang et al., 2006) significantly increased when fresh samples were dried. On the other hand, other authors have highlighted that unprocessed materials (i.e. berries and corn) had a higher total phenolic content than the dried ones (Asami et al., 2003; Wojdylo et al., 2009). Among other things, the contradictory effect of drying could be linked to the different heat and light sensitivities of the phenolic compounds and the degradative enzymatic activity in the raw materials. Therefore, it seems necessary to assess the influence of drying on each material's shelf life, the drying costs and operation times, the extraction efficiency and whether there are any losses in the phenolic compounds during processing. Hence, it will be possible to conveniently decide if it is worth drying prior to extraction or not.

As regards drying techniques, some authors have reported no significant differences between the total phenolic content in hot air and freeze dried products (Chang et al., 2006). On the contrary, other studies have confirmed that freeze drying increases the extraction of the bioactive compounds in different products as compared to air drying (Dorta et al., 2012; Kwok et al., 2004; Pinela et al., 2012). This could be due to the fact that freeze drying is based on the dehydration by sublimation of a frozen product (Ratti, 2001). Therefore, the rupture of vegetable tissue as a consequence of ice crystal formation during raw material freezing would favor the further extraction process. Moreover, the low processing temperatures used in freeze drying would preserve the phenolic compounds from degradation. Meanwhile, high temperatures and prolonged heat treatments have generally been responsible for the degradation of heat labile polyphenols by causing irreversible chemical changes (Li et al., 2006; Lin et al., 1998; Mejia-Meza et al., 2008). However, Kim et al. (2006)

reported that heat treatments (100-150 °C) of grape seeds were a suitable means of increasing the amount of phenolic compounds. Other researchers have proposed the use of mild drying temperatures which help to retain the integrity of fruit polyphenols. Thus, the extracts obtained from orange peel or pulp which had been previously dried at 60 °C showed greater antioxidant capacity than those obtained from samples dehydrated at higher (i.e. 80 and 90 °C) or even lower temperatures (i.e. 30 and 40 °C) (Garau et al., 2007). This could be linked to the fact that low temperatures imply longer drying times during which the phenolic compounds are exposed to drying air thus favoring their degradation. Therefore, it is important to take into account not only the drying temperature but also the combination of time-temperature during the dehydration step. Notwithstanding this, in some cases it has been possible to use a low air drying temperature (30 °C) to obtain extracts with a higher content of polyphenols than when applying higher temperatures (70-100 °C). In this sense, considering the high cost of freeze drying, it could be suggested that low temperature drying might be an acceptable alternative for some materials. For example, in the case of sweet potato leaves dried at 30 °C, the phenolic content was only reduced by 12 % compared to the ones which were freeze dried (Jeng et al., 2014).

Considering what has been mentioned above, it seems obvious that the raw material plays a key role in how much the drying process influences the phenolic content since the same drying technique can give rise to different results. In this sense, the influence of the different characteristics of the raw material has also been evaluated in the literature. On the one hand, the influence of cell wall composition has been proven. In some cases, the effect of freeze drying has been seen to be less damaging on the tissue structure than other drying methods (Yousif et al., 1999) and is able to keep the tissue structure almost intact. However, in aromatic herbs rich in phenolic compounds that present a woody structure, such as rosemary or thyme, the preservation of the natural and rigid structure might act as a barrier for the release of phenolic compounds from vegetable matrices, resulting in extracts with lower antioxidant capacity (Hossain et al., 2010). On the other hand, the geometry of samples can influence their final phenolic content. For instance, dried muscadine

pomace discs, 4 mm thick, retained the phenolics better than those 2 mm thick. This could be due to the fact that in 4 mm thick discs, the heat transfer occurs at a slower rate and, therefore, the heat-sensitive phenolics are less affected than those in 2 mm thick samples (Vashisth et al., 2011). The effect of the physical form of the samples (whole or powder) on the antioxidant activity has also been proven. For instance, extracts obtained from whole or powder grape seed exhibited a different concentration of different phenolic compounds (Kim et al., 2006), one form or another being more suitable depending on the phenolic compound evaluated and the heating process conditions. Moreover, the different phenolic composition of the raw materials and their sensitivity as well as the location of the polyphenols (i. e. in orange peel or pulp) can influence the final phenolic potential of the extracts (Garau et al., 2007).

In conclusion, the influence of drying on the phenolic bioactive content of vegetable materials can be conditioned not only by processing (method and conditions) but also by the properties of the raw materials, such as sample preparation or initial phenolic profile. Therefore, the exploitation of new phenolic sources requires that each material be submitted to an exhaustive study beforehand into the convenience or not of applying drying as a pre-treatment prior to the phenolic extraction.

## 1.4. Extraction of phenolic bioactive compounds

There are a large number of interesting phenolic compounds present in plant and vegetable tissues to be exploited for industrial applications. Some of these biophenols remain attached to cell walls while others are in the cytoplasmatic vacuoles. This fact must be taken into account when designing of the extraction operations. Hence, the choice of extracting method, the type of solvent, the processing time or the temperature are critical parameters that must be defined in order to achieve a high-level extraction performance (Robards, 2003). Moreover, the quality of the polyphenolic extracts and their antioxidant capacity depends not only on the quality of the starting biomass (geographical origin, climatic conditions, harvesting

date and storage conditions), but also on the technological processes involved in their manufacture (Nkhili et al., 2009).

### 1.4.1. Conventional methods

The solid–liquid extraction process, as a mass transfer process of the target compounds from a vegetable material to a particular solvent, plays a key role in the recovery of those phenolics and antioxidants retained in the vegetable matrices (Pinelo et al., 2006). The extraction of these compounds from plants or seeds has conventionally been based on the optimum combination of solvent, heat and/or agitation in order to achieve an increased mass transfer rate.

The traditional extraction methods mainly involve maceration, soxhlet and agitation systems. Maceration, the extraction of compounds in a solvent without agitation, has been employed for the extraction of non-volatile plant compounds used in pharmaceutical products and in the food industry; for instance, in the extraction of anthocyanins from grape skins during red wine manufacturing (Kelebek et al., 2006). However, this process has important limitations, such as intensive labour needs and long extraction times. Thus, an agitation technique is widely applied in order to increase the turbulence, favour the phenolic leaching and so, raise the extraction rate.

Soxhlet extraction is another well-established extraction technique. In this case, the material is left in a flask and the solvent passes back into the plant's solid bed. The operation is repeated until complete extraction is achieved and the extracted solute is separated from the solvent using distillation. Nevertheless, this technique is not suitable for thermo-sensitive compounds (Luque de Castro & García-Ayuso, 1998).

Besides other processing variables which have been extensively studied, such as extraction time, temperature or raw material/solvent ratio, the solvent used plays a key role in the extraction of phenolic compounds. Taking into account the polarity of the target compounds, a vast range of solvents has been used for the extraction of polyphenols from dry or fresh materials. Water, methanol, ethanol, as well as aqueous alcohol mixtures, are the most common solvents (Nour et al., 2014; Reis et al., 2012; Sánchez-Maldonado et al., 2014; Tubtimdee & Shotipruk, 2011). Methanol

contributes to the disruption of cell walls and the inhibition of enzyme action, and its mixture with water provides a very good solvent for most phenolic compounds (Waterman & Mole, 1994). Moreover, it can be easily evaporated to facilitate the recovery of extracted phenolics. However, ethanol is more lipophilic and it is superior when dealing with polymeric and hydrophobic compounds. Extractions with other solvents, such as acetone, ethyl acetate and hexane (Dey & Kuhad, 2014; Meneses et al., 2013; Razali et al., 2012), have also been tested.

The resistance to phenolic extraction encompasses the natural resistance of the plant structure to solvent penetration, the compound's resistance to being dissolved or suspended in the solvent and the external resistance of the diffusion of phenolics+solvent to the bulk liquid. Therefore, long operating times, high energy demand and elevated solvent consumption (Cissé et al., 2012; Shirsath et al., 2012) are required in conventional extraction systems. These facts, linked to the increasing human use of phenolic compounds from natural sources, has promoted the development of new extraction methods based on non-toxic solvents and the application of auxiliary energy sources for the purposes of accelerating the extraction processes.

### 1.4.2. New technologies

There are some emerging techniques available for the extraction of phenolic compounds from plant materials, four of which should be mentioned: pressurized solvent extraction (Vergara-Salinas et al., 2015), microwave assisted extraction (Setyaningsih et al., 2015), supercritical fluid extraction (Kazan et al., 2014) and ultrasound assisted extraction (Ghitescu et al., 2015). Moreover, the combination of these novel techniques has also been tested as a means of improving the extraction processes. For example, the application of ultrasound in supercritical fluid extraction has been proposed as a mechanism with which to intensify the process and increase its yield (Riera et al., 2004; Rodríguez et al., 2014b).

Pressurized solvent extraction using water as the solvent is one of the most interesting methods, since water is non-toxic, non-flammable, environmentally safe and inexpensive. It enables the rapid extraction (less than 30 min) of interesting

compounds in a closed and inert environment under high pressures (no higher than 20 MPa) and over a wide range of temperatures (25–200 °C). Pressurized solvent extraction is efficient, rapid, selective, and reliable (Björklund et al., 1999). Its major advantage over low pressure (conventional) extraction methods is that pressurized solvents remain in a liquid state well above their boiling points. Hence, it is possible to perform short-time (5–15 min) extractions at high temperatures, but being cautious with thermo-sensitive compounds. Moreover, the pressure applied during extraction could contribute to the disruption of the solid matrix, which could enhance the mass transfer of the solute from the sample to the solvent. Thus, the compound solubility and the kinetics of desorption from matrices would be improved (Richter et al., 1997; Santos, 2011). The set-up in the pressurized liquid extraction equipment provides protection for oxygen and light-sensitive compounds. Nevertheless, one drawback of using pressurized fluid technologies is that pressure requires expensive equipment (Smith, 2002). Therefore, it is important to evaluate whether the improvement in the extraction process compensates for the high investment costs (Ramos et al., 2002).

Microwave assisted extraction is a process which uses electromagnetic energy to efficiently heat solvents so that the solutes can be readily partitioned from the sample matrix into the solvent. As a result, compared to conventional methods, shorter extraction times (from several hours to minutes), high quality extracts with better target compound recovery and a reduction in solvent volume and energy are achieved (Ballard et al., 2010; Chan et al., 2011). Moreover, chemical substances absorb microwaves to different extents and this makes it possible to selectively extract target compounds from complex food matrices (Eskilsson & Björklund, 2000; Hemwimon et al., 2007). Notwithstanding this, the efficiency of the process depends on the extraction time, extraction temperature, solid–liquid ratio and the type and composition of the solvent used (Pizarro et al., 2007; Rostagno et al., 2007; Song et al., 2011).

Supercritical fluid extraction is based on the use of a supercritical fluid as solvent. The supercritical phase is attained by increasing the pressure and temperature, reaching a state of aggregation at which no distinction between the gas and liquid can be observed (Starmans & Nijhuis, 1996). The transport properties of

these fluids favor higher extraction yields by penetrating into porous solid materials more effectively than liquid solvents (Riera et al., 2004). Carbon dioxide is the most frequently used solvent because it is non-toxic, non-flammable, odourless, environment-friendly and easily separated from the extract by depressurization. It also has a good extraction capacity due to its high penetration power (Khansary et al., 2015) and low critical temperature, which allows it to be used in the extraction of thermal sensitive and reactive compounds (Piantino et al., 2008). By changing the process pressure and/or temperature, it is possible to modify the solvation power of the fluid and, therefore, achieve a remarkably high degree of selectivity (Tabernero et al., 2010). However, due to its non-polarity, $CO_2$ may not be efficient for the extraction of polar (e.g. phenolic) molecules. Thus, in some cases it is convenient to modify the solvent polarity by adding co-solvents (Pereira & Meireles, 2010; Serra et al., 2010). Using ethanol, methanol or water enhances the solvating power of $CO_2$, which may increase the selectivity and extraction yield of target compounds (Díaz-Reinoso et al., 2006; Lang & Wai, 2001; Reverchon & De Marco, 2006). Moreover, other variables, such as flow rate and extraction time, need to be considered.

Ultrasound assisted extraction has been used for the extraction of plant components in order to shorten the extraction time, reduce solvent consumption, increase extraction yields and improve the quality of the extracts (Lijun & Weller, 2006). This technique is of great interest due to its simplicity and efficiency (Huang et al., 2009b). In addition, it is not as expensive as other technologies (Rostagno el at., 2003). Ultrasound assisted extraction is based on the use of sound waves with frequencies above 20 kHz in liquid mediums. The enhancement of the extraction is mainly attributed to the effects produced by the acoustic cavitation (creation, growth and implosion of gas bubbles), which are originated in the solvent by the ultrasonic waves when the power applied is above a certain threshold (Wang et al., 2008). However, three interconnected mechanisms can be distinguished through which power ultrasound increases the efficiency of the extraction process by providing improved mass transfer: (i) breakdown of plant cells via the formation of microjets due to asymmetrical bubble collapse near a solid surface, which increases the permeability of the plant tissue and releases the intracellular material; (ii) increase in

analyte solubility and solvent penetration, caused by the localized increase in temperature and pressure at the zone of the bubble implosion; and (iii) enhanced external mass transfer as a consequence of the microstreaming generated by ultrasound (Mason et al., 2011). Ultrasonic waves may also cause some chemical effects which are rather undesirable due to the changes in chemical composition, possible degradation of targeted compounds and production of free radicals within the gas bubbles (Paniwnyk et al., 2001). In any case, extraction yields will depend on the effect of the different experimental operating conditions such as time, temperature, ultrasonic power and frequency, raw material and/or solvent.

## 1.5. Use of natural phenolic extracts in the food industry

During the last few years, the incorporation of bioactive compounds into food products and formulations has been studied for the purposes of finding real applications and making use of natural extracts rich in phenolics with potential benefits for human health.

One of the most widely explored research topics has been the use of phenolic extracts as new ingredients for the nutritional fortification of both liquid and solid food products. As a result, several functional products with a higher phenolic content and greater antioxidant activity have been developed. Some examples of fortified liquid products include, tomato juices with extracts from vegetable byproducts (artichoke, cauliflower, carrot, celery and onion) (Larrosa et al., 2002), espresso coffee brewed with hazelnut skin phenolic extract (Contini et al., 2012), milk beverages fortified with phenolic compounds extracted from olive vegetation water (Servili et al., 2011) or fruit juices and smoothies with phenolics from barley and brewers' spent grain (McCarthy et al., 2013). As regards the fortification of solid products, it is possible to highlight the infusion of grape seed phenolics into fruits and vegetables (Rózek et al., 2010), the addition of grape and callus extracts to yogurt (Karaaslan et al., 2011), the enrichment of ice cream with pomegranate peel phenolics (Çam et al., 2014), the incorporation of a green tea extract into bread (Wang & Zhou, 2004), the formulation of novel foods such as a dry apple product enriched with a green tea

extract (Lavelli et al., 2010) and the addition of vegetable extracts (cocoa and grape seed) to enrich dry fermented sausages, such as "fuet" and "salchichón" (Ribas-Agustí et al., 2014).

If the number of studies carried out into natural phenolic extracts and their compounds is taken into consideration, they can be regarded as promising (and cheap) additives with which to increase the antioxidant potential of food products. Notwithstanding this, the food industry has also investigated them with other purposes in mind. For instance, polyphenolic copigments from rose petals (Mollov et al., 2007) and grape pomace (De Souza et al., 2014) to improve the color stability of foodstuffs, phenolic compounds (caffeic, ferulic and chlorogenic acids) commonly found in fruits and vegetables to reduce the allergenic capacity of peanut products (Chung & Champagne, 2009) and eucalyptus leaves and almond skin extracts as a partial alternative to sulfur dioxide during the ageing of white wines in oak barrels (González-Rompinelli et al., 2013). Moreover, the role of natural extracts in lipid oxidation has been studied both in oils and meat products. In this way, natural antioxidants have been postulated as substitutes for synthetic antioxidants in the food industry to lower the lipid oxidation and preserve the quality in products such as cooked beef (Ye et al., 2015) and frozen chicken nuggets (Teruel et al., 2015). As regards vegetable oils, fortification with phenolic extracts from fruit and sesame cake, among other sources, has resulted in a lower extent of thermooxidative degradation during frying (Aladedunye & Matthäus, 2014) and a good stabilization during storage (Mohdaly et al., 2011), respectively.

Natural phenolic compounds are put to other uses which, although related to the food industry are not directly concerned with food formulations. For example, the application of plant extracts in aquaculture as appetite stimulators, growth promoters and immunostimulants (Reverter et al., 2014), and the development of antioxidant active films for food packaging using, for example, barley husks and curcuma (Bitencourt et al., 2014; Pereira de Abreu et al., 2012); in some cases these films are even biodegradable (Marcos et al., 2014). Furthermore, in agriculture, several natural extracts rich in phenolic compounds have shown a capacity to reduce the initial fungal contamination in shredded cabbage (Kocic-Tanackov et al., 2014), improve

the growth and productivity of tomatoes during summer (Kanechi et al., 2013) and prevent the postharvest decay of apples caused by postharvest pathogens (Daniel et al., 2015).

## 1.6. Extract stability

As previously explained, there is a wide range of studies which attempt to find useful and novel applications for natural phenolic compounds and extracts. Nevertheless, in order to achieve their successful inclusion into the food industry, it is indispensable to pay attention to other aspects related to extract/compound stability during their processing and storage or, even, to their resistance to human gastrointestinal digestion.

Thermal treatments are applied in several food processing operations, mainly to ensure the products' food safety. Thus, it is necessary to evaluate whether the phenolic compounds of extracts obtained from natural sources are able to remain stable at high temperatures. For example, in this sense, Zhang et al. (2013) found that immersion in a hot water bath or processing in autoclave had a positive effect on some chemical constituents and the antioxidant activity of natural extracts obtained from garlic. In addition, it has been proven that the stability and, therefore, the bioactive potential of extracts obtained from plant materials (drumstick, mint and carrot) could vary with pH and storage temperature (Arabshahi-D et al., 2007).

Drying and encapsulation can also play a key role, not only in the stability of the extracts during storage, but also in the protection and preservation of food formulations to which bioactive compounds are added. On the one hand, it is becoming more and more common to use dried extracts since solid forms possess several advantages over fluid forms, the main ones being the fact that transport and storage are easier and a higher concentration of phenolic compounds is achieved (Moreira et al., 2009; Oliveira et al., 2006). On the other hand, some authors propose microencapsulation by means of spray drying in order to improve the shelf life and stability (Chatterjee & Bhattacharjee, 2013). However, due to drying conditions being essential (Cortés-Rojas et al., 2015) and since the advisability of encapsulation can be

conditioned by the subsequent application of the extract, it is important to explore the advantages and drawbacks of extract dehydration through different drying methods.

In addition to focusing on extract stability during storage and processing, scientific studies have also explored the resistance of bioactive compounds under human gastrointestinal digestion. Thus, both *in vitro* (Tenore et al., 2015) and *in vivo* (Bansode et al., 2014) tests have been used to estimate the amount of ingested compounds from a phenolic extract which, potentially, will be able to provide health benefits in humans. The majority of the studies assess the bioaccessibility (fraction of compounds available for intestinal absorption) and bioavailability (fraction of compounds available for use in normal physiological functions and for storage) of target compounds (Parada & Aguilera, 2007). Nevertheless, the gastrointestinal release of encapsulated antioxidants (Tavano et al., 2014) is another interesting research topic that has opened up in this field.

In conclusion, it is important to consider the most appropriate technological conditions and processing factors which can modify the stability and activity of antioxidant extracts for their use in food. Moreover, it is convenient to assess the bioacessibility and/or bioavailability of phenolic compounds.

## 1.7. Conclusions

- Byproducts from the food industry are potential sources of phenolic compounds to be taken into account in order to increase the value of food processing wastes and obtain alternative natural ingredients.

- Olive oil industry byproducts are rich in phenolic compounds, such as oleuropein, verbascoside and luteolin, with several bioactive properties. However, these promising vegetable materials have not yet been exploited on an industrial scale.

- Dehydration of vegetal matrices prior to the extraction step can affect the antioxidant potential of extracts, depending on the nature of the raw material, the drying method and the processing conditions.

- The use of novel technologies, such as power ultrasound, in the extraction of phenolics could have a positive impact on the extraction process in terms of time, yield and costs.

- The efficient and effective exploitation of natural phenolic resources requires a thorough study of the possible effects that raw material pretreatments and extraction conditions can induce on the quality of the extracts. Moreover, as one of the aims of using these natural phenolic compounds is that of providing people with health benefits, it is necessary to assess their bioaccessibility and bioavailability.

- Natural extracts with a high phenolic content possess a wide range of interesting applications in the food industry: for example, the development of functional foods, as the replacement for synthetic additives or phytosanitary products and as a means of improving food packaging.

# 2. OBJECTIVES

The main objective of this doctoral Thesis was to determine the influence of the main processing stages involved in the obtaining of natural extracts with high antioxidant potential from byproducts originating in the olive oil industry. In order to achieve this goal, the following partial objectives were established:

1. To assess the influence of raw material processing (drying and freezing) on the antioxidant potential (phenolic content and antioxidant capacity) of the extracts obtained from olive leaves and olive pomace.

2. To address the intensification of olive leaf phenolic compounds extraction by means of the application of high power ultrasound, evaluating the effect on both extraction kinetics and extract quality.

3. To determine the influence of raw material processing (drying and extraction method) on the bioaccessibility of polyphenols present in the olive leaf extracts.

4. To evaluate the impact of the drying technique and the storage conditions on the stability of olive leaf extracts.

5. To address the feasibility of infusing olive leaf extracts in a previously dehydrated food solid matrix, such as apple, as a potential application for natural extracts to develop functional foodstuff.

The objectives abovementioned constitute the general purposes pursued in this Thesis, more details about specific aims are given in the chapters of the Results and Discussion section.

# 3. METHODOLOGY

## 3.1. Working plan

The working plan of the present Doctoral Thesis (Fig. 1) was designed on the basis of the objectives proposed. Thus, the experimental plan was organized in four different tasks giving rise to the four chapters in which the results section has been structured. These four tasks correspond with the main processing stages involved in the exploitation of olive oil byproducts as sources of phenolic compounds: pretreatment and processing of raw material (*Chapter 1*), extraction process of polyphenols (*Chapter 2*), stability of extracts under different processing conditions (*Chapter 3*) and, finally, the subsequent application of the bioactive extracts obtained (*Chapter 4*).

Firstly, it was evaluated how pretreatments of raw material, prior to the phenolic extraction, could affect the antioxidant potential of olive oil byproducts (*Chapter 1*). For this purpose, two olive oil byproducts, olive leaves and pomace, were used and the freezing, the further drying process and the thermal overheating of the raw material were addressed. Thus, in the first set of experiments, the influence of freezing and drying method of *olive leaves* was assessed. For this end, three batches of olive leaves were obtained depending on freezing conditions: (i) frozen in liquid $N_2$, (ii) conventionally frozen at -28 ºC and (iii) non-frozen (fresh leaves). On the one hand, fresh leaves and a part of liquid $N_2$ frozen leaves were hot air dried at two temperatures, 70 and 120 °C. On the other hand, conventionally (-28 °C) and the other part of liquid $N_2$ frozen leaves were freeze dried. Another set of experiments were carried out by using, in this case, *olive pomace* to study the effect of air drying temperature (from 50 to 150 °C) on antioxidant potential. Finally, a last set of experiments was performed to evaluate the influence of thermal overheating of the raw material. For that purpose, olive pomace was maintained at 150 °C for different times ranging from 5 to 60 min. Drying kinetics were analyzed by using diffusion and Weibull models. A conventional extraction was carried out with all processed materials (leaves and pomace) and total phenolic content, antioxidant capacity and phenolic composition were determined.

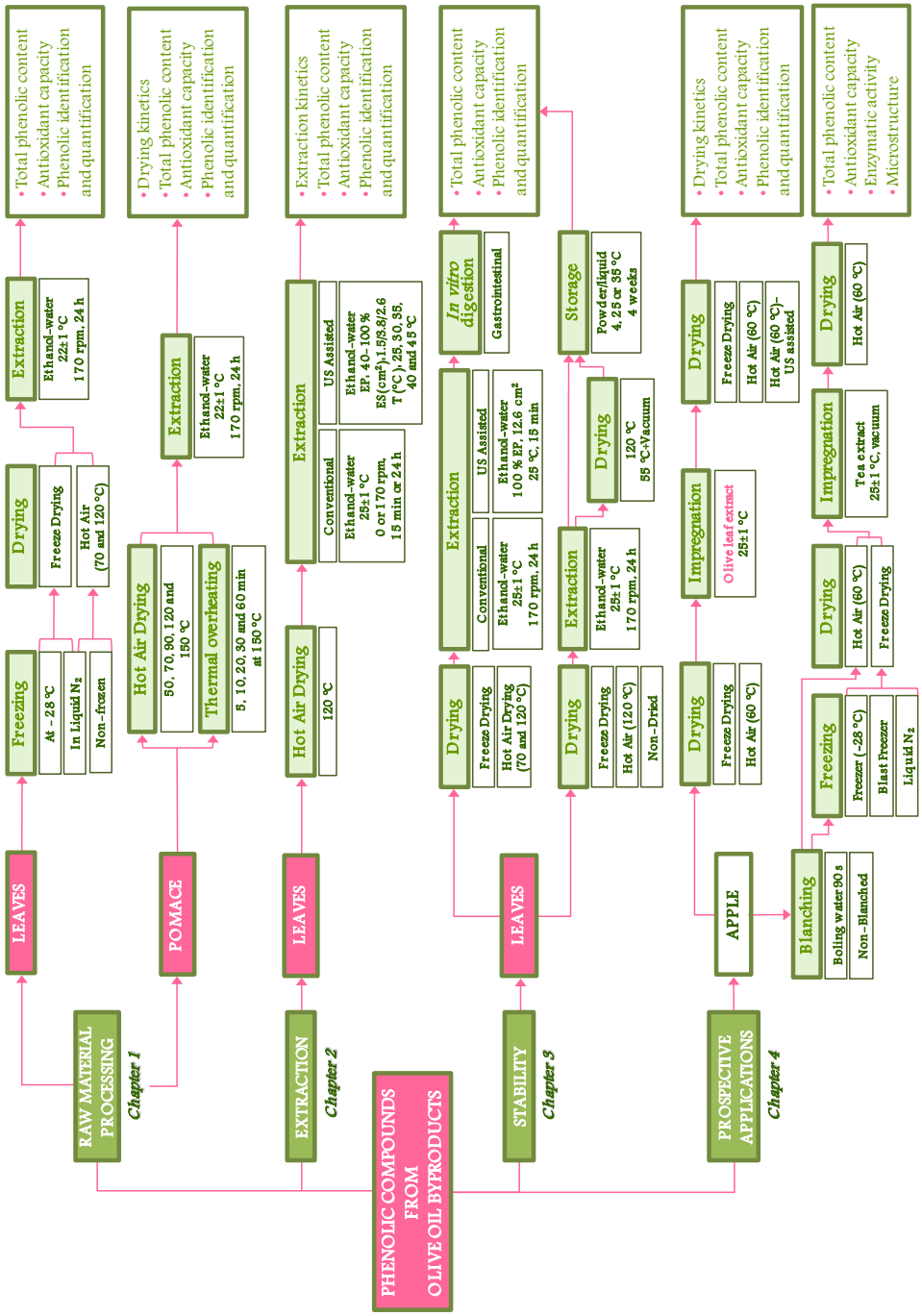


**Fig. 1.** Working plan.

Secondly, the extraction of phenolic compounds from olive leaves was addressed (*Chapter 2*) with the aim of not only improving the process but also providing high quality extracts. Hence, a *conventional solid-liquid extraction process* was compared to an alternative method which includes a novel technique for process intensification, the *ultrasound assisted extraction*. This work included a parametric study of some of the main process variables which can affect the ultrasound extraction. The parameters taken into account were the electric power supplied to the ultrasonic transducer (40, 60, 80 and 100 % of the total electric power of the ultrasonic system used, 400 W), the emitter surface (12.6, 3.8 and 1.5 $cm^2$) and the extraction temperature (25, 30, 35, 40, 45 and 50 °C). Extraction experiments were monitored by measuring the total phenolic content and antioxidant capacity. Extraction kinetics obtained were modeled with Naik equation. Moreover in the final extracts, the main polyphenols were identified and quantified by HPLC–DAD/MS–MS.

Thirdly, the *stability* of olive leaf extracts (*Chapter 3*) was assessed by performing two different set of experiments. In the first one, different extracts obtained by combining drying of raw material (hot air drying at 70 and 120 °C or freeze drying) and extraction (conventional or ultrasound assisted) methods were subjected to an *in vitro gastrointestinal digestion*. The total phenolic content, the antioxidant activity and the main polyphenols present in extracts were measured during the *in vitro* digestion. Thus, it was possible to estimate the bioaccessibility of the main olive leaf phenolic compounds and study if processing conditions (drying and extraction) of raw material could modify the extract behavior during *in vitro* digestion. In the second set of experiments, the impact of both *raw material and extract drying and storage conditions* on the bioactive potential and stability of olive leaf extracts was analyzed. For this purpose, fresh, hot air (120 °C) and freeze dried leaves were used to obtain liquid extracts which were stored at 4 °C for 4 weeks. Moreover, a part of the extracts obtained from hot air dried leaves was dehydrated by using two different techniques: hot air at 120 °C and vacuum drying at 55 °C. A batch of these powder extracts was re-diluted with the solvent used in the extraction. Afterward, both powder and re-diluted batches were stored at 4, 25 and 35 °C for

4 weeks. Extracts stability was determined by means of antioxidant capacity, the total phenolic content and the concentration of the main phenolic compounds.

Finally, the last task of this Thesis was designed with the aim of going in depth in one of the ***prospective applications*** of the natural extracts, the development of dried stable products enriched with bioactive compounds (***Chapter 4***). In a first stage, the influence of the ***drying method on the retention of olive leaf polyphenols*** infused in a solid matrix (apple) was addressed. Thus, fresh apple samples were dried under different conditions (freeze drying or hot air drying at 60 °C). The drying was carried out to facilitate the further infusion (25 °C) of olive leaf extracts into the solid matrix. Finally, to achieve a stable final product, the impregnated samples were also dried with different techniques (freeze drying and hot air drying at 60 °C with and without ultrasound application). Drying kinetics of infused products were mathematically described using diffusion models. The increase in the bioactive content of final samples was established from the total phenolic content, antioxidant capacity and quantification of the main olive leaf polyphenols. The second stage was planned in order to clarify the ***role of the structure and the enzymatic activity of dried matrixes*** used in the impregnation. In this case, different dried materials were obtained by combining different pretreatments (blanching, conventional freezing at -28 °C, blast freezing at -30 °C or freezing in liquid $N_2$ at -196 °C) and drying methods (freeze drying and hot air drying at 60 °C). Then, all samples were vacuum impregnated with a tea extract and dehydrated for the final stabilization by hot air drying at 60 °C. The total phenolic content, antioxidant capacity, polyphenol oxidase and peroxidase activity and the microstructure (SEM) were determined in all dehydrated materials.

It is important to highlight that the research work has been carried following the same order as the chapters are presented in the Results and Discussion section. Therefore, the experimental conditions tested in some of the chapters were defined taking into account the results obtained in previous chapters. In each one of the research works included in the Results section, the specific methodology used is

detailed. Therefore in this section, only olive oil byproducts used as raw material and a general overview of the methodology will be described.

## 3.2. Raw material

Olive leaves and olive pomace were the two byproducts from olive oil industry selected as phenolic compounds sources. Both byproducts came from olive trees (*Olea europaea*) of the Serrana variety, characteristic from the region of Alto Palancia (Castellón, Spain). A short description of the origin and harvest of each raw material can be found as follows.

### 3.2.1. Olive leaves

The olive leaves employed for the present Thesis were collected from 15 years old trees, which are located in the area of "El Portillo" (UTM coordenates 718.082.40, 4.406.115.18, Segorbe, Castellón, Spain). Overall, 100 of the 200 trees were sampled in a familiar smallholding (Fig. 2). Considering that olive leaves as byproduct are mainly originated from tree pruning, they were randomly collected from the crown of the trees avoiding the influence of growing factors as sun exposure. Thus, homogeneous batches of leaves were obtained.

**Fig. 2.** Olive cultivation in Segorbe (Castellón, Spain).

Olive leaves were always processed in less than 48 h and stored in plastic bags at 4 °C.

### 3.2.2. Olive pomace

Olive pomace, also known as olive cake or orujo, is a solid byproduct made up from pieces of pit, skin and pulp (Fig. 3a). In this work, it was provided by an oil factory located in Altura (Castellón, Spain) where olive oil is produced through a traditional pressing system. Thus, the byproduct was directly collected after the oil extraction without being mixed with other solvents. The olive pomace was placed in plastic bags which were vacuum sealed and stored at 4 °C until being used. The experimental work related to the antioxidant potential of olive pomace was conducted by using the raw material as a whole (Fig. 3a). However, for modeling the drying kinetics, it was necessary to separate the byproduct into two fractions: pulps+peels (Fig. 3b) and pits (Fig. 3c).


**Fig. 3.** Olive pomace (a) composed of pulps and peels (b) and pits (c).

## 3.3. Methodology

In order to study the impact of processing on the exploitation of phenolic compounds from olive oil byproducts, different unit operations were addressed, which are summarized in this section. In addition, this section also includes the analysis carried out in order to characterize the content of bioactive compounds, microstructure and enzymatic activity.

### 3.3.1. Raw material pretreatments

Pretreatments, alone or combined, were applied in both olive oil byproducts as sources of phenolic compounds, and apple as vegetable food matrix used for the infusion of olive polyphenols.

*BLANCHING*

Blanching was carried out by immersing the samples in boiling water for 90 s and so, reducing the enzymatic activity of food matrixes. Hence, blanched apple samples were obtained to be further processed (frozen and/or dried).

*FREEZING*

Different freezing methods were performed to study the effect of freezing on not only the phenolic compounds extraction but also the capacity of apple samples to retain infused phenolic compounds. These methods differed in the freezing rate and, therefore, the size of ice crystal obtained.

On the one hand, olive leaves were frozen by a conventional method (-28 °C) and by immersion in liquid $N_2$ at -196 °C.

On the other hand, apple samples (food matrixes) were frozen by three different methods: conventional freezing at -28 °C, blast freezing at -30 °C and freezing in liquid $N_2$ (-196 °C).

*DRYING*

Several drying methods were performed throughout this work with different purposes.

Firstly, drying was applied to extend the shelf–life of olive leaves and pomace and study the impact of raw material drying on the further phenolic extraction. Hence, olive leaves were dehydrated by freeze drying and hot air drying at two different temperatures (70 and 120 °C). Meanwhile, olive pomace was only air dried in a wide range of experimental conditions (temperatures from 50 to 150 °C, and drying times from 5 to 60 min).

Secondly, drying was performed to stabilize liquid extracts of olive leaf and evaluate its impact on the antioxidant potential of the extracts. In this case two dehydration methods were applied: air drying at 120 °C and vacuum drying at 55 °C.

Thirdly, drying was applied to improve the infusion of olive leaf extracts into solid matrixes. For this fact, apple samples were air dried at 60 °C and freeze dried.

Finally, the drying of impregnated samples (apples with antioxidant extract) was carried out with the aim of obtaining a final stable product rich in phenolic compounds. In this case, samples were dried by three methods: freeze drying and air drying at 60 °C with and without ultrasound application.

### 3.3.2. Phenolic compounds extraction

In general terms, a conventional solid–liquid extraction method with agitation of the mixture solvent–raw material (olive leaves or pomace) was used for the extraction of phenolic compounds. Notwithstanding, the application of power ultrasound was additionally tested as a new technology to intensify the extraction process. Thus, a parametric study of the main process variables involved in the ultrasound assisted extraction was conducted and the electric power supplied to the ultrasonic transducer, the emitter surface and the extraction temperature considered.

Two different solvents were used depending on the further processing step. Extracts used for apple impregnation were obtained by using water whereas in the other extraction experiments a mixture of ethanol–water (80:20, v/v) was used.

### 3.3.3. Storage and *in vitro* digestion

The stability of olive leaf extracts under different environments was investigated.

On the one hand and taking into account future applications of olive leaf extracts in food industry, the stability of phenolic compounds under human gastrointestinal digestion was estimated by an *in vitro* simulation. Thus, pepsin and pancreatin–bile solutions were added to the extracts and the pH conditions were adapted to gastric or intestinal digestion. This way allowed determining the sensitivity of extracts to the digestion conditions and the individual bioaccessibility of main polyphenols, this last being defined as the ratio between the amount of a specific compound before and after digestion.

On the other hand, liquid and powder forms of olive leaf extracts obtained at different experimental conditions were stored protected from light at 4, 25 and 35 °C for 4 weeks.

### 3.3.4. Infusion of phenolic compounds

With the aim of developing a solid foodstuff rich in bioactive compounds, extracts from olive leaf or tea, probably one of the most widely consumed sources of phenolic compounds, were infused in a vegetable matrix (apple) by impregnation. Two types of impregnation were carried out. In the first one, apple samples were just immersed in olive leaf extract until the equilibrium was reached (difference between two consecutive weights less than 0.02 g). In the second one, sample impregnation with tea extract was conducted in two steps, a vacuum period of 14 h followed by 55 min at atmospheric pressure.

### 3.3.5. Analysis of antioxidant potential, enzymatic activity and microstructure

Antioxidant potential was assessed by measuring the phenolic content and the antioxidant capacity, as described as follows:

### *PHENOLIC CONTENT*

The phenolic content was evaluated by both a general method to determine the total phenolic content (Folin–Ciocalteu) and the identification and quantification of the main phenolic compounds present in the extracts or solid matrixes by high-pressure liquid chromatography-diode array detector/ion trap mass spectrometry (HPLC-DAD/MS–MS).

### *ANTIOXIDANT CAPACITY*

Two methods, based on different chemical principles, were used to determine the antioxidant capacity. The ferric-reducing ability power (FRAP) method, which is a simple method used to estimate the reduction of a ferric-tripyridyltriazine complex, and the trolox equivalent antioxidant capacity (TEAC) method, which measures the reduction of the radical cation of ABTS caused by antioxidants.

### *ENZYMATIC ACTIVITY*

Processing can affect some properties of food matrixes which can play a key role in the retention of phenolic compounds. Thereby, the measurement of the oxidative enzymatic activity of peroxidase and polyphenol oxidase in apples samples were carried out before the extract impregnation and after the stabilization of enriched samples.

### *MICROSTRUCTURE*

Structure of solid matrixes may also be able to condition not only the infusion of polyphenols but also their stability to further processing conditions. For this reason, the microstructure of fresh-dried and infused-dried apple was analyzed. Scanning electron microscopy (SEM) was the technique used for this purpose.

# 4. RESULTS AND DISCUSSION

# CHAPTER 1

## *Processing of raw material*

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# *Influence of freezing and dehydration of olive leaves (var. Serrana) on extract composition and antioxidant potential*

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# Influence of freezing and dehydration of olive leaves (var. Serrana) on extract composition and antioxidant potential


## ABSTRACT

In this work, the effect of the methods used for the freezing and drying of olive leaves on the polyphenol content and antioxidant capacity of the extracts was addressed. Thus, different methods were used to dry olive leaves (fresh or frozen by conventional (-28 °C) or $N_2$ freezing): hot air drying at 70 or 120 °C and freeze drying. The extracts were characterized by determining the phenolic content, antioxidant capacity and HPLC-DAD/MS-MS profile.

Drying had a significant ($p<0.05$) influence on the antioxidant potential of olive leaf extracts. Both the drying and freezing methods significantly ($p<0.05$) influenced the concentration of the main polyphenols identified. Hot air drying provided a higher phenolic content, especially in oleuropein, than freeze drying. Thus, drying at 120 °C was the best processing condition. Freezing reduced the antioxidant potential as compared to fresh leaves, probably due to oxidase activation, although its influence was not dependent on the freezing method.


***Keywords***: byproduct, freeze drying, hot air drying, polyphenols, oleuropein.

## 1. Introduction

Olive cultivation has traditionally played an important role in the human diet because of the nutritional value and beneficial properties of olive oil (Roche et al., 2000). Olive tree (*Olea europaea* L.) pruning generates an average of 7 kg of leaves per year, which represents approximately 10 % of the olive fruit mass (Tabera et al., 2004). This byproduct is mainly used in animal feeds or removed by burning. However, olive leaves have a high content of phenolic bioactive compounds; in some cases, this content is similar to or even higher than in fruit (Japón-Luján & Luque de Castro, 2007). Therefore, olive leaf extracts can protect against nitrite and nitrosamine-related cancer and exhibit wide antioxidant, antitumor (Taamalli et al., 2012) and antiviral activities, among other properties (Micol et al., 2005).

As taking into account that there is considerable significance emphasis placed on the recovery, recycling and upgrading of byproducts (Wijngaard et al., 2012) and since they are highly popular considering the fact that they enjoy great with consumers demand due to the health benefits they provide, olive leaf byproducts can be used to develop functional foods and health-promoting agents (Lee et al., 2009). Nevertheless, this stage requires the development of efficient drying and extraction processes (Cárcel et al., 2010).

The immediate drying of olive leaves is the most important operation in post-harvest processing in order to avoid quality losses and to prevent possible degradation during storage. Moreover, leaves are often dried before extraction to reduce their moisture content and to avoid any interference of water in the polyphenol leaching (Soysal & Öztekin, 2001). The breakdown of cellular constituents during drying could also facilitate the release of bound phenolic compounds (Chism & Haard, 1996). Thus, the raw material drying has been considered as a very useful means of increasing the amount of phenolic compounds and the antioxidant capacity of extracts (Hossain et al., 2010). Air drying at room temperature is the traditional technique used to preserve medicinal herbs because low temperatures are thought to protect the bioactive components from degradation. However, this operation is not controlled and constitutes a slow process which may lead to quality loss and

subsequently affect the extracts (Fennell et al., 2004; Keinanen & Julkunen-Titto, 1996). For that reason, hot air drying is mostly used on an industrial scale, since it shortens the processing time and can be controlled. However, during hot air drying, vegetables undergo physical, structural, chemical and nutritional changes that can affect quality attributes like texture, color, flavor and nutritional value (Di Scala & Crapiste, 2008; Kubola et al., 2013). On the other hand, vacuum freeze drying has been considered the best method for water removal, giving rise to dried products of the highest quality (Genin & René, 1995; Irzyniec et al., 1995). Nevertheless, despite many advantages, freeze drying has always been recognized as the most expensive process for manufacturing a dehydrated product (Ratti, 2001) and it requires a previous freezing that, in certain ways, could also affect quality. Therefore, the choice of the most adequate method with which to obtain a quality product, minimizing operational costs and time consumption, is the key to a successful operation. The aim of this work was to study how the methods for the freezing and drying of olive leaves affect the antioxidant potential of extracts. The study aims to choose an appropriate drying process in order to obtain extracts rich in bioactive compounds.

## 2. Materials and methods

### 2.1. Raw material

Olive leaves (*O. europaea*, var. Serrana) were collected on a farm located in Segorbe (Castellón, Spain), packaged and stored at 4 °C until drying or freezing. In every case, the samples were processed in less than 48 h. The initial moisture content was determined by drying in a vacuum chamber at 70 °C until constant weight was reached (AOAC, 1997).

### 2.2. Drying experiments

The olive leaves were dried by following two different methods: hot air drying (HAD) and freeze drying (FD).

In HAD experiments, olive leaves were dehydrated at 70 °C for 50 min (HAD-70) and at 120 °C for 12 min (HAD-120). Experiments were conducted in a forced air laboratory drier (FD, Binder, Tuttlingen, Germany), using an initial mass load of 150 g, an air flow of 0.094 $m^3/s$ and an air velocity of 0.683 m/s.

FD experiments were conducted at an initial temperature of -48 ± 2 °C and the shelf temperature was set at 22 ± 2 °C. FD required 48 h and, during this time, pressure was kept at 1.4 $10^{-1}$ mbar in a freeze dryer chamber (LIOALFA 6-50, Telstar, Madrid, Spain) and the initial mass load was 40 g.

In both HAD and FD, the dehydration process was finalized when the samples lost 40 ± 1 % of the initial weight. After drying, olive leaves were packaged in plastic bags and stored at 4 °C until extraction.

## 2.3. Freezing experiments

In order to study the influence of olive leaf freezing on the extract antioxidant properties, two different methods of olive leaf freezing were tested in freeze drying (FD) experiments: conventional at -28 °C for 24 h (C) and liquid nitrogen freezing ($N_2$). Moreover, a portion of the leaves that were frozen in liquid $N_2$ were dried by hot air drying (70 and 120 °C), like the fresh ones (F).

## 2.4. Extraction set-up

The dried olive leaves were milled, thus obtaining a powder, and sieved to select a particle diameter of under 0.05 mm. The extraction was carried out in sealed containers, protected from light and immersed in a thermostatic shaking water bath (SBS40, Stuart, Staffordshire, UK). The solvent used was a solution of ethanol-water (80:20, v/v). The ratio between the weight of the olive leaves and the solvent volume used was 3.75 g/30 mL. During extraction, the mixture was stirred (170 rpm) at 22 ± 1 °C for 24 h. Afterwards, extracts were centrifuged for 10 min at 5000 rpm (Medifriger BL-S, J.P. Selecta, Barcelona, Spain), filtered (nylon filters of 0.45 μm) and stored in opaque vials at 4 °C until analyzed. At least 3 extraction replicates were made for each different dehydration or freezing condition.

## 2.5. Total phenolic content measurement (TPC)

The phenolic content was determined by the Folin-Ciocalteu method (Singleton et al., 1999). Briefly, 100 μL of sample were mixed with 200 μL of Folin-Ciocalteu's phenol reagent (Sigma-Aldrich, Madrid, Spain) and 2 mL of distilled water. After 3 min at 25 °C, 1 mL of $Na_2CO_3$ (Panreac, Barcelona, Spain) solution ($Na_2CO_3$-water 20:80, w/v) was added to the mixture. The reaction was kept in the dark at room temperature for 1 h. Finally, the absorbance was read at 765 nm using a spectrophotometer (Helios Gamma, Thermo Spectronic, Cambridge, UK). The measurements were carried out in triplicate, unless otherwise stated. The standard curve was previously prepared using solutions of a known concentration of gallic acid hydrate (Sigma-Aldrich, Madrid, Spain) in ethanol-water (80:20, v/v). Results were expressed as mg of gallic acid (GAE) per g of dry weight of olive leaves.

## 2.6. Antioxidant capacity measurement (AC)

The antioxidant capacity of extracts was determined using two different methods (FRAP and TEAC) based on different chemical principles in order to compare the obtained results.

### 2.6.1. Ferric-reducing ability power (FRAP)

The FRAP method, which is a simple method used to estimate the reduction of a ferric-tripyridyltriazine complex, was applied following the procedure described by Benzie & Strain (1996) with some modifications. Briefly, 900 μL of freshly prepared FRAP reagent were mixed with 30 μL of distilled water and 30 μL of test sample or ethanol-water (80:20, v/v) as appropriate reagent blank and kept at 37 °C for 30 min. The FRAP reagent contained 2.5 mL of a 10 mM TPTZ (Fluka, Steinheim, Germany) solution in 40 mM HCl (Panreac, Barcelona, Spain) plus 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ (Panreac, Barcelona, Spain) and 2.5 mL of 0.3 M acetate buffer (Panreac, Barcelona, Spain), pH 3.6 (Pulido et al., 2000). Readings at the maximum absorption level (595 nm) were taken using a spectrophotometer (Helios Gamma, Thermo Spectronic, Cambridge, UK). Four replicates were made for each measurement. The antioxidant capacity was evaluated through a calibration curve, which was

previously determined using ethanol solutions (ethanol-water 80:20, v/v) of known Trolox (Sigma-Aldrich, Madrid, Spain) concentrations and expressed as mg Trolox per g of dry weight of olive leaves.

### 2.6.2. Trolox equivalent antioxidant capacity (TEAC)

The TEAC method, which measures the reduction of the radical cation of ABTS caused by antioxidants, was performed as previously described by Laporta et al. (2007). Briefly, ABTS radical cation ($ABTS^{+\bullet}$) was produced by reacting ABTS (Sigma-Aldrich, Europe) stock solution with 2.45 mM potassium persulfate (final concentration) and leaving the mixture in the dark at room temperature for 12-24 h before use. The $ABTS^{+\bullet}$ solution was diluted with distilled water until an absorbance value of 0.714 ± 0.02 at 734 nm was reached. For the photometric assay, the absorbance of 200 μL of the $ABTS^{+\bullet}$ solution or blank was measured (Spectrostar Omega, BMG Labtech, Offenburg, Germany). Afterwards, 20 μL of the antioxidant extract or blank (ethanol-water 80:20, v/v) were added and, after 29 min at 734 nm, the final absorbance was measured (Spectrostar Omega, BMG Labtech, Offenburg, Germany). The antioxidant capacity was determined from the difference of absorbance, using a Trolox calibration curve (Sigma-Aldrich, Madrid, Spain). Four replicates were made for each extract. The antioxidant capacity results were expressed as mg of Trolox per g of dry weight of olive leaves.

## 2.7. Identification and quantification of polyphenols by HPLC-DAD/MS-MS

In order to identify and quantify the main polyphenols, olive leaf extracts were analyzed using an HPLC instrument (Agilent LC 1100 series; Agilent Technologies, Inc., Palo Alto, CA, USA) controlled by the Chemstation software. The HPLC instrument was coupled to an Esquire 3000+ (Bruker Daltonics, GmbH, Germany) mass spectrometer equipped with an ESI source and ion-trap mass analyzer, and controlled by Esquire control and data analysis software. A Merck Lichrospher 100RP-18 (5 μm, 250 x 4 mm) column was used for analytical purposes.

Separation was carried out through a linear gradient method using 2.5 % acetic acid (A) and acetonitrile (B), starting the sequence with 10 % B and programming the

gradient to obtain 20 % B at 10 min, 40 % B at 35 min, 100 % B at 40 min, 100 % B at 45 min, 10 % B at 46 min and 10 % B at 50 min. For the LC-MS pump to perform accurately, 10 % of organic solvent was pre-mixed in the water phase. The flow-rate was 1 mL/min and the chromatograms monitored at 240, 280 and 330 nm. Mass spectrometry operating conditions were optimized in order to achieve maximum sensitivity values. The ESI source was operated in negative mode to generate $[M-H]^-$ ions under the following conditions: desolvation temperature at 365 °C and vaporizer temperature at 400 °C; dry gas (nitrogen) and nebulizer were set at 12 L/min and 4.83 bar, respectively. The MS data were acquired as full scan mass spectra at 50–1100 m/z by using 200 ms for the collection of the ions in the trap.

The main compounds were identified by HPLC-DAD analysis, comparing the retention time, UV spectra and MS/MS data of the peaks in the samples with those of authentic standards or data reported in the literature. Only the main olive leaf polyphenols were quantified using commercial standards: oleuropein (Extrasynthese, Genay Cedex, France), luteolin-7-O-glucoside (Phytolab, Vestenbergsgreuth, Germany), neohesperidin (Sigma-Aldrich, Madrid, Spain) and apigenin (Nutrafur, Murcia, Spain). A purified extract (96.85 %) provided by Universidad Miguel Hernández (Elche, Spain) was used to quantify verbascoside. The quantitative evaluation of the compounds was performed with a calibration curve for each polyphenol, using ethanol (oleuropein), methanol (verbascoside and luteolin), methanol-water (neohesperidin) or dimethyl sulfoxide (apigenin) solutions of known concentration. The polyphenol concentrations were expressed as mg polyphenol per g of dry weight of olive leaves.

## 2.8. Statistical analysis

An analysis of variance (ANOVA) was applied in order to determine the significant effects of the variables under study, adopting a significance level of 95 %. In addition, a principal component analysis (PCA) was carried out in order to identify significant relationships among polyphenolic content, antioxidant capacity and drying/freezing conditions. The PCA uncovers combinations of the original variables (these combinations are known as latent variables or principal components-PCs)

which describe the dominant patterns and the main trends in the data, also determining the main sources of variability and establishing the relationship between samples and variables. In the PCA, the content of the main polyphenols was expressed as mg per g of dry weight of olive leaves using the calibration curves described in section 2.7. The statistical analysis was performed using Statgraphics-Plus software 5.1 (Statistical Graphics, Rockville, MD, USA).

## 3. Results and discussion

### 3.1. Effect of drying on antioxidant potential

Different methods for the drying of olive leaves were evaluated in order to extract olive leaf polyphenols efficiently, bearing in mind that the previous dehydration step could affect the antioxidant activity. Thus, freeze drying (FD) and hot air drying (HAD) at two different temperatures (70 and 120 °C) were compared.

As can be observed in Table 1, the drying method had a significant ($p<0.05$) influence on TPC and AC. The extracts obtained from fresh olive leaves dried using hot air at 120 °C (HAD-120) showed the highest TPC and AC. The reduction of the drying temperature from 120 to 70 °C led to a significant ($p<0.05$) decrease of TPC (24 %). The same fact was also observed in the case of AC, which, as the temperature was reduced, fell by 14 and 27 % for FRAP and TEAC, respectively. The fact that the drying rate improved at high temperatures could explain why the best results were achieved drying the olives leaves at 120 °C. Thus, high temperatures (120 °C) required shorter drying times than mild temperatures (70 °C) to achieve the same final moisture content, while providing more bioactive compounds. In addition, higher temperatures may inactivate the enzymatic oxidation caused by polyphenol oxidases, which are widely distributed among the different leaf tissues (Ortega-García et al., 2008), either directly or by diminishing water activity. Supporting these results, Bahloul et al. (2009) found that, in solar drying at temperatures of between 40 and 60 °C, the total phenols and the radical scavenging activity of olive leaves were significantly ($p<0.05$) influenced by drying air conditions and tended to decrease the longer the drying time. Thus, the drying time-temperature

combination could explain phenolic degradation, since short drying times at high temperatures seem to preserve the phenol content better than long drying processes at mild temperatures (García-Pérez et al., 2010). However, the opposite behavior has also been observed. Thus, when the effect of the drying temperature on the polyphenol content and antioxidant activity of red grape pomace peels was studied (Larrauri et al., 1997), increasing the temperature up to 100 and 140 °C led to a significant ($p<0.05$) reduction in the antioxidant potential of the bio-waste. These contradictory conclusions about the effect of the drying temperature on bioactive properties could probably be ascribed to the different structure and properties of the plant material and the nature of the bioactive compounds. In all likelihood, high temperatures (120 °C) have little impact on secoiridoids and flavones (oleuropein, luteolin) and a greater one on anthocyanins, such as those derived from red grape peels. Therefore, an optimum combination of drying time/temperature should be established for each product, as well as its polyphenolic profile, with which to minimize the degradation of bioactive compounds during the dehydration process.

**Table 1.** Effect of drying and freezing method on the total phenolic content and the antioxidant capacity of olive leaf extracts.

| Drying | Material | Total phenolic content (mg GAE/g d.w.) | Antioxidant Capacity (mg Trolox/g d.w.) | |
|---|---|---|---|---|
| | | | FRAP | TEAC |
| HAD-70 | Fresh | 45 ± 2 $^{a}$ | 94 ± 2 $^{a}$ | 6.08 ± 0.09 $^{a}$ |
| HAD-120 | Fresh | 59 ± 3 $^{b}$ | 109 ± 5 $^{b}$ | 8.3 ± 0.5 $^{b}$ |
| FD | C-Frozen | 36.3 ± 1.4 $^{c}$ | 75.7 ± 0.9 $^{c}$ | 4.5 ± 0.4 $^{c}$ |
| FD | $N_2$-Frozen | 37 ± 2 $^{c}$ | 80 ± 3 $^{c\,d}$ | 4.9 ± 0.4 $^{c\,d}$ |
| HAD-70 | $N_2$-Frozen | 37 ± 2 $^{c}$ | 81 ± 2 $^{d}$ | 5.5 ± 0.2 $^{a\,d}$ |
| HAD-120 | $N_2$-Frozen | 49 ± 2 $^{d}$ | 94.2 ± 1.2 $^{a}$ | 7.7 ± 0.4 $^{b}$ |

$^{a-d}$ Show homogeneous groups in the same column established from LSD (Least Significance Difference) intervals ($p<0.05$).

It is widely recognized that freeze drying is the dehydration method which best preserves the properties of the raw material. Thus, Dorta et al. (2012) highlighted that freeze dried samples of mango peel and seed provided a higher extraction yield than others dried by hot air. Mao et al. (2006) concluded that the TPC was higher in

freeze dried than hot air dried daylily flowers. However, contrary to what might be expected, in this study FD was the worst dehydration method for obtaining olive leaf extracts, providing both the lowest AC and TPC. If compared with HAD-120 (Table 1), FD provoked a significant ($p<0.05$) decrease in TPC (39 %) and AC (31 or 46 % depending on the method used, FRAP or TEAC, respectively).

During hot air drying, the high temperatures place great stress on the cell walls, while during freeze drying, the cell stress is ascribed to cell damage by ice crystals. In both cases, drying makes the release of phenolic compounds into the solvent easier due to the cell wall breakdown related to water removal (Hossain et al., 2010). The drying not only facilitates the extraction of phenols, but also the release of other intra-cellular compounds, such as the oxidative enzymes, which would reduce the antioxidant potential during the extraction process. Nevertheless, the high temperatures involved during hot air drying could deactivate the enzymes (Chism & Haard, 1996), thus avoiding or minimizing the phenolic degradation. This phenomenon could explain the fact that HAD extracts have a higher content of phenolic compounds than FD ones, since the low temperatures used during freeze drying would preserve enzymes in a latent state, thereafter facilitating their oxidative effect during thawing and extraction. Moreover, as already mentioned, the thermal deactivation also explains the higher antioxidant capacity in samples dried at 120 °C than in those dried at 70 °C, since the higher the temperature, the more intense the enzyme deactivation.

In order to gain insight into the influence of drying on the antioxidant potential of olive leaf extracts, bioactive compounds were identified by HPLC-DAD/MS-MS (Fig. 1 and Table 2). Regardless of the drying method, the same polyphenolic profile was identified in every extract. Although the profile was similar to those reported in literature for olive leaf extracts (Benavente-García et al., 2000), hydroxytyrosol, a known oleuropein derivative, was not found in our extracts. This result might be because of the different drying methods used in those studies or to a different olive tree variety (Scognamiglio et al., 2012).

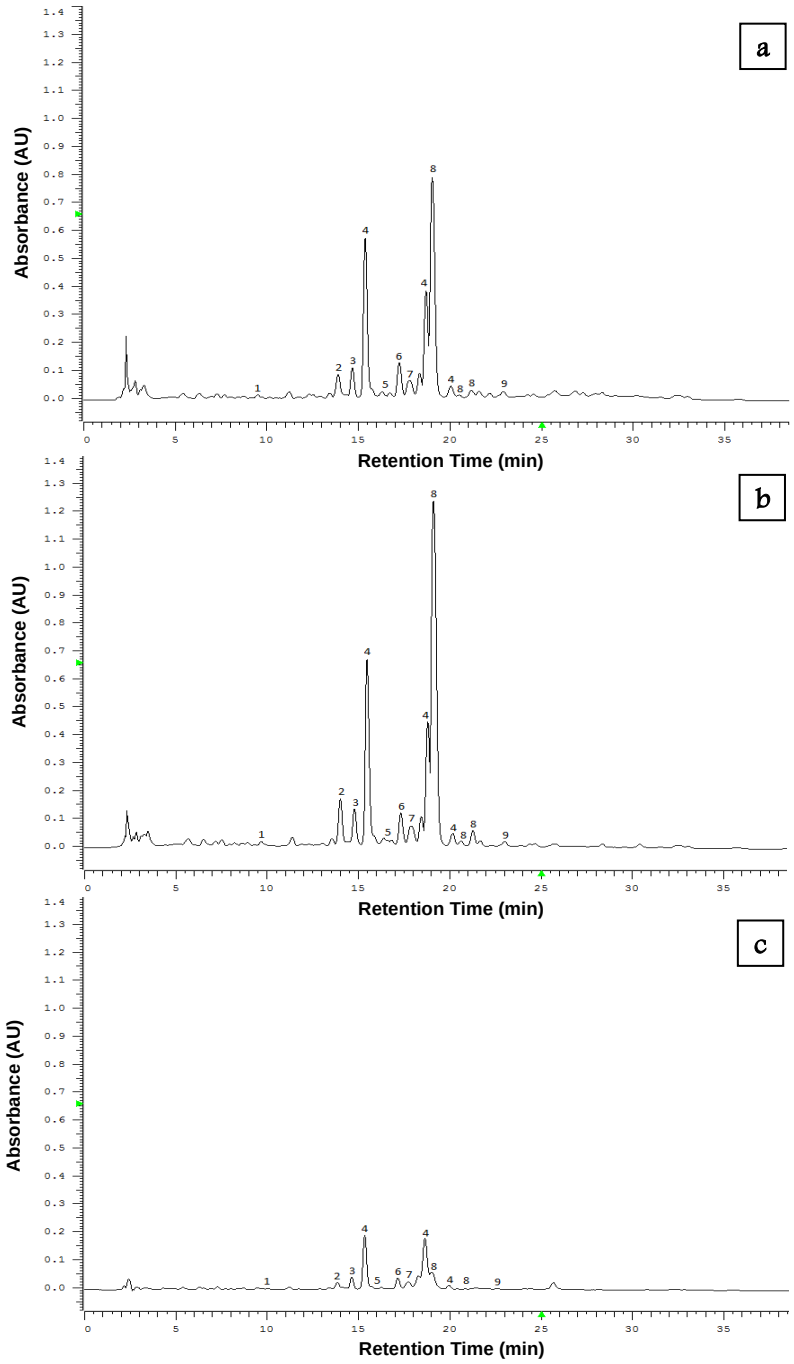


**Fig. 1.** HPLC chromatograms at 280 nm of extracts obtained from fresh olive leaves dried by hot air drying at 70 °C (a; HAD-70) or 120 °C (b; HAD-120) and extracts obtained from conventionally frozen leaves prior to freeze drying (c; C-FD).

**Table 2.** Relevant analytical data of compounds identified in olive leaf extracts by HPLC-ESI-MS/MS.

| Peak no. | Phenolic compound | $[M-H]^-$ m/z | MS/MS | Retention time (min) |
|---|---|---|---|---|
| 1 | Apigenin-6,8-diglucoside | 593 | 503, 473, 383, 353 | 9.7 |
| 2 | Verbascoside | 623 | 461 , 179 | 14.02 |
| 3 | Neohesperidin | 609 | 301 | 14.78 |
| 4 | Luteolin-7-O-glucoside | 447 | 285 | 15.47 |
| | Luteolin-7-O-glucoside (isomer) | 447 | 285 | 18.80 |
| | Luteolin-7-O-glucoside (isomer) | 447 | 285 | 20.16 |
| 5 | Oleuropein glucoside | 701 | 539, 377, 307 | 16.39 |
| | Oleuropein glucoside (isomer) | 701 | 539, 377, 307 | 16.59 |
| | Oleuropein glucoside (isomer) | 701 | 539, 377, 307 | 16.83 |
| 6 | Apigenin rutinoside | 577 | 269 | 17.32 |
| 7 | Luteolin-7-O-rutinoside | 593 | 285 | 17.92 |
| 8 | Oleuropein | 539 | 377, 197, 153 | 19.11 |
| | Oleuropein (isomer) | 539 | 377, 197, 153 | 20.63 |
| | Oleuropein (isomer) | 539 | 377, 197, 153 | 21.27 |
| 9 | Ligstroside (derivate) | 553 | 341, 257, 181, 137 | 23.03 |

Chromatograms corresponding to various extracts showed noticeable differences in the compound areas depending on the drying method used (Fig. 1). Quantification (Table 3) highlighted that drying had a significant influence ($p<0.05$) on the content of the three main compounds found: verbascoside (2), luteolin-7-O-glucoside (4) and oleuropein (8). The highest concentration of these compounds corresponded with the extracts showing the highest TPC and AC, which were obtained from HAD-120 leaves. As FD did not seem to facilitate the extraction of olive leaf phenols, the oleuropein content was reduced by 96 and 97 % compared with HAD-70 and HAD-120, respectively. As far as we are concerned, the improvement in the oleuropein content brought about by prior air drying at high temperatures has not previously been reported in the literature, and constitutes a relevant finding for industrial purposes. Papageorgiou et al. (2008) also stated that freeze drying reduced the content of some phenolic compounds in aromatic plants.

**Table 3.** Effect of drying and freezing method on the content (mg/g d.w.) of polyphenols identified in olive leaf extracts.

| Drying | HAD-70 | HAD-120 | FD | FD | HAD-70 | HAD-120 |
|---|---|---|---|---|---|---|
| Material | Fresh | Fresh | C-Frozen | $N_2$-Frozen | $N_2$-Frozen | $N_2$-Frozen |
| Oleuropein | 69 ± 4 [a] | 108.6 ± 1.6 [b] | 3 ± 0.9 [c] | 16.44 ± 1.14 [d] | 30.1 ± 0.8 [e] | 48 ± 2 [f] |
| Oleuropein glucoside [•] | 1.29 ± 0.6 [a] | 2.11 ± 0.04 [a] | 1.8 ± 0.4 [a] | 2.2 ± 0.6 [a] | 2.53 ± 0.9 [a] | 3.4 ± 0.4 [a] |
| Verbascoside | 1.5 ± 0.2 [a] | 2.7 ± 0.2 [b] | 0.3 ± 0.1 [c] | 1.26 ± 0.12 [d] | 1.9 ± 0.2 [e] | 1.89 ± 0.09 [e] |
| Luteolin-7-O-glucoside | 8.9 ± 0.2 [a] | 10.6 ± 0.2 [b] | 2.6 ± 0.3 [c] | 9.7 ± 1.6 [a b] | 10.9 ± 0.8 [b] | 11.1 ± 0.5 [b] |
| Luteolin-7-rutinoside [••] | 0.91 ± 0.04 [a] | 1.142 ± 0.009 [a] | 0.48 ± 0.03 [b] | 0.9 ± 0.2 [a b] | 0.98 ± 0.04 [a] | 0.99 ± 0.05 [a] |
| Apigenin 6,8-diglucoside [•••] | 0.12 ± 0.05 [a] | 0.118 ± 0.002 [a] | 0.09 ± 0.02 [a] | 0.16 ± 0.02 [a b] | 0.262 ± 0.112 [b] | 0.11 ± 0.02 [a] |
| Apigenin rutinoside [•••] | 0.7 ± 0.02 [a] | 0.736 ± 0.005 [a] | 0.333 ± 0.114 [b] | 0.59 ± 0.07 [a b] | 0.66 ± 0.03 [a] | 0.66 ± 0.04 [a] |
| Neohesperidin | 0.78 ± 0.04 [a] | 1.13 ± 0.13 [b] | 0.39 ± 0.02 [c] | 0.73 ± 0.08 [a c] | 0.99 ± 0.08 [a b] | 0.87 ± 0.06 [a b] |
| Ligstroside [•] | 2.13 ± 0.13 [a] | 1.35 ± 0.08 [b] | 0.6 ± 0.3 [c d] | nd [d] | 0.9 ± 0.2 [b c] | 0.8 ± 0.2 [b c] |

[a–f] Show homogeneous groups in the same row established from LSD (Least Significance Difference) intervals ($p<0.05$).

[•] Content expressed as equivalents of oleuropein (mg/g d. w.).

[••] Content expressed as equivalents of luteolin-7-O-glucoside (mg/g d. w.).

[•••] Content expressed as equivalents of apigenin (mg/g d. w.).

Moreover, HAD temperature significantly ($p<0.05$) influenced the polyphenolic concentration of olive leaf extracts. Thereby, the reduction of the drying temperature from 120 to 70 °C decreased the concentrations of polyphenols, such as oleuropein (36 %), verbascoside (44 %) and luteolin-7-O-glucoside (16 %). Other minor phenolic compounds, such as ligstroside, oleuropein glucoside, apigenin rutinoside or neohesperidin, were also found in olive leaf extracts but were less affected by the drying method than the most abundant ones. This fact could be explained by the different sensitivity of each polyphenol to the stress conditions caused by drying or due to a competitive mechanism of polyphenols in relation to oxidases.

Although freeze drying does not seem to be an adequate dehydration method for improving the extraction of phenols from olive leaves, it is recognized that ice crystals formed within the plant matrix during the freezing pre-treatment are able to improve extraction efficiency in other materials. Ice crystals can destroy the cell structure, permitting the removal of inner components, facilitating the contact with solvent and, consequently, improving extraction (Asami et al., 2003). Thus, taking this assumption into account, the influence the method used to freeze olive leaves has on the antioxidant potential was evaluated.

## 3.2. Influence of freezing on antioxidant potential

### 3.2.1. Influence of freezing method (liquid $N_2$ or conventional freezing)

In order to study whether the freezing method might influence antioxidant potential or not, extraction experiments were carried out using freeze dried leaves that had previously been frozen either by liquid $N_2$ ($N_2$-FD) or, conventionally, in a freezer at -28 °C (C-FD).

It is recognized that conventional freezing, due to the fact that it is slower, generates bigger ice crystals than liquid $N_2$ freezing, which is almost instant. In general terms, ice formation involves cellular damage, which should facilitate the extraction of polyphenols. Thereby, it could be assumed that the larger the ice crystal, the better the release of bioactive compounds. However, in this case, crystal size did not affect the extraction process. Although $N_2$-FD samples presented higher TPC and

AC than C-FD ones (Table 1), the differences between the extracts were not significant ($p<0.05$). Asami et al. (2003) found a significant influence of freezing and freeze drying on the TPC of marionberry, strawberry and corn, which disagree with the results found for the olive leaves. This fact could be ascribed to the particular structure of olive leaves, which present a high content of cellulosic components in the cell wall, probably protecting the inner cell components from mechanical damage.

Neither C-FD nor $N_2$-FD samples were able to provide extracts with similar antioxidant characteristics to those obtained by HAD (70 and 120 °C). As already mentioned, FD does not seem to be an adequate drying technique with which to promote the extraction of polyphenols from olive leaves. Although FD might be very useful for a good appearance or for moisture reconstitution in dried products, when it is a question of leaching bioactive compounds, it does not promote the release of the phenolic compounds into the extracts, leading to lower AC (Hossain et al., 2010).

Despite the fact that the identified bioactive compound profile was nearly the same regardless of the freezing method used (Fig. 1c, 2 and Table 2), some differences were found in peak areas. $N_2$-FD samples presented a significantly ($p<0.05$) higher content of the majority of polyphenols quantified (Table 3): oleuropein, verbascoside and luteolin-7-O-glucoside. Compared to C-FD, the $N_2$-FD treatment increased the oleuropein and verbascoside content by 448 and 320 %, respectively. However, the concentrations reached were still far below ($p<0.05$) the ones quantified in extracts obtained from leaves dried by HAD. These results may also be explained by considering the already mentioned influence of the enzymatic degradation of polyphenols during extraction. Dehydration may affect oxidative enzymes, not only by reducing the water activity but also by thermal deactivation (Jones, 1981; Suhaj, 2006), the latter only being manifested in HAD. Thus, when freeze dried leaves come into contact with moisture from any source, as happens during aqueous extraction, the oxidative enzymes are activated and their degradative action starts again (Hossain et al., 2010). In HAD samples, the high temperatures applied during drying would reduce the oxidative action of the enzymes during extraction.

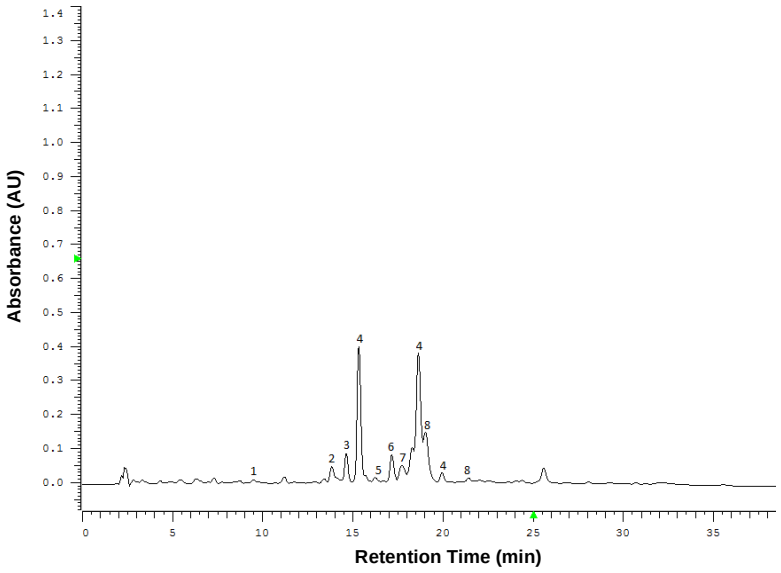


**Fig. 2.** HPLC chromatogram at 280 nm of an olive leaf extract obtained from $N_2$ frozen leaves prior to freeze drying ($N_2$-FD).

On the other hand, by using $N_2$ instead of conventional freezing, freeze dried leaves showed oleuropein glucoside, luteolin and apigenin contents similar to fresh HAD samples. This fact highlights that these compounds were more resistant to enzymatic degradation.

### 3.2.2. Influence of freezing on hot air drying (HAD)

In order to determine the effect that freezing has and quantify its capacity to reduce the antioxidant potential of olive leaves, HAD-120 and HAD-70 were carried out using olive leaves frozen by liquid $N_2$. In the extracts obtained, TPC and AC analysis and the identification and quantification of phenolic compounds were performed (Tables 1 and 3).

Freezing prior to HAD significantly ($p<0.05$) reduced TPC; this was 18 and 17 % lower than fresh HAD samples at 70 and 120 °C, respectively. Both antioxidant determination methods used (FRAP and TEAC) highlighted the fact that extracts obtained from fresh HAD leaves presented a higher AC than frozen HAD samples. However, the differences were only significant ($p<0.05$) in the case of the FRAP method. The differences observed in the TPC and AC of fresh and frozen samples

were similar at both air drying temperatures tested (70 and 120 °C). Therefore, it can be seen that the freezing effect was not influenced by the air drying temperature. In any case, regardless of the drying temperature, frozen HAD leaves showed a higher antioxidant potential than the freeze dried ones.

Considering that the high temperatures applied in HAD should be able to deactivate oxidative enzymes, why would freezing prior to HAD reduce the antioxidant potential of fresh leaves? The explanation could be linked to some changes that take place during the thawing period (Cannac et al., 2007; El-Kest & Marth, 1992), which should occur during sample handling and the first moments of drying. Thus, before water activity reduction and enzyme deactivation by HAD, the freezing process seems to promote the structural damage of cells, and therefore the degradative action of oxidative enzymes on polyphenols.

Compared to fresh hot air dried leaves (Fig. 1a and 1b), all the frozen dried ones (Fig. 3) showed a similar phenolic profile. Oleuropein was the polyphenol most seriously affected by freezing (Table 3) which reduced the oleuropein content by 56 % on average and was quite similar at both temperatures tested (70 and 120 °C). Despite the reduction in the oleuropein content, hydroxytyrosol (a product derived from oleuropein) was not detected in the extracts. Other iridoid glucosides, such as ligstroside, were also negatively affected by freezing. The rest of the phenolic compounds were less sensitive to freezing prior to HAD (Table 3). The content of some compounds, such as verbascoside and luteolin-7-glucoside, increased when fresh leaves were frozen before HAD.

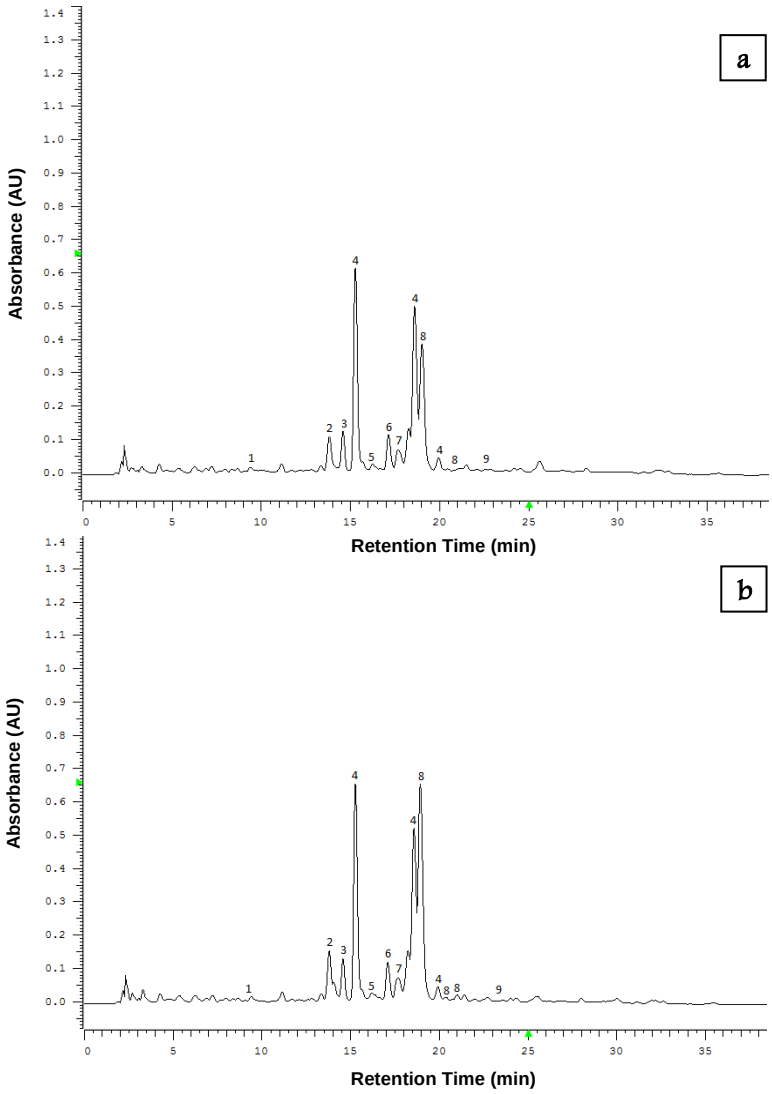


**Fig. 3.** HPLC chromatograms at 280 nm of olive leaf extracts obtained from $N_2$ frozen leaves prior to hot air drying at 70 °C (a; $N_2$-HAD-70) or 120 °C (b; $N_2$-HAD-120).

## 3.3. Principal component analysis (PCA)

In previous sections, a close positive correlation between TPC and AC was described. Therefore, polyphenolic compounds could be considered key contributors to the AC of olive leaf extracts. Then, aiming both to identify the bioactive compounds that are the main contributors to the antioxidant potential and also to summarize the main differences among the different olive leaf extracts, a principal components

analysis (PCA) was performed. For that purpose, AC and TPC (Table 1) measurements and the polyphenol composition (Table 3) of the extracts were included in the PCA.

Two principal components (PC) were extracted from the statistical analysis (with eigenvalues>1), which together allow 81.9 % of the total variance in extract composition to be explained. Fig. 4 shows the projection of the measured variables in all the extracts on the plane defined by PC1 and PC2.

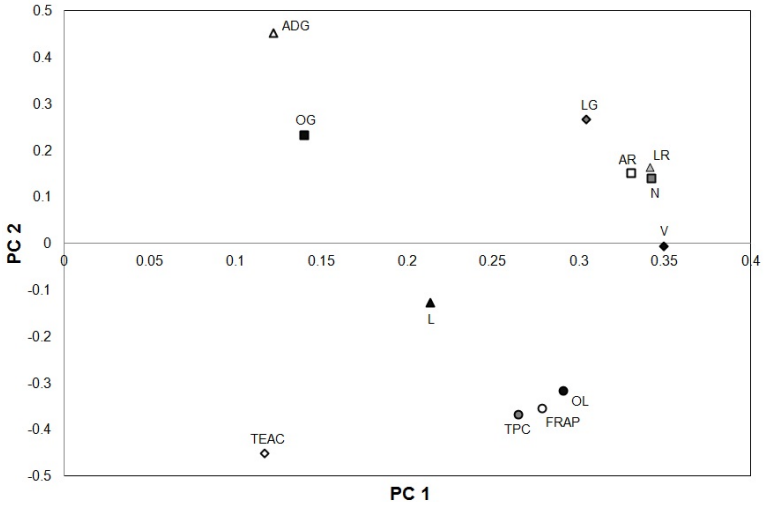


**Fig. 4.** Principal component analysis. Correlation scatterplot of the variables with principal component 1 (PC1) and principal component 2 (PC2). TEAC: antioxidant capacity measured by TEAC method; FRAP: antioxidant capacity measured by FRAP method; TPC: total phenolic content; O; oleuropein; OG: oleuropein glucoside; L: ligstroside (derivative); V: verbascoside; N: neohesperidin; AR: apigenin rutinoside; ADG: apigenin-6,8-diglucoside; LR: luteolin-7-O-rutinoside; LG: luteolin-7-O-glucoside.

For PC1, which explains 59.4 % of the experimental variability, all the variables showed a positive correlation, although the highest ones were found for TPC, AC (FRAP) and the concentration of some polyphenols, such as verbascoside, oleuropein, neohesperidin, luteolin derivatives and apigenin rutinoside. In the case of PC1, no negative correlations were found since every polyphenol contributed both to TPC and AC measured by means of the FRAP method. PC2 (explained variance 22.5 %) could be mainly related with the antioxidant capacity, measured using the TEAC method.

The highest correlations were found for AC (TEAC and FRAP), TPC and oleuropein content. Thus, oleuropein can be considered as the polyphenol which has the greatest influence on TEAC. The negative correlations indicate that the lower the oleuropein content, the lower the TEAC measured. On the other hand, the positive correlation found for apigenin-6,8-diglucoside in PC2 shows that the presence of this component does not lead to an increase in the antioxidant capacity measured using TEAC (Fig. 4).

When the scores of each different extract were examined in a two-dimensional plot of the two principal components (Fig. 5), it was found that the samples could be separated into four groups depending on the drying method, the freezing method and the combination of both. As can be observed, the freeze dried leaf samples are clearly separated from the fresh samples dried at 120 °C, the former being the worst and the latter the best processing method with which to achieve a high phenolic content, respectively. Freeze dried samples had a negative correlation with TPC and AC (FRAP) (PC1), which confirms that it is not an appropriate method for facilitating the release of olive leaf bioactive compounds. Fig. 5 also shows that freezing with liquid $N_2$ would be a good pre-treatment prior to HAD-120 or FD in order to obtain a high content of apigenin-6,8-diglucoside, since these dried samples had a positive correlation with PC2.).

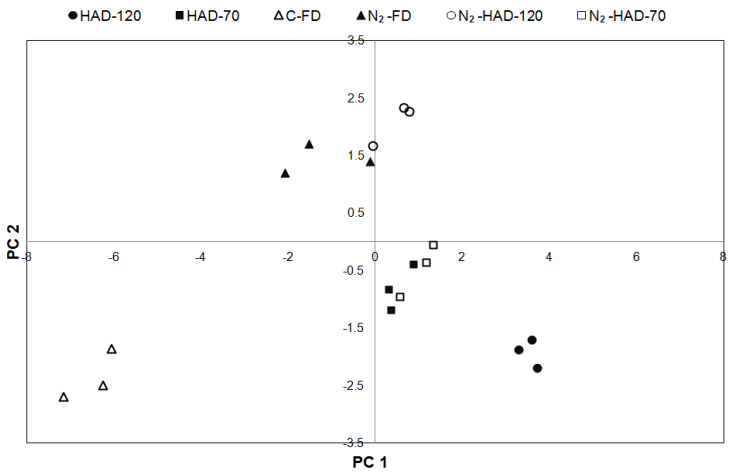


**Fig. 5.** Representation of samples of olive leaf extracts on the plane defined by the first and second principal components, PC1 (59.4 %) and PC2 (22.5 %).

## 4. Conclusions

The antioxidant potential of olive leaf extracts has been shown to be largely dependent on operations prior to extraction. Thus, major polyphenols identified in extracts, such as oleuropein, verbascoside and luteolin-7-O-glucoside, have been influenced by both leaf freezing and drying techniques. Regardless of the freezing technique, freeze dried leaves did not provide extracts with a high antioxidant potential. Thereby, the hot air drying of fresh leaves at high temperatures (120 °C) was the most appropriate method with which to improve the antioxidant potential and oleuropein content of extracts, which for industrial purposes, could be a relevant finding not previously reported in literature since it is a more affordable dehydration technique than freeze drying.

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# *Influence of air temperature on drying kinetics and antioxidant potential of olive pomace*

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# Influence of air temperature on drying kinetics and antioxidant potential of olive pomace


## ABSTRACT

This work aims to evaluate the influence of olive pomace drying (a solid byproduct of the olive oil industry) on both antioxidant potential and drying kinetics. The two main fractions of olive pomace (pits, PI and pulps+peels, P+P) were characterized by image analysis and density measurement. The drying process was analyzed in experiments carried out at different temperatures (from 50 to 150 °C) and mathematically described from the diffusion and Weibull models. The antioxidant potential of the extracts (ethanol-water 80:20 v/v, 22 ± 1 °C, 170 rpm for 24 h) obtained from the dry product was analyzed by measuring the total phenolic content and antioxidant capacity and the main polyphenols were quantified by HPLC-DAD/MS-MS.

The drying behavior of olive pomace was well described by considering the diffusion in the PI and P+P fractions separately and the influence of temperature on effective moisture diffusivities was quantified by an Arrhenius type equation. The antioxidant potential was only mildly influenced by the drying temperature. However, long drying times at the highest temperature tested (150 °C) significantly ($p<0.05$) increased the antioxidant potential.


***Keywords:*** phenolic content, antioxidant capacity, drying kinetics, diffusion, olive pomace.

## 1. Introduction

The olive (*Olea europaea*) is an evergreen tree traditionally cultivated for the production of oil and table olives. As regards both wealth and tradition, the olive oil industry is a relevant one, especially in the Mediterranean countries where 97 % of the world's olive production is harvested. Spain is the leading country in terms of the total crop surface and the number of productive trees (Niaounakis & Halvadakis, 2004).

Nowadays, the olive oil industry generates a great environmental impact due to the production of high polluting residues (Baeta-Hall et al., 2005). Several studies have stated the negative effects of these forms of waste on soil's microbial populations (Paredes et al., 1987), aquatic ecosystems (DellaGreca et al., 2001) and even on the air (Rana et al., 2003). However, olive polyphenols, such as oleuropein, verbascoside or hydroxytyrosol, are present not only in olive oil but also in oil waste products, exhibiting among other things, antiviral, antitumoral and antioxidant activities (Della Ragione et al., 2000; Liu et al., 2003; Micol et al., 2005). One of the most problematic olive oil waste products is pomace (the solid byproduct made up from pieces of pit, skin and pulp), also known as cake. Actually, it is used for animal feed, residual oil extraction, energy recovery, soil amendment or the extraction of valuable polyphenols (Roig et al., 2006). A previous dehydration stage reduces the pomace water content to 5-6 % (wet basis), aiming to stabilize the byproduct and so avoiding undesirable degradation during storage. Moreover, in the particular case of bioactive compound extraction, drying avoids the interference of water in the polyphenol release (Soysal & Öztekin, 2001), improving the extraction yield. For industrial purposes, hot air drying is the most widely used method, since it allows an accurate control of the process variables. Traditionally, low air temperatures are used as a means of better protecting the bioactive compounds from degradation during drying. However, drying at low temperatures constitutes a slow process in which metabolic reactions may be long lasting, leading to quality loss (Fennell et al., 2004). Thereby, certain studies also suggest the use of high temperatures for the industrial drying of olive pomace (Göğüs & Maskan, 2006). High temperatures speed up the drying kinetics, which could be interesting for the purpose of increasing productivity on an

industrial scale (Ahmad-Qasem et al., 2013a), but at the same time it could promote the oxidative degradation of polyphenols (Gomes & Caponio, 2001) and requires the use of a great amount of energy. For this reason, the main aim of this work was to assess the influence of the air temperature on the drying kinetics and antioxidant potential of olive pomace, two aspects which have not previously been considered together.

## 2. Materials and methods

### 2.1. Raw material

The raw material used in this work was olive pomace from a traditional pressing system for obtaining olive oil, provided by an oil factory located in Altura (Castellón, Spain). The pomace was collected just after the pressing operation and immediately vacuum packaged and stored at 4 °C. The initial moisture content was determined by drying in a vacuum chamber at 70 °C until reaching constant weight (AOAC method nº 934.06, AOAC, 1997).

It could be considered that olive pomace is mainly composed of two main fractions: pits (PI) and pulps + peels (P+P). Homogeneous samples of olive pomace were taken, both fractions were separated by hand and their corresponding mass fraction ($X$) calculated and characterized by image analysis (Table 1). RGB images were taken (Fig. 1a and 1c) and processed using Image J software (Research Service Branch, National Institute of Mental Health, US, available as freeware from http://rsbweb.nih.gov/ij/). Images were converted to the binary system (Fig. 1b and 1d) using an automatic threshold. Finally, the particles were counted and their surface ($S$, $mm^2$) calculated considering the scale reference. From another set of experiments, the initial moisture content of both fractions was also determined, as already explained for the olive pomace.

The bulk density ($\rho$) of both the PI and P+P fractions, as well as that of the fresh olive pomace, was determined at 20 °C by liquid displacement using water, a

volumetric standard picnometer (48.89 mL) and an analytical balance (PB 303-S, Mettler Toledo).

**Table 1.** Characterization of pit (PI) and pulp + peel (P+P) fractions of olive pomace.

| | PI | P+P |
|---|---|---|
| $\rho$ (kg/L) | 1.30 ± 0.05 | 1.5 ± 0.2 |
| $W_o$ (g w/g d.w) | 0.234 ± 0.004 | 0.66 ± 0.05 |
| $X$ | 0.424 ± 0.005 | 0.576 ± 0.005 |
| $r_m$ (mm) | 1.80 ± 0.02 | 0.311 ± 0.015 |
| $Y_1$ ($S_p$ > 10 mm$^2$) | 0.584 | 0.502 |
| $Y_2$ (1 < $S_p$ < 10 mm$^2$) | 0.381 | 0.384 |
| $Y_3$ (0.25 < $S_p$ < 1 mm$^2$) | 0.021 | 0.063 |
| $Y_4$ ($S_p$ < 0.25 mm$^2$) | 0.015 | 0.051 |

$\rho$ = density, $W_o$ = initial moisture content, $X$ = mass fraction, $r_m$ = characteristic dimension (thickness in PI and radius in P+P fraction), $Y$ = sub-fraction of particles with a specific surface ($S_p$, $Y_1+Y_2+Y_3+Y_4 = 1$).

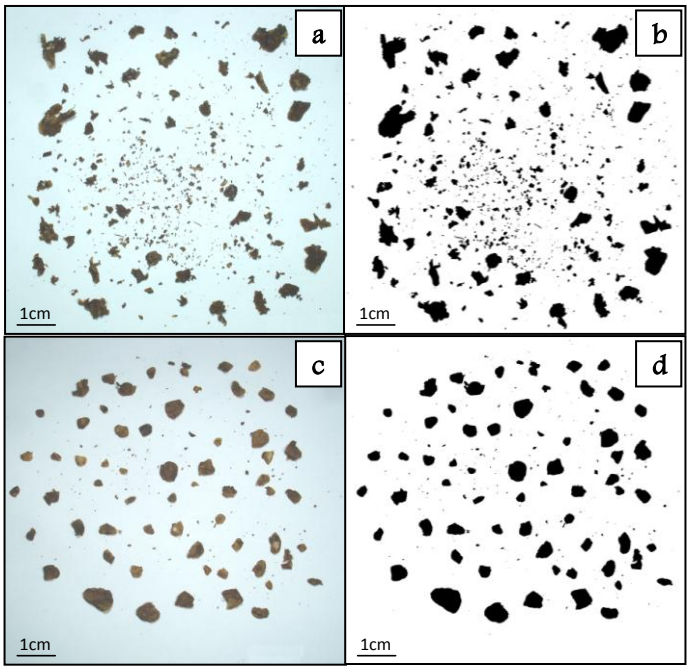


**Fig. 1.** RGB images of pulp + peel (P+P, a) and pit (PI, c) fractions of olive pomace and binary conversion (P+P, b and PI, d) using Image J.

## 2.2. Drying experiments

Drying experiments were conducted in a forced air laboratory drier (FD, Binder, Tuttlingen, Germany), using a horizontal air flow of 0.094 $m^3/s$ and an air velocity of 0.683 m/s. Each run was carried out with an initial mass load of 40 g of olive pomace, uniformly distributed in a monolayer (4 ± 1 mm thick, 0.083 $g/cm^2$).

Two different sets of experiments were designed. In the first one, the variable to be considered was that of the air temperature in order to determine its influence on both the drying kinetics and antioxidant potential of the extracts obtained from the dried product. For this purpose, the drying experiments were carried out at different air drying temperatures: 50, 70, 90, 120 and 150 °C. During the process, the samples were weighed (XS204, Mettler Toledo, Barcelona, Spain) at pre-set times. The drying experiments finalized when the sample weight loss reached 30 ± 1 %. This fact was established by previous experiments, ensuring that the water activity was below 0.4 and the obtained product was stable.

In the second set of experiments, the variable to be studied was the drying time. For that purpose, drying experiments were carried out at 150 °C and for different drying times: 5, 10, 20, 30 and 60 min. It should be highlighted that, in this set of experiments the effective drying period took place between 5 and 10 min, from which mass transfer could be considered negligible. Therefore, this involves overexposing the olive pomace to a high temperature (150 °C).

The drying experiments for each experimental condition tested were carried out three times, at least.

## 2.3. Modeling of hot air drying kinetics

The experimental drying kinetics were determined from the initial sample mass and the weight loss measured during drying. Previous approaches to the modeling of the drying kinetics of olive pomace have been addressed through the use of deep beds, assuming in the modeling that the sample is as thick as the bed is high and that it behaves like an infinite slab (Göğüs & Maskan, 2006). In addition, Vega-Gálvez et al. (2010) molded olive cake into a rectangular form and conducted drying

experiments in monolayer at different temperatures in order to identify an effective moisture diffusivity in this particular body. However, the drying of the individual particles of olive pomace has not previously been addressed. This could be considered a complicated issue, since olive pomace is a heterogeneous material made up mainly of pits, peels and pulp pieces, which represents a handicap when using a diffusion model where the samples are assumed to be homogeneous. In this work, therefore, the monolayer drying of this byproduct has been studied in order to identify the drying behavior of olive pomace at particle level, and further studies should be performed to address the drying of the bulk of the olive pomace. For that purpose, two different approaches were considered.

On the one hand, a diffusion model for the olive pomace was used by considering the diffusion in both fractions of the olive pomace to be different: Pits (PI) and pulps + peels (P+P). It was assumed that pits could be considered as geometrically spherical particles, while peels + pulps could behave like infinite slabs. Eqs. (1) and (2) show the solution of diffusion models for spheres and infinite slabs, respectively, considering:

- Homogenous and isotropic solids.
- Constant effective diffusivity.
- Negligible shrinkage.
- Uniform initial moisture and temperature.
- The solid surface at equilibrium with the drying air.
- Solid symmetry.

$$W_{PI} = W_e + (W_c - W_e)\left[\sum_{n=1}^{\infty} \frac{6}{n^2\pi^2} exp\left(-\frac{D_e^{PI}}{R^2} n^2\pi^2 t\right)\right] \quad (1)$$

$$W_{P+P}(t) = W_e + (W_c - W_e)\left[\sum_{n=0}^{\infty} \frac{8}{(2n+1)^2\pi^2} exp\left(-\frac{D_e^{P+P}(2n+1)^2\pi^2 t}{4L^2}\right)\right] \quad (2)$$

where $W$ is the average moisture content (dry basis), subscripts $c$ and $e$ refer to the critical and equilibrium states, $t$ (min) the drying time, $D_e$ is the effective moisture diffusivity ($m^2/s$), which was considered to be different in both PI and P+P fractions. The characteristic diffusion paths, radius ($R$) and thickness ($L$), were experimentally determined. The average radius of pit pieces was obtained from image analysis. For that purpose, the radius of individual particles, which was calculated from the measurement of the surface ($S_p = \pi R^2$), was computed and weighed to obtain an average value. Whereas the average particle thickness of the pulp + peel fraction was calculated from the measurement of the total particles surface ($S_t$), also obtained by image analysis, the mass ($M$) and the density ($\rho$) (Eq. (3)):

$$L = \frac{M}{\rho S_t} \tag{3}$$

Considering the diffusion in PI and P+P fractions to be different, the moisture content of olive pomace could be calculated from Eqs. (1) and (2) by using a compositional model (Eq. (4)). Similar diffusion models have been used for analyzing mass transfer phenomena in geometrically complex plant tissues, such as grape stalk (García-Pérez et al., 2006) and broccoli (Sanjuán et al., 2001).

$$W = X_{PI} W_{PI} + X_{P+P} W_{P+P} \tag{4}$$

where $X$ is the mass fraction of PI and P+P fractions ($X_{PI} + X_{P+P} = 1$).

On the other hand, the Weibull empirical model (Cunha et al., 1998; Simal et al., 2005) was also used for the mathematical description of the drying kinetics of olive pomace. The Weibull model (Eq. (5)) is a probability function used to explain the behavior of changeable complex systems (Cunha et al., 1998). Initially, it was used to predict material failures caused by fatigue. In food technology, it has been used for the description of degrading processes, since the degradation of food can be considered as a system fault under certain stress conditions (Blasco, 2003), such as when exposed to hot air. Thus, the Weibull model adapted to a drying process is presented in Eq. (5):

$$\psi(t) = \frac{W(t) - W_e}{W_c - W_e} = exp\left(-\left(\frac{t}{\beta}\right)^{\alpha}\right) \tag{5}$$

where $\Psi$ represents the dimensionless moisture content, $\alpha$ the dimensionless parameter related to the shape, assimilated to the behavior index of the product during drying, and $\beta$ (min $^{-1}$) is the kinetic parameter inversely related ($1/\beta$) with the process rate.

The identification of the model parameters ($\alpha$ and $\beta$ in the Weibull model, and $D_e$ for PI and P+P fractions in the diffusion model) was carried out by minimizing the sum of the squared differences between the experimental and calculated moisture content of olive pomace samples using the Solver tool from Excel™ (Microsoft Corporation, Seattle, USA). For each drying condition tested, the parameter identification was simultaneously carried out with all the replicates. The explained variance (VAR) was computed (Eq. (6)) to determine the goodness of the model's fit to experimental data:

$$\text{VAR} = 1 - \frac{S_{xy}^2}{S_y^2} \tag{6}$$

where $S^2_{xy}$ is the variance of the estimation and $S^2_y$ the variance of the sample. In addition, the mean relative error (MRE) was calculated (Eq. (7)) to establish the difference between the experimental ($W_{EXPi}$) and calculated ($W_{CALi}$) data:

$$\text{MRE} = \frac{100}{N}\left(\sum_{i=1}^{N}\frac{W_{EXPi} - W_{CALi}}{W_{EXPi}}\right) \tag{7}$$

where $N$ is the number of experimental data.

Moreover, in order to evaluate the influence of temperature on the kinetic parameters, an Arrhenius type equation (Meziane, 2011) was used (Eqs. (8) and (9)):

$$\frac{1}{\beta} = \frac{1}{\beta_0}\cdot exp\left(-\frac{E_a}{R\cdot T}\right) \tag{8}$$

$$D_e = D_0\cdot exp\left(-\frac{E_a}{R\cdot T}\right) \tag{9}$$

where $1/\beta_0$ (min $^{-1}$) and $D_0$ (m$^2$/s) are the pre-exponential factors, $E_a$ (kJ/mol) the activation energy, $R$ (kJ/mol K) the universal gas constant and $T$ (K) the drying temperature.

## 2.4. Extraction experiments

Dried olive pomace was milled (Blixer 2, Robot Coupe USA, Inc., Jackson, MS, USA) and sieved (Metallic mesh 0.05 mm, Filtra Vibración, Barcelona, Spain) to obtain particles with a diameter of under 0.05 ± 0.01 mm. The extraction was carried out in sealed containers protected from light and immersed in a thermostatic shaking water bath (SBS40, Stuart, Staffordshire, UK) working at 170 rpm. The solvent used was a solution of ethanol-water (80:20, v/v) and the ratio between the weight of the olive pomace and the solvent volume, 20 g/30 mL. The extraction process was carried out at 22 ± 1 °C for 24 h, which was based on previous works (Ahmad-Qasem et al., 2013a, 2013b). In addition, another set of extraction experiments was carried out by varying the extraction times from 5 to 48 h to monitor the extraction process, which is relevant in the case of industrial operations where a high level of productivity needs to be attained. In this case, extraction experiments were conducted with fresh and dried olive pomace at 50 and 150 °C.

Every extract was centrifuged for 10 min at 5000 rpm (Medifriger BL-S, J.P. Selecta, Barcelona, Spain), filtered (nylon filters of 0.45 μm) and stored in opaque vials at 4 °C until analysis. At least 3 replicates were made for each different condition tested.

## 2.5. Total phenolic content (TPC) and antioxidant capacity (AC) measurement

The phenolic content and antioxidant capacity of the extracts obtained were determined by the Folin-Ciocalteu (Singleton et al., 1999) and Ferric-Reducing Ability Power (FRAP) methods, respectively (Benzie & Strain, 1996; Pulido et al., 2000). These methods are exhaustively described by Ahmad-Qasem et al. (2013a, 2013b).

The TPC was expressed as mg of gallic acid (GAE) per g of dry weight of olive pomace (g d.w.), while the AC was expressed as mg of Trolox per g of dry weight of olive pomace (g d.w.).

## 2.6. Identification and quantification of polyphenols by HPLC-DAD/MS-MS

In order to identify and quantify the main polyphenols, the olive pomace extracts were analyzed using an HPLC instrument (Agilent LC 1100 series; Agilent Technologies, Inc., Palo Alto, CA, USA) controlled by the Chemstation software. The HPLC instrument was coupled to an Esquire 3000+ (Bruker Daltonics, GmbH, Germany) mass spectrometer equipped with an ESI source and ion-trap mass analyzer, and controlled by Esquire control and data analysis software. A Merck Lichrospher 100RP-18 (5 μm, 250 x 4 mm) column was used for analytical purposes.

Separation was carried out through a linear gradient method using 2.5 % acetic acid (A) and acetonitrile (B), starting the sequence with 10 % B and programming the gradient to obtain 20 % B at 10 min, 40 % B at 35 min, 100 % B at 40 min, 100 % B at 45 min, 10 % B at 46 min and 10 % B at 50 min. For the LC-MS pump to perform accurately, 10 % of organic solvent was pre-mixed in the water phase. The flow-rate was 1 mL/min and the chromatograms monitored at 240, 280 and 330 nm. Mass spectrometry operating conditions were optimized in order to achieve maximum sensitivity values. The ESI source was operated in negative mode to generate $[M-H]^-$ ions under the following conditions: desolvation temperature at 365 °C and vaporizer temperature at 400 °C; dry gas (nitrogen) and nebulizer were set at 12 L/min and 4.83 bar, respectively. The MS data were acquired as full scan mass spectra at 50–1100 m/z by using 200 ms for the collection of the ions in the trap.

The olive pomace compounds were identified by HPLC-DAD analysis, comparing the retention time, the UV spectra and the MS/MS data of the peaks. Only the luteolin-7-O-glucoside was quantified using a commercial standard (Phytolab, Vestenbergsgreuth, Germany). The quantitative evaluation was performed with a calibration curve using methanol solutions of known concentrations. The polyphenol concentration was expressed as mg luteolin-7-O-glucoside per g of dry weight of olive pomace (g d.w.).

## 3. Results and discussion

### 3.1. Hot air drying kinetics at different temperatures

In order to evaluate the influence of air temperature on drying kinetics, experiments were carried out at temperatures ranging from 50 to 150 °C. In these experiments, the initial moisture content of olive pomace was reduced from 0.33 to 0.05 kg water/kg fresh olive pomace (30 % of the initial weight loss). Despite the fact that olive pomace is a heterogeneous material, an adequate repeatability and a small experimental variability was found in the experimental drying kinetics (Fig. 2).

As can be observed in Fig. 2, the air temperature significantly affected ($p<0.05$) the drying rate: the higher the temperature, the shorter the processing time. Thus, the drying times needed to achieve a 30 % loss of the initial weight ranged from 40 min at 50 °C to 10 min at 150 °C and increasing the drying temperature from 90 to 150 °C shortened the drying time by 50 %. These results agreed with those reported by other authors who studied olive pomace drying by means of different techniques. Thus, Ruiz-Celma et al. (2008), who studied the infrared drying of wet olive husk (pomace), found that a rise in temperature from 80 to 140 °C reduced the drying time by a third.

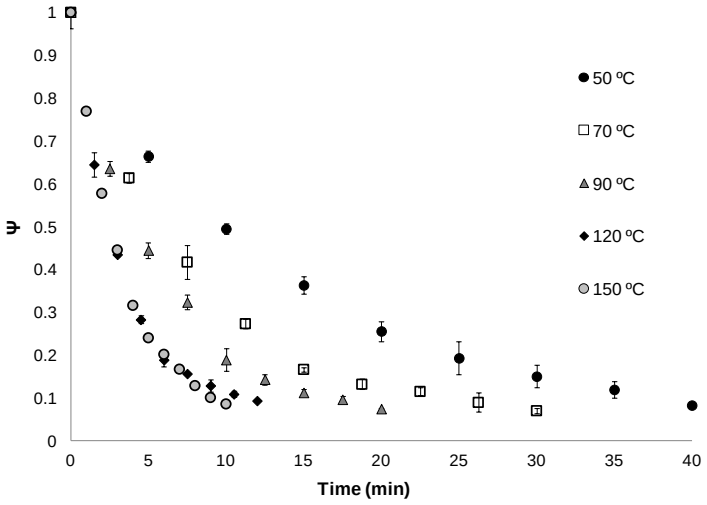


**Fig. 2.** Experimental (average ± standard deviation) drying kinetics of olive pomace at different temperatures.

The experimental data showed that, for the air temperatures tested, the drying only took place during the falling rate period. As an example, the drying rate for two particular temperatures tested (50 and 120 °C) is shown in Fig. 3. Therefore, the initial moisture content was assumed to be equal to the critical one. These results agreed with previous works, like those published by Göğüs & Maskan (2006) who studied olive pomace behavior during hot air drying at temperatures ranging between 60 and 80 °C or Ruiz-Celma et al. (2008) working on infrared drying at temperatures from 80 to 140 °C. However, Kadi & Hamlat (2002) found a constant drying period during the hot air drying of olive pomace. These contradictory results could be linked to the sample layer thickness used in each experimental design. Thus, the constant rate period cited by Kadi & Hamlat (2002) could be attributed to a thick sample layer, which leads to air saturation, but not to the behavior of the particle.

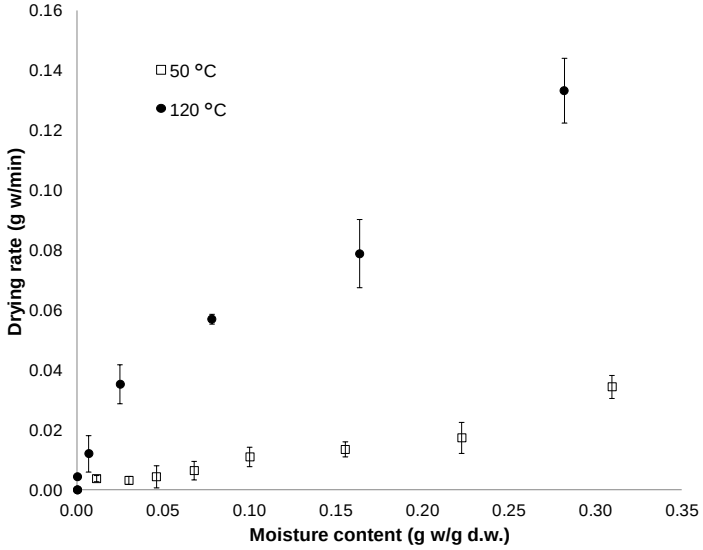


**Fig. 3.** Evolution of drying rate (average ± standard deviation) during drying of olive pomace at 50 and 120 °C.

Modeling of drying kinetics was addressed from the diffusion and Weibull (Cunha et al., 1998) models, as explained in section 2.3. The modeling not only aimed to quantify the influence of the temperature on the drying kinetics but also to better characterize the olive pomace drying at particle level. The characterization of PI and

P+P fractions is shown in Table 1, while the results of drying kinetics modeling are included in Table 2.

**Table 2.** Modelling of drying kinetics of olive pomace carried out at different temperatures and identified parameters of Weibull and Diffusion models.

| | Drying temperature (°C) | | | | |
|---|---|---|---|---|---|
| | **50** | **70** | **90** | **120** | **150** |
| *Weibull* | | | | | |
| $\alpha$ | 0.88 | 0.85 | 0.88 | 0.85 | 0.99 |
| $\beta$ (s) | 14.4 | 8.5 | 6.2 | 3.7 | 3.6 |
| % VAR | 99.4 | 99.5 | 99.6 | 99.4 | 99.7 |
| % MRE | 9.1 | 11.0 | 8.4 | 10.1 | 4.6 |
| *Diffusion* | | | | | |
| $D_e^{PI}$ ($m^2/s$) | $1.17 \times 10^{-7}$ | $1.60 \times 10^{-7}$ | $2.69 \times 10^{-7}$ | $2.90 \times 10^{-7}$ | $2.92 \times 10^{-7}$ |
| $D_e^{P+P}$ ($m^2/s$) | $3.58 \times 10^{-11}$ | $6.48 \times 10^{-11}$ | $8.45 \times 10^{-11}$ | $1.59 \times 10^{-10}$ | $1.60 \times 10^{-10}$ |
| % VAR | 98.8 | 99.1 | 99.03 | 98.9 | 96.9 |
| % MRE | 11.9 | 10.9 | 9.5 | 9.2 | 11.3 |

A much higher effective moisture diffusivity ($D_e$) was found in the PI fraction than in the P+P one (Table 2). Thus, $D_e$ ranged from $1.17 \times 10^{-7}$ to $2.92 \times 10^{-7}$ $m^2/s$ for the PI fraction and between $3.58 \times 10^{-11}$ and $1.60 \times 10^{-10}$ $m^2/s$ for the P+P fraction. The temperature was found to have a significant influence on $D_e$ values: the higher the temperature, the higher the $D_e$. The fact that the $D_e$ figures found in the PI fraction are higher suggests that its structure has a low water retention capacity, which leads to a higher water removal rate than in the P+P fraction. This fact could be explained by taking the lower density of the PI fraction into account (Table 1) while the low $D_e$ values of the P+P fraction could be ascribed to the water proof capacity of peels, which constitutes a natural protection of olive fruit from dehydration . To our knowledge, there are no references in the literature to the $D_e$ in the PI and P+P fractions since this issue has not been previously addressed. However, the values identified in this work for both fractions are similar to others reported in literature for olive pomace drying in deep beds, thin layers or regular-shaped bodies. Thus, Meziane (2011) in working on fluidized bed drying (thickness 41-33 mm) at

50-80 °C, reported $D_e$ figures from 0.68 x $10^{-7}$ to 2.15 x $10^{-7}$ $m^2/s$, which were similar to those reported by Göğüs & Maskan (2006) for tray drying (thickness 6-12 mm) at 60-80 °C. However, lower $D_e$ figures have also been reported. Thus, in the case of tray drying (thickness 4-12 mm) at 80-110 °C, Doymaz et al. (2004) found values ranging from 4.89 x $10^{-10}$ to 9.89 x $10^{-10}$ $m^2/s$ while Montero et al. (2011) identified values ranging from 9.1 x $10^{-11}$ $m^2/s$ to 1.4 x $10^{-10}$ $m^2/s$ for solar drying at 20-50 °C (thickness 20-40 mm). The great differences found in the literature for the $D_e$ figures of olive pomace could be ascribed not only to the effect of the thickness of the sample being dried but also to the highly heterogeneous nature of this product.

The diffusion model proposed in this work fitted the experimental data closely, providing similar explained variance (VAR) and mean relative errors (MRE) to the Weibull model (Table 2). In overall terms, the VAR and MRE were close to 99 % and 10 %, respectively. The only significant difference between both models was found at 150 °C (Fig. 4), at which temperature the Weibull model fitted the drying kinetic much better than the diffusion (Table 2); the VAR was found to fall from 99.7 to 96.9 %.

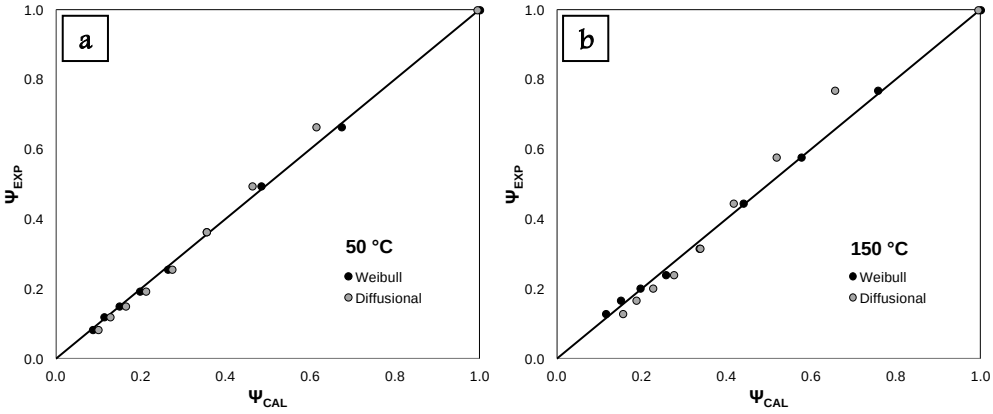


**Fig. 4.** Experimental and calculated dimensionless moisture content using Weibull and diffusion models. Experiments carried out at 50 (a) and 150 °C (b).

This could suggest that, at high temperatures, diffusion was less important and other significant mass transport phenomena appeared. In this sense, it was found that

the $\alpha$ Weibull parameter, related to product behavior, was not significantly ($p<0.05$) affected by temperature in the range of 50 to 120 °C, reaching an average value of 0.87 ± 0.02. However, the value identified at 150 °C (0.99) was significantly ($p<0.05$) higher. Therefore, although it could be stated that the behavior of olive pomace remained stable during drying over the temperature range of 50 to 120 °C, water removal phenomena seemed to change at 150 °C.

The drying temperature also affected the identified kinetic parameter ($\beta$) of the Weibull model. Thus, $1/\beta$ increased when the air drying temperature rose, showing that, over the range studied, the higher the temperature applied, the faster the drying. The influence of temperature on $D_e$ of the PI and P+P fractions and the $1/\beta$ parameter was well described from an Arrhenius-type relationship (Fig. 5) over the range of 50 to 120 °C. In every case, the value identified at 150 °C departed from the trend observed at the other temperatures, as can be seen in Fig. 5. So, excluding the kinetic data at 150 °C, the identified activation energies ($E_a$) from the $1/\beta$ Weibull parameter were 20.3 kJ/mol for olive pomace 21.9 kJ/mol for the P+P fraction and 14.6 kJ/mol for the PI fraction. The $E_a$ figure reported for olive pomace was in the same order of magnitude as that obtained by other authors. Thus, Meziane (2011) reported a value of 36.8 kJ/mol (50-80 °C), Göğüs & Maskan (2006) 25.7 kJ/mol (60-80 °C) and Doymaz et al. (2004) 26.71 kJ/mol (80-110 °C).

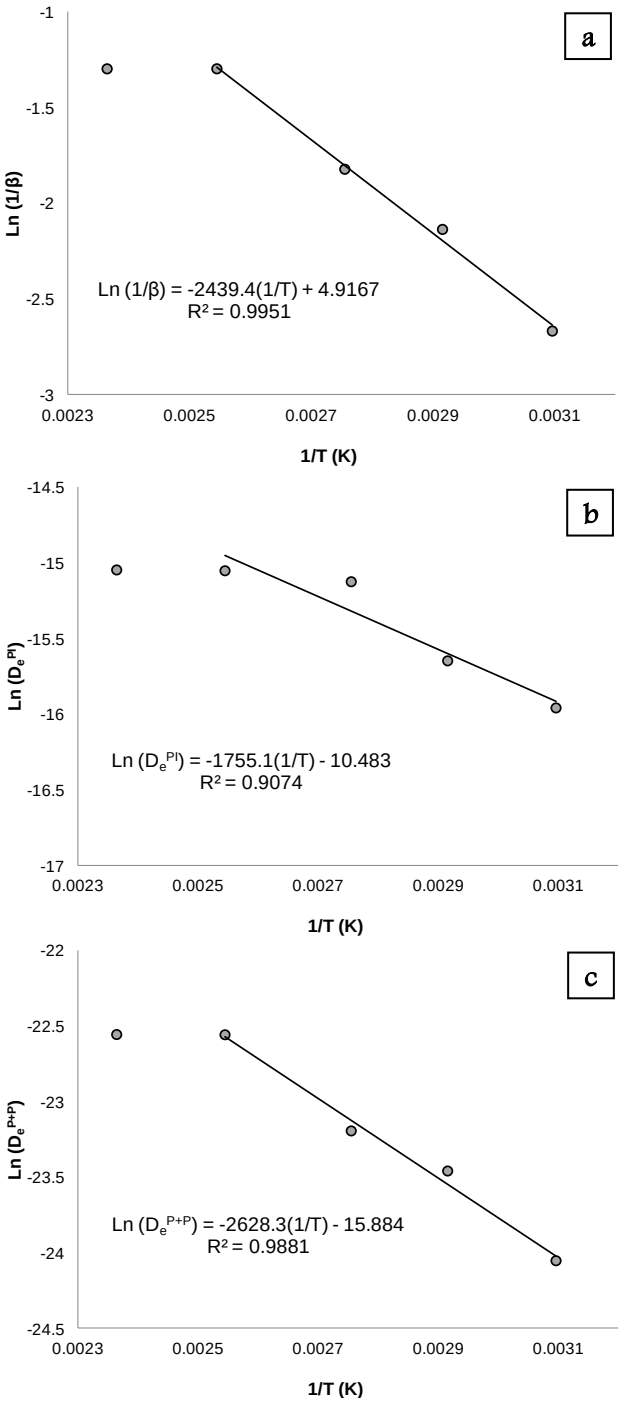


**Fig. 5.** Fit of an Arrhenius type equation to the kinetic parameter of the Weibull model ($1/\beta$, a) and effective moisture diffusivities for pit ($D_e^{PI}$, b) and pulp + peel ($D_e^{P+P}$, c) fractions.

## 3.2. Antioxidant potential affected by drying temperature

Olive pomace is susceptible to spoilage due to the fact that it presents a high level of enzymatic and microbial activity. This must be considered when it is used as a potential source of bioactive compounds. Drying stabilizes the raw material during storage and limits some degradative reactions but, in a certain way, it can influence the bioactive potential of olive pomace. Hence, the total phenolic content (TPC) and the antioxidant capacity (AC) were assessed in the extracts obtained from olive pomace dried at temperatures ranging between 50 and 150 °C and were compared with those obtained from fresh pomace.

As can be observed in Fig. 6, once the drying temperature exceeded 70 °C, it was noticeable that there was a slight tendency of the TPC to increase as the temperature rose. This could be attributed to the formation of new phenolic compounds at high temperatures (90-150 °C), due to the fact that non-enzymatic interconversion leads to the availability of precursors of phenolic molecules (Que et al., 2008). However, the statistical analysis highlighted the fact that the influence of temperature on TPC was not significant ($p<0.05$). Moreover, the dried material exhibited a similar TPC to that shown by fresh pomace. Different results have been found in literature when using similar biomaterials. Thus, Khanal et al. (2010) reported that drying temperatures over 60 °C had a negative effect on the phenolic content of grape and blueberry pomace.

As regards the AC of extracts, the drying temperature of pomace had a significant ($p<0.05$) effect on the antioxidant potential. Olive pomace dried at 150 °C provided the extracts with the highest AC (Fig. 6), it being 15.5 % higher than the one obtained from pomace dried at 50 °C. Furthermore, compared with fresh pomace, drying at 150 °C increased the AC of extracts by 12.8 %. Samples dried over the range of 50-120 °C did not exhibit significant ($p<0.05$) differences compared with the fresh product. Studying the effect of the drying air temperature on the antioxidant capacity of polyphenolic compounds in mulberry leaves, Katsube et al. (2009) also observed an increase in the antioxidant capacity when the air temperature rose from 70 to 110 °C.

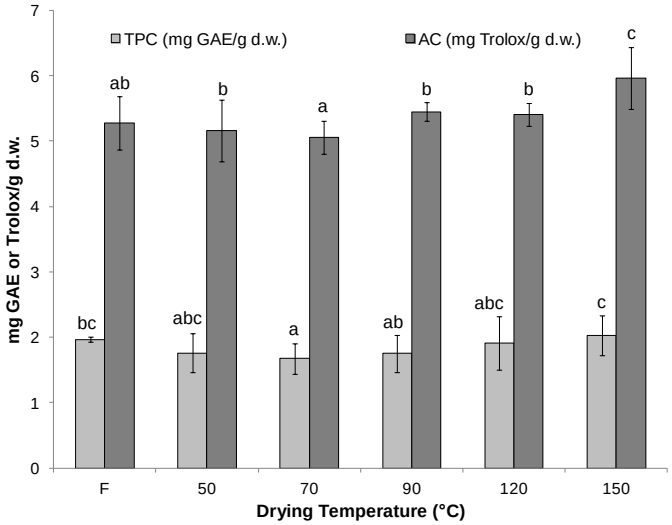


**Fig. 6.** Experimental TPC and AC (average ± standard deviation) of extracts of olive pomace dried at different temperatures. Superscript letters show homogeneous groups established from Least Significance Difference (LSD) intervals ($p<0.05$).

The effect of the drying conditions on the antioxidant properties of different byproducts and materials has been evaluated in several research studies. In overall terms, it can be stated that there is great controversy over the most suitable drying conditions. Thus, the use of mild drying temperatures (60 °C) and intermediate drying times is reported as the most suitable for orange peel (Garau et al., 2007) or mulberry (Katsube et al., 2009). On the contrary, Harbourne et al. (2009) found that, over the range of 30-70 °C, the drying air temperature did not influence the phenolic constituents of meadowsweet and willow. Other authors state that the use of high temperatures (90 °C) allows extracts to be obtained with a high antioxidant potential (Vega-Gálvez et al., 2009). These different conclusions concerning the effect of the drying temperature on bioactive properties could probably be ascribed to the different nature of the raw material processed.

In the case of the present study, the highest drying temperature tested, 150 °C, seemed to be the most suitable drying conditions under which to obtain the highest AC of the extracts. It should be remarked that it is not only the temperature but also the length of exposure to heat which can influence the extract properties (Erbay & Icier, 2009), since the short treatments at high temperatures may promote the

presence of bioactive compounds in the extracts (García-Pérez et al., 2010). However, since in a certain way drying also involves thermal treatment, the impact of the heating time during drying at high temperatures was further studied and the results are presented in the following section.

### 3.3. Antioxidant potential affected by drying time at high temperature

A new set of drying experiments at 150 °C was carried out varying the drying time of olive pomace from 5 to 60 min. It should be noticed that after approximately 8 min of drying, see section 3.1, samples could be considered dried (water activity less than 0.4). Thus, longer processing times are unnecessary for water removal and represent an additional overheating due to the product being overexposed to high air temperatures. Once processing finalized, the TPC and AC of the extracts obtained from these dried samples were measured.

Although phenolic compounds are considered as heat sensitive antioxidants (Erbay & Icier, 2009), the results showed that there was a significant ($p<0.05$) increase in the TPC as the drying time lengthened . As can be observed in Fig. 7, at a drying time of over 10 min the samples exhibited a significantly ($p<0.05$) higher TPC than the fresh material, and the longer the drying time, the higher the TPC of the extracts. In such a way, the highest TPC was obtained after a drying time of 30 min, representing a 39.9 % increase compared to what was observed in the fresh olive pomace extracts. The increase in the drying time, from 30 to 60 min, did not significantly ($p<0.05$) affect the TPC. Thereby, it is not advisable to heat olive pomace longer than 30 min, since this would reduce the productivity and increase both the processing costs and the energy consumption. As far as this aspect is concerned, the literature throws up contradictory results. It is widely recognized that polyphenols are heat labile, thus, it is reported that heat treatments cause irreversible chemical changes (Mejía-Meza et al., 2008). In this way, Kyi et al. (2005) highlighted the fact that, when cocoa beans were dried at temperatures over the 40-60 °C range, the concentration of total polyphenols declined drastically when the drying time was extended, additionally observing that the higher the temperature, the lower the residual amount of polyphenols. On the contrary, the positive influence that heating

has on the antioxidant capacity has also been observed in microwave treatments. Hence, Hayat et al. (2010) stated that, in mandarin pomace, the sum of the content of the individual phenolic acids in the free fraction significantly increased as the drying time lengthened.

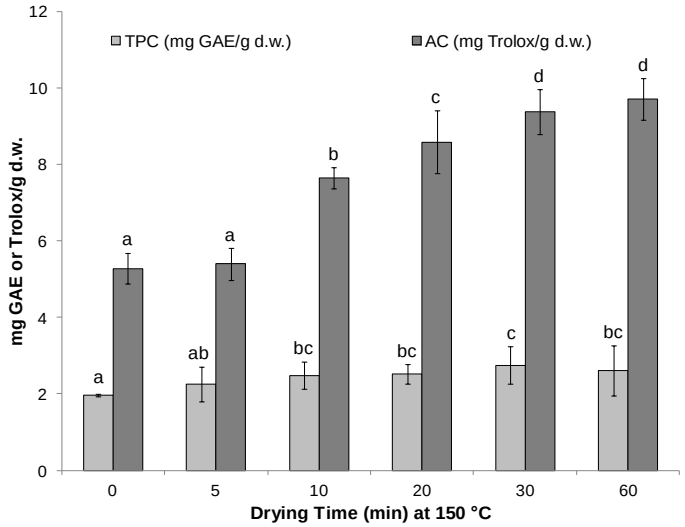


**Fig. 7.** Experimental TPC and AC (average ± standard deviation) of extracts of olive pomace dried at 150 °C for different times. Superscript letters show homogeneous groups established from Least Significance Difference (LSD) intervals ($p<0.05$).

As regards the AC measurements, overheating the olive pomace also significantly ($p<0.05$) increased the AC of extracts (Fig. 7). For drying times longer than 5 min, the extracts exhibited a significantly ($p<0.05$) higher AC than the extracts obtained from fresh olive pomace; from 5 min of drying onwards, the longer the overheating, the higher the AC. However, it is important to highlight that, as in TPC measurement, no significant ($p<0.05$) differences were found between extracts obtained from pomace treated for 30 and 60 min. Therefore, drying at 150 °C for 30 min seemed to be the best processing conditions under which to obtain the highest AC. It should be remarked upon that, under these conditions (150 °C and 30 min) and compared with the fresh product, AC increased almost twice as much (78 %) as TPC (40 %).

Vashisth et al. (2011) observed that the drying time had no influence on the antioxidant capacity of muscadine pomace at 70 and 80 °C. Considering the fact that

long drying times at low air temperatures (30-40 °C) promote a decrease in the antioxidant capacity (Garau et al., 2007) and in view of the results obtained in this work, it could be reasonable to consider that there is a temperature threshold from which point onwards the drying time increases the content of antioxidant compounds. This behavior could be explained by considering that high temperatures promote the inactivation of oxidative enzymes (Sanjuán et al., 2000), avoiding the degradation of antioxidants for later processing, which includes the extraction stage. Furthermore, at high temperatures, the generation and accumulation of Maillard-derived melanoidins with a varying degree of antioxidant activity could also enhance the antioxidant properties of extracts (Que et al., 2008).

In order to clarify the effect the overheating had on the increase in antioxidant phenolic compounds, the composition of the extracts was analyzed by HPLC-DAD/MS-MS. The HPLC-DAD profile of the samples was quite complex and a large variety of peaks were detected at UV wavelengths. Nevertheless, the fact that ionization occurred in only a few of them was probably due to the presence of organic polymers in pomace samples, which complicated the identification of the polyphenolic profile of the samples. Among the phenolic compounds identified, minor quantities of secoiridoids, such as oleuropein and ligstroside, were detected. The main polyphenol to be identified and quantified was luteolin, which was selected as a marker to be quantified in the different extracts obtained from samples subjected to different drying times. The drying treatment at 150 °C led to an increase in the luteolin concentration as compared to fresh material (Fig. 8); additionally, the longer the drying time, the higher the luteolin content. Thus, as shown in Fig. 8, increasing the drying time from 10 to 60 min led to a rise in the luteolin content of approximately 100 %. Therefore, these results highlighted the relationship between the previously observed enhancement of antioxidant potential and the increase in the content of some individual polyphenols, such as the flavone luteolin.

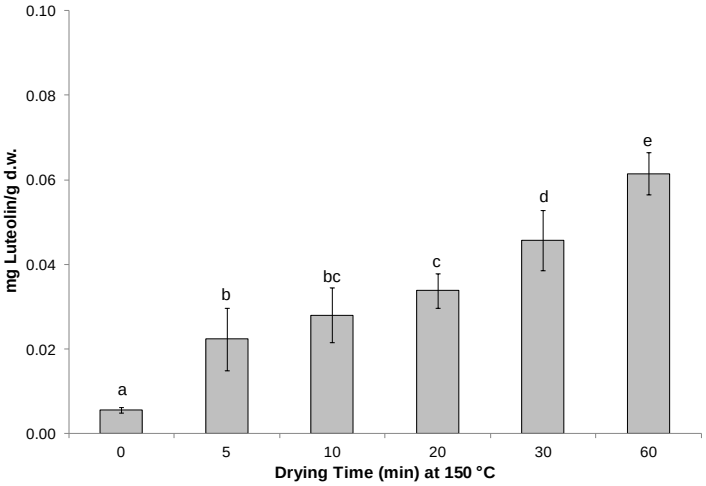


**Fig. 8.** Luteolin-7-O-glucoside content (average ± standard deviation) of extracts of olive pomace dried at 150 ºC for different times. Superscript letters show homogeneous groups established from Least Significance Difference (LSD) intervals ($p<0.05$).

## 3.4. Monitoring of the extraction process

In experiments carried out to assess how the drying temperature of olive pomace or the drying time at high temperatures affected the antioxidant potential of extracts, an extraction time of 24 h was considered enough to reach equilibrium conditions according to previous studies (Ahmad-Qasem et al., 2013a, 2013b). In order to test the feasibility of using shorter extraction times as a means of improving productivity, which could be relevant for industrial purposes, another set of experiments was performed using the fresh and dried pomace at the lowest (50 °C) and the highest (150 °C) temperatures tested. Thus, separate extraction experiments were conducted, varying the extraction time from 5 to 48 h, and replicated at least three times.

As observed in Fig. 9, most of the phenolic compounds were extracted from the solid matrix during the first 5 h of contact with the solvent. Afterwards, some slight variation of TPC was found, which was especially noticeable in the case of fresh material and olive pomace dried at 50 °C. Thus, increasing the extraction time from 5 to 48 h led to an observed rise in the TPC of fresh and dried pomace of 23.4 and 24.8 % at 50 °C, respectively. However, for the material dried at 150 °C, the

difference in the TPC at these extraction times was almost negligible. This fact highlights another noticeable benefit of drying at high temperatures (150 °C), which is the degradation of the raw structure which promotes a sharp rise in the TPC of the solvent. From experimental results, it is also evident that 24 h is a reasonable extraction time after which to evaluate the TPC of olive pomace, since equilibrium is reached. Nevertheless, if further industrial applications are considered, it seems reasonable to choose a short extraction time, like 5 h, in order to increase productivity, thereby generating large volumes of extracts with a high TPC after short treatment times.

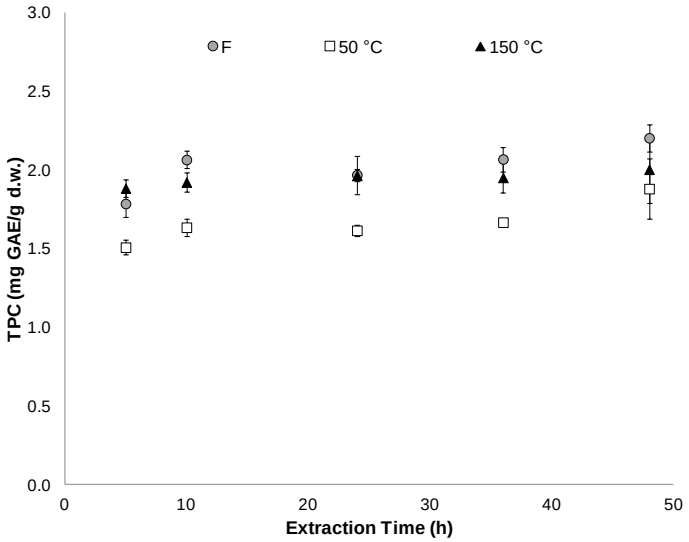


**Fig. 9.** TPC of extracts obtained from fresh and dried olive pomace (at 50 and 150 °C) at different extraction times. Average ± standard deviation.

## 4. Conclusions

A compositional diffusion model considering a different effective diffusivity in pit and pulp + peel fractions provided a good description of the drying behavior of olive pomace. Effective diffusivity for the pit fraction was higher than in that of the pulps + peels and increased as the air temperature rose. Although the influence of the drying temperature on the antioxidant potential was only mild, long drying times at

the highest temperature tested (150 °C) significantly increased the antioxidant potential.

Further studies should analyze the deep bed drying of olive pomace in order to validate the developed model and confirm the effect of temperature on the antioxidant potential of olive pomace.

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# CHAPTER 2

## *Phenolic extraction*

Innovative Food Science and Emerging Technologies,
2013, 17, 120–129.

# *Kinetic and compositional study of phenolic extraction from olive leaves (var. Serrana) by using power ultrasound*

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# Kinetic and compositional study of phenolic extraction from olive leaves (var. Serrana) by using power ultrasound

## ABSTRACT

Power ultrasound is being used as a novel technique for process intensification. In this study, the feasibility of using power ultrasound to improve the phenolic extraction from olive leaves was approached taking both compositional and kinetic issues into account and also determining the influence of the main process parameters (the electric power supplied, emitter surface and temperature). For this purpose, the extraction kinetics were monitored by measuring the total phenolic content and antioxidant capacity and mathematically described by Naik's model, and HPLC-DAD/MS-MS was used to identify and quantify the main polyphenols. The electric power supplied and the emitter surface greatly affected the effective ultrasonic power applied to the medium, and hence the extraction rate. However, the influence of temperature on ultrasound assisted extraction was not clear. Compared with conventional extraction, ultrasound assisted extraction reduced the extraction time from 24 h to 15 min and did not modify the extract composition.

***Keywords***: olive leaves, byproducts, antioxidant capacity, polyphenols, ultrasonics.

## 1. Introduction

Olive (*Olea europaea* L.) is one of the most important crops in the Mediterranean countries, one which has traditionally played an important role in human diet because of the high nutritional value of olive oil (Ryan et al., 2001). Olive fruit is rich in phenolic compounds with bioactive properties providing, among other things, antiviral, antitumoral and antioxidant activity (Della Ragione et al., 2000; Liu et al., 2003). Nowadays, the harvesting of olive fruit and the pruning of olive trees generate an important number of byproducts, such as branches and leaves, both mainly used as animal feed or to be removed by burning. However, bioactive compounds have been found in these byproducts (Japón-Luján & Luque de Castro, 2007) which exhibit similar antioxidant potential to those found in olive fruit (Malik & Bradford, 2006). Therefore, the extraction of phenolic compounds could represent an interesting means of increasing the value of these byproducts (Guinda et al., 2004; Tabera et al., 2004).

The conventional extraction of bioactive compounds from plants or seeds has been carried out by maceration using liquid solvents, which is considered a slow process requiring long extraction times. The extraction rate may be improved by choosing the best combination of process variables, such as the type of solvent or level of agitation (Rodríguez-Bernaldo de Quirós et al., 2010). Using high temperatures does lead to a kinetic improvement, but it is limited by the fact that polyphenols are sensitive to high temperatures. Thus, although heat treatments can improve extraction kinetics, they reduce both the phenolic content and antioxidant capacity. Recent studies into future industrial applications have addressed some alternatives to conventional extraction, such as supercritical extraction with $CO_2$ (Bensebia et al., 2009), ultrasound assisted (Knorr et al., 2004; Zhang et al., 2009), microwave-assisted (Hayat et al., 2009) or superheated liquid extraction (Japón-Luján & Luque de Castro, 2006).

Ultrasound assisted extraction is considered one of the most interesting techniques by which to intensify the extraction of valuable compounds from vegetal materials (Vilkhu et al., 2008). This is due to the fact that it is not only a simple,

efficient and inexpensive alternative to conventional extraction procedures (Huang et al., 2009), but it also induces mechanical effects in the medium being applied. In liquids, ultrasound enhances mass transfer mainly by inducing cavitation. The implosion of gas bubbles in liquid generates high localized pressures and micro-streaming, causing plant tissue disruption and improving the release of intracellular substances into the solvent (Knorr et al., 2002). Ultrasound also produces other effects coupled to cavitation, like interfacial instabilities and successive compressions and expansions that can influence both external and internal mass transfer. Two common ultrasonic devices are employed in solid/liquid extraction, namely baths and probe-type systems. Although ultrasound baths are more widely used, probe-type systems offer the advantage of providing more intense and localized ultrasonic application, which heightens the effects in solid-liquid systems (Priego-Capote & Luque de Castro, 2004). In addition, probes allow a wider choice of process parameters than ultrasonic baths, which is highly interesting for research purposes. The effectiveness of ultrasound application is directly related to the ability of the ultrasonic probe to introduce energy into the solvent medium. This fact mainly depends on how well the emitter surface fits the solvent medium and product being treated, which is extremely complicated to predict, and therefore should be determined in each specific application. Other process parameters, such as electric amplitude supplied to the ultrasonic transducer, sonication time, temperature, solvent composition (Herrera & Luque de Castro, 2005) or number of extraction steps (Jerman et al., 2010) could also affect the ultrasound assisted extraction process. Ultrasound assisted extraction from olive leaves has previously been reported by Japón-Luján et al. (2006) and Sánchez-Ávila et al. (2007), who for analytical purposes studied, optimized and characterized the extract composition using different process parameters (Esclápez et al., 2011). However, the compositional study should be accompanied by a thorough analysis of the kinetics taking into account the effective power applied to the medium, a fact which is not included in previous research and which is highly relevant for industrial applications. Thereby, the aim of this work was to address the power ultrasound assisted extraction of olive leaf bioactive compounds by evaluating the influence of some process parameters (the

electric amplitude, the emitter surface and temperature) on both the extraction kinetics and the extract composition.

## 2. Materials and methods

### 2.1. Raw material

Olive leaves (*O. europaea*, var. Serrana) were collected on a farm located in Segorbe (Castellón, Spain) in February (approximately 2 months after the fruit harvest), packaged, stored at 4 °C and processed in less than 48 h. The initial moisture content was determined by drying until constant weight in a vacuum chamber at 70 °C (AOAC, 1997).

### 2.2. Drying experiments

The olive leaves, with an initial moisture content of 39.2 ± 0.9 % (kg water/kg total), were dried at 120 °C in a forced air laboratory drier (FD, Binder, Tuttlingen, Germany) according to Ahmad-Qasem et al. (2013). Samples were dried until constant weight, which corresponded to a loss of 40 ± 1 % of the initial weight. After drying, the olive leaves were stored at 4 °C until subjected to extraction.

### 2.3. Extraction experiments

#### 2.3.1. Olive leaf sample preparation

In order to perform the extraction experiments, dried olive leaves were milled (Blixer 2, Robot Coupe USA, Inc., Jackson, MS, USA). The obtained powder was sieved (Metallic mesh 0.05 mm, Filtra Vibración, Barcelona, Spain) to select particles with a diameter of less than 0.05 mm and a density of 426.2 kg/m$^3$. Thus, using this small particle diameter, it was possible to increase the active surface area of the olive leaf sample.

### 2.3.2. Extraction solution and extract preparation

The solvent (extracting medium) used was an 80:20 (v/v) ethanol-water solution. The extracts obtained were centrifuged for 10 min at 5000 rpm (Medifriger BL-S, J.P. Selecta, Barcelona, Spain), filtered (nylon filters of 0.45 μm) and stored in opaque vials at 4 °C until analyzed. The extraction kinetic was monitored in both ultrasound assisted extraction experiments as well as in conventional solid-liquid maceration. Both extraction methods are described in the following sections.

### 2.3.3. Ultrasound assisted extraction (USAE)

#### 2.3.3.1. Experimental set-up and characterization of ultrasonic field

The experimental set-up used to carry out the ultrasonic assisted extraction experiments is shown in Fig. 1. During the experiments, the temperature was held constant and measured with a Pt100 sensor located in the centre of the extraction vessel and wired to a process controller (E5CK, Omron, Hoofddorp, The Netherlands). A peristaltic pump (302 S, Watson-Marlow, Postfach, Germany), driven by the controller, recirculated a glycol solution (10 % glycol) at -10 °C from the cooling reservoir, equipped with a chiller (Frigedor, J.P. Selecta, Barcelona, Spain), through a jacketed extraction vessel. Ultrasound was continuously applied (cycle 100 %) using a probe system (UP400S, Dr. Hielscher, Teltow, Germany), which allows the tip probe to be changed, thus being able to test different emitter surfaces. The ultrasonic emitter was immersed 1 cm into the solution. In order both to avoid the negative effect of light on phenolic compounds and to preserve the original composition of extracts, the extraction vessel was protected from light in every experiment.

A calorimetric procedure was used to determine the effective ultrasonic power transferred into the medium for every condition tested (Raso et al., 1999). For this purpose, the temperature of the solvent was logged every 3 s for the first 3 min of ultrasound application without controlling the temperature. Thus, using the temperature rise caused by cavitation, the ultrasonic power applied ($P$, W) was calculated as:

$$P = M \cdot C_p \left( \frac{dT}{dt} \right) \quad (1)$$

where $M$ (kg) is the solvent mass, $C_p$ (J/kg °C) the heat capacity and $dT/dt$ the slope of the logged temperature-time curve. The ultrasonic power was measured, at least in triplicate, for every condition tested.

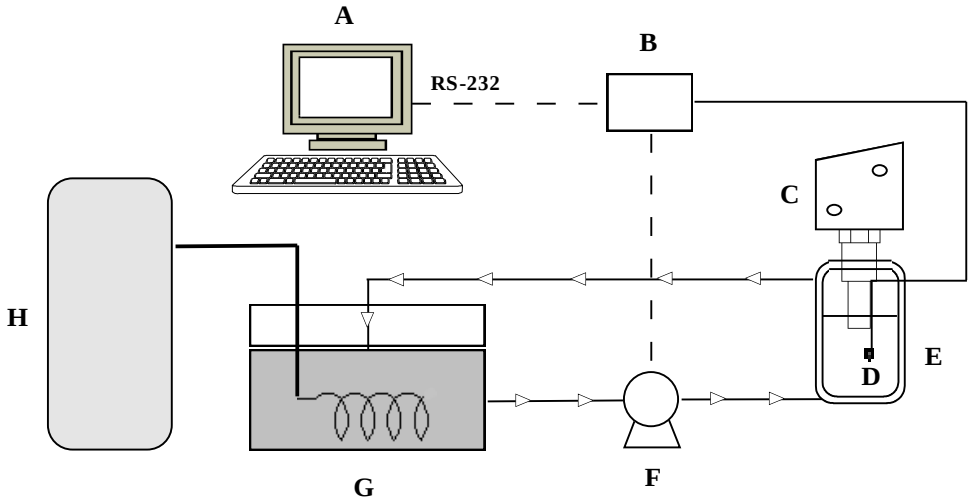


**Fig. 1.** Experimental set-up for ultrasonic assisted extraction of olive leaf phenolic compounds. A: Computer; B: Process controller; C: Ultrasonic probe system; D: Temperature sensor (Pt100); E: Jacketed extraction vessel; F: Peristaltic pump; G: Glycol reservoir; H: Chiller.

### 2.3.3.2. Parametric study

A parametric study was performed in order to identify the influence of process variables in the ultrasonic assisted extraction. The parameters taken into account were the electric power supplied to the ultrasonic transducer, the emitter surface and the extraction temperature. The first two parameters affect the ultrasonic intensity applied to the medium that could produce a different extension of ultrasound effects, while the extraction temperature could have an effect on both the extraction kinetic and final yield.

A first set of experiments was carried out supplying different levels of electric power to the transducer (40, 60, 80 and 100 % of the total power of the system,

400 W) using an emitter surface of 12.6 $cm^2$. Afterwards, using the electric power which provided the extracts with the highest antioxidant capacity, the influence of the emitter surface (12.6, 3.8 and 1.5 $cm^2$) on the extraction yield was evaluated in a second set of experiments. Both extraction tests were carried out at 25 °C for 15 min. Finally, a third set of experiments was carried out for 15 min at 6 different extraction temperatures (25, 30, 35, 40, 45 and 50 °C). In this case, the electric power supplied and the emitter surface were fixed by the first two experiments.

Each extraction experiment was carried out using a ratio of olive leaf mass to solvent volume of 6.25 g/200 mL (0.031 g/mL). In order to determine the extraction kinetics, the samples were taken (2 mL) at preset times (0, 3, 6, 9, 12 and 15 min) replacing the extract volume with new solvent. At least 3 replicates were made for each extraction condition tested.

### 2.3.4. Conventional extraction

In order to determine conventional extraction kinetics, experiments were carried out without (static extraction, ST) and with agitation (CVE) at 170 rpm in a thermostatic shaking water bath (Stuart, Staffordshire, UK). From previous experiments, it was stated that this level of agitation was enough to maintain a high degree of turbulence in the medium. The same ratio between olive leaf mass and solvent volume (0.031 g/mL) was used as in section 2.3.3.2. In addition, kinetics were also monitored by taking samples (2 mL) at preset times (0, 3, 6, 9, 12 and 15 min) and replacing the extract volume with new solvent.

Moreover, additional conventional extraction experiments were carried out using the ratio of olive leaf mass to solvent volume (0.125 g/mL) proposed as optimum by other authors (Japón-Luján & Luque de Castro, 2006; Sánchez-Ávila et al., 2009). These experiments were prolonged until equilibrium was reached, which needed nearly 24 h. During extraction, the samples were also stirred at 170 rpm using the thermostatic shaking water bath. In this case, the extraction kinetic was not evaluated and only the final extract (24 h) was analyzed.

Every conventional extraction test was carried out at 25 ± 1 °C in sealed containers protected from light. At least, 3 extraction replicates were made for each extraction condition.

## 2.4. Quality evaluation of olive leaf extracts

### 2.4.1. Total phenolic content (TPC)

The TPC was determined by the Folin-Ciocalteu method (Singleton et al., 1999). Briefly, 100 μL of sample were mixed with 200 μL of Folin-Ciocalteu's phenol reagent (Sigma-Aldrich, Madrid, Spain) and 2 mL of distilled water. After 3 min at 25 °C, 1 mL of $Na_2CO_3$ (Panreac, Barcelona, Spain) solution ($Na_2CO_3$-water 20:80, w/v) was added to the mixture. The reaction was kept in dark at room temperature for 1 h. Finally, absorbance was read at 765 nm using a spectrophotometer (Helios Gamma, Thermo Spectronic, Cambridge, UK). Measurements were taken at least in triplicate. A standard curve of gallic acid (Sigma-Aldrich, Madrid, Spain) was previously prepared using solutions of a known concentration in ethanol-water (80:20, v/v) solution. Results were expressed as mg gallic acid (GAE)/g of dry weight of olive leaves.

### 2.4.2. Antioxidant capacity (AC)

The AC was determined by the Ferric-reducing ability power method (FRAP) in order to monitor the extraction kinetics. Moreover, the Trolox equivalent antioxidant capacity (TEAC) method was also used to compare the quality of USAE and CVE extracts.

#### 2.4.2.1. Ferric-reducing ability power (FRAP)

The FRAP method was applied following the procedure described by Benzie & Strain (1996), with some modifications. Briefly, 900 μL of FRAP reagent were used; this had been freshly prepared and heated to 37 °C and mixed with 30 μL of distilled water and 30 μL of test sample or ethanol-water (80:20, v/v) used as an appropriate reagent blank. The FRAP reagent contained 2.5 mL of a 10 mM TPTZ (Fluka,

Steinheim, Germany) solution in 40 mM HCl (Panreac, Barcelona, Spain) plus 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ (Panreac, Barcelona, Spain) and 2.5 mL of 0.3 M acetate buffer (Panreac, Barcelona, Spain), pH 3.6 (Pulido et al., 2000). Readings at the maximum absorption level (595 nm) were taken using a spectrophotometer (Helios Gamma, Thermo Spectronic, Cambridge, UK). At least 4 replicates were made for each measurement. The AC was evaluated through a calibration curve that had been previously determined using the extracting solvent (ethanol-water 80:20, v/v) of a known Trolox (Sigma-Aldrich, Madrid, Spain) concentration and expressed as mg Trolox/g dry matter.

### 2.4.2.2. Trolox equivalent antioxidant capacity (TEAC)

The TEAC method was performed as previously described by Laporta et al. (2007). Briefly, an ABTS radical cation ($ABTS^{+\bullet}$) was produced by reacting ABTS (Sigma-Aldrich, Europe) stock solution with 2.45 mM potassium persulfate (final concentration) and keeping the mixture in the dark at room temperature for 12-24 h before use. The $ABTS^{+\bullet}$ solution was diluted with distilled water until an absorbance value of 0.714 ± 0.02 at 734 nm was reached. For the photometric assay, an absorbance of 200 µL of the $ABTS^{+\bullet}$ solution, or blank, was measured in a spectrophotometer (Spectrostar Omega, BMG Labtech, Offenburg, Germany). Then 20 µL of antioxidant extract, or blank, were added and, after 29 min, the final absorbance was measured at 734 nm (Spectrostar Omega, BMG Labtech, Offenburg, Germany). The AC was determined from the difference between the initial and final absorbance and the calibration curve of Trolox (Sigma-Aldrich, Madrid, Spain). At least 3 replicates were made for each extract. The AC results were expressed as mg Trolox/g dry matter.

## 2.4.3. Identification and quantification of polyphenols by HPLC-DAD/MS-MS

In order to identify and quantify the main polyphenols present in the USAE and CVE extracts, these were analyzed using a HPLC instrument (Agilent LC 1100 series; Agilent Technologies, Inc., Palo Alto, CA, USA) controlled by the Chemstation software. The HPLC instrument was coupled to an Esquire 3000+ (Bruker Daltonics,

GmbH, Bremen, Germany) mass spectrometer equipped with an ESI source and ion-trap mass analyzer, and controlled by Esquire control and data analysis software. A Merck Lichrospher 100RP-18 (5 μm, 250 x 4 mm) column was used for analytical purposes.

Separation was carried out through a linear gradient method using 2.5 % acetic acid (A) and acetonitrile (B), starting the sequence with 10 % B and programming gradient to obtain 20 % B at 10 min, 40 % B at 35 min, 100 % B at 40 min, 100 % B at 45 min, 10 % B at 46 min and 10 % B at 50 min. In order to ensure the LC-MS pump performed accurately, 10 % of organic solvent was premixed in the water phase. The flow-rate was 1 mL/min and the chromatograms were monitored at 240, 280 and 330 nm. The mass spectrometry operating conditions were optimized in order to achieve maximum sensitivity values. The ESI source was operated in negative mode to generate $[M-H]^-$ ions under the following conditions: a desolvation temperature of 365 °C and a vaporizer temperature of 400 °C; dry gas (nitrogen) and nebulizer were set at 12 L/min and 70 psi, respectively. The MS data were acquired as full scan mass spectra at 50–1100 m/z by using 200 ms for the collection of the ions in the trap.

The main compounds were identified by means of a HPLC-DAD analysis, comparing the retention time, UV spectra and MS/MS data of the peaks in the samples with those of authentic standards or data reported in literature.

Only the main olive leaf polyphenols were quantified using commercial standards: oleuropein (Extrasynthese, Genay Cedex, France) and luteolin-7-O-glucosyde (Phytolab, Vestenbergsgreuth, Germany). A purified verbascoside standard (96.85 %), obtained from Universidad Miguel Hernández (Elche, Spain), was used for quantification. The quantitative evaluation of compounds was performed with a calibration curve for each polyphenol, using ethanolic (oleuropein) or methanolic (verbascoside and luteolin) solutions of known concentrations. USAE and CVE extracts were analyzed at least in triplicate and results were expressed as mg polyphenol/g dry matter.

## 2.5. Modeling of extraction kinetics and statistical analysis

The monitoring of the total phenolic content (TPC) and antioxidant capacity (AC) of extracts during extraction allowed the extraction kinetics to be evaluated. The Naik model was used to mathematically describe the extraction kinetics (Naik et al., 1989):

$$Y = \frac{Y_\infty \cdot t}{B + t} \quad (2)$$

where $Y$ represents the extraction yield (TPC or AC) (mg gallic acid (GAE) or mg Trolox/g dry matter of olive leaves), $t$ (min) the extraction time, $Y_\infty$ the extraction yield at equilibrium and $B$ (min) the extraction time needed to reach half of $Y_\infty$. The Excel™ Solver tool (Microsoft Corporation, Seattle, WA, USA) was used to identify the model parameters ($Y_\infty$ and $B$) that minimized the sum of the squared differences between the experimental and calculated $Y$. The explained variance (VAR) was used to determine the goodness of the model fit to the experimental data:

$$\mathrm{VAR} = 1 - \frac{S_{xy}^2}{S_y^2} \quad (3)$$

where $S^2_{xy}$ is the variance of the estimation and $S^2_y$ the variance of the sample. Moreover, the mean relative error (MRE) was calculated to establish the difference between the experimental ($Y_{EXPi}$) and calculated ($Y_{CALi}$) data:

$$\mathrm{MRE} = \frac{100}{N}\left(\sum_{i=1}^{N} \frac{Y_{\mathrm{EXPi}} - Y_{CALi}}{Y_{\mathrm{EXPi}}}\right) \quad (4)$$

where $N$ is the number of experimental data.

Analysis of Variance (ANOVA) was performed using Statgraphics® Centurion XV (Statpoint Technologies Inc., Warrenton, VA, USA) in order to identify significant ($p<0.05$) differences among the extracts, while the Fisher's Least Significant Difference (LSD) intervals were used for comparison of means.

# 3. Results and discussion

## 3.1. Ultrasonic assisted extraction (USAE)

USAE was addressed in depth in order to estimate how the process parameters affect the ultrasonic field intensity and to identify an adequate combination of parameters with which to improve antioxidant extraction from olive leaves. First of all, the ultrasonic field was characterized as a means of establishing the energy applied to the medium by different emitters and electric powers. Moreover, a parametric study was carried out into the main process parameters that affect the ultrasound application.

### 3.1.1. Ultrasonic field characterization

The intensity reached in the ultrasonic field during the different tests was measured by means of calorimetry, as was explained in section 2.3.3.1. Thus, it was possible to assess the effective power transferred by the transducer into the medium (ethanol-water 80:20, v/v) and choose the proper combination of electric power supplied to the transducer and emitter surface. From experimental results, it was observed that the greater the supply of electric power to the transducer, the more the ultrasonic power applied to the medium (Table 1). This relationship was linear for all the emitters tested.

**Table 1.** Ultrasonic power (W) applied to the medium as a function of the percentage of the total electric power (400 W) supplied to the ultrasonic transducer and the emitter surface of the probe tip.

| Tip diameter (cm) | Emitter surface ($cm^2$) | Electric power supplied to transducer | | | |
|---|---|---|---|---|---|
| | | 40 % | 60 % | 80 % | 100 % |
| 4.0 | 12.6 | 12.6 ± 0.3 | 18.5 ± 0.5 | 23.7 ± 0.3 | 28.4 ± 0.6 |
| 2.2 | 3.8 | 24 ± 2 | 32.4 ± 0.2 | 41.75 ± 1.13 | 51.47 ± 1.13 |
| 1.4 | 1.5 | 11.85 ± 0.17 | 16.9 ± 0.6 | 27.6 ± 1.5 | 33.3 ± 0.5 |

The emitter surface also had a significant ($p<0.05$) influence on the ultrasonic power applied to the medium. For every level of electric power supplied to the

transducer, the ultrasonic power achieved by the 3.8 $cm^2$ emitter (intermediate surface) was nearly double that reached when using other emitters (12.6 and 1.5 $cm^2$). Therefore, this emitter achieved the best coupling between the ultrasonic probe and the medium and led to the maximum figure of the effective ultrasonic power 51.47 W (100 % of the electric power and emitter surface of 3.8 $cm^2$). In this case, it should be remarked that the yield electric/ultrasonic was only of approximately 13 % (51 W/400 W), which indicates that the energy conversion degree was low and there exists a wide range for the improvement of the ultrasonic devices.

### 3.1.2. Parametric study

#### 3.1.2.1. Electric power supplied

First of all, the effect of the electric power supplied to the transducer was monitored in olive leaf extraction kinetics by taking TPC and AC measurements. Different percentages of electric power, from 40 to 100 % of the total, were tested using an ultrasonic probe with a 12.6 $cm^2$ emitter. Thus, as is shown in Table 1, the effective ultrasonic power applied ranged from 12.6 to 28.4 W.

The extraction kinetics are shown in Fig. 2 for the different experimental conditions. As can be observed, the more the electric power supplied, the higher the TPC or AC of the extract. Thereby, the best results were obtained supplying 100 % of the total electric power to the ultrasound transducer, which corresponded with the highest ultrasonic power applied (28.4 ± 0.6 W) to the medium (Table 1). Since the acoustic energy transmitted into the medium is directly related to the extension of the ultrasonic effects, the more the ultrasonic power applied, the greater the cavitation intensity. Cavitation makes it easier for the solvent to penetrate into the matrix and eases interface transport (Luque de Castro & Priego-Capote, 2006), increasing the extraction efficiency of antioxidant compounds present in the sample (Dash et al., 2005).

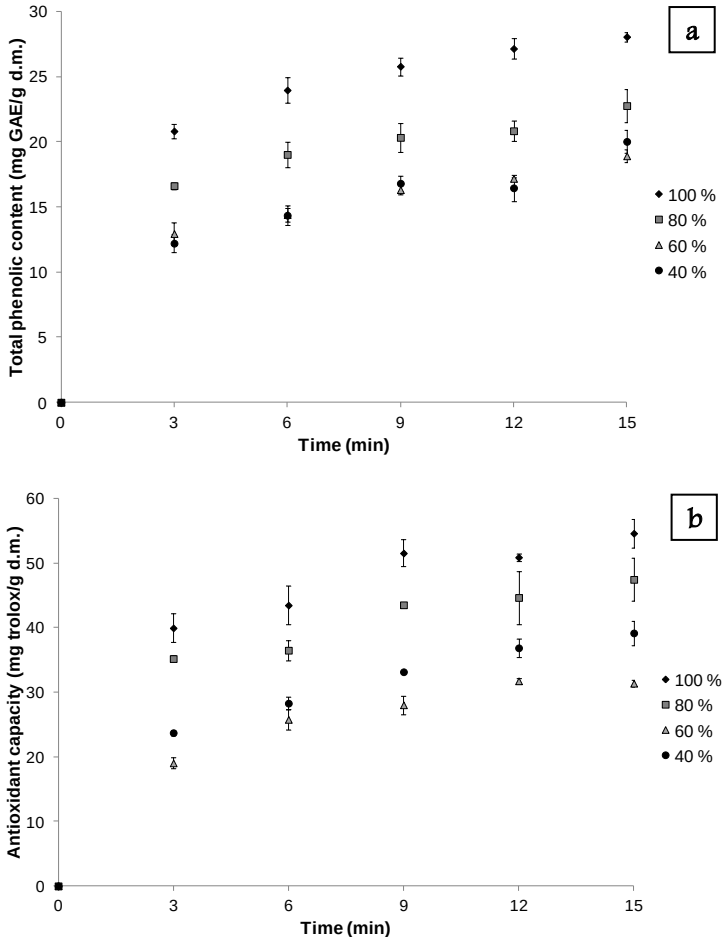


**Fig. 2.** Evolution of the total phenolic content (a) and antioxidant capacity (b; FRAP) of olive leaf extracts obtained by applying ultrasound at different electric powers supplied to the transducer (emitter surface 12.6 $cm^2$ and 25 °C extraction temperature).

The statistical analysis confirmed that the electric power applied only had a significant influence ($p<0.05$) on the final extracts, those obtained after 15 min of extraction, when it was above a certain threshold, which was 18.5 ± 0.5 W (60 % electric power) for TPC and 23.7 ± 0.3 W (80 % electric power) for AC. No influence of the ultrasound application was observed when less power was applied. These results agree with the ones reported by Cárcel et al. (2007a, 2007b), who also found that the ultrasound effect on mass transfer during the osmotic treatment of apple was only significant ($p<0.05$) when the ultrasonic power applied was above 10.8 $W/cm^2$

(Cárcel et al., 2007a) and 50 W/cm$^2$ during meat brining (Cárcel et al., 2007b). However, another study into the ultrasound assisted extraction of the triterpenic fraction of olive leaves concluded that irradiation power was not a significant ($p<0.05$) factor within the range under study (10-50 % electric power, 450 W) (Sánchez-Ávila et al., 2007). It is likely that in this case, the ultrasonic power range applied was too low, which prevented any significant differences from being observed.

Naik's model was used to quantify the influence of the ultrasonic power applied on the evolution of TFC and AC of olive leaf extracts during extraction process (Table 2). The model provided a close fit of experimental kinetics: the percentage of explained variance (VAR) was over 92 % and the mean relative error (MRE) lower than 9 %. The TPC and AC of extracts at equilibrium ($Y_\infty$) increased as the level of ultrasonic power applied rose, until reaching the maximum level for the highest ultrasonic power tested (28.4 ± 0.6 W, 100 % electric power). As far as the initial extraction rate is concerned ($R_0$), it also increased as the level of power applied went up in both the TPC and AC. Therefore, ultrasound quickened the extraction process, which allowed the final TPC and AC of the extracts to increase, the effect being dependent on the electric power applied. Thereby, the highest electric power (100 %) was chosen to evaluate the influence of other process variables, such as the emitter surface of the ultrasonic probe and the temperature.

**Table 2.** Identified parameters of Naik's model. Influence of process parameters on the total phenolic content and antioxidant capacity (FRAP) of olive leaf extracts.

| Extraction variables | | Total phenolic content | | | | |
|---|---|---|---|---|---|---|
| | | $Y_\infty$ (mg GAE/g d. m.)[a] | $B$ (min)[b] | $R_O$[c] | VAR (%)[d] | MRE (%)[e] |
| Electric Power (%) | 40 | 21.6 | 2.6 | 8.2 | 95.3 | 6.3 |
| | 60 | 21.9 | 2.3 | 9.5 | 95.4 | 6.3 |
| | 80 | 23.0 | 1.2 | 19.6 | 97.2 | 4.6 |
| | 100 | 29.1 | 1.2 | 24.1 | 98.1 | 3.4 |
| Emitter surface ($cm^2$) | 1.5 | 27.0 | 0.4 | 64.5 | 97.9 | 3.9 |
| | 3.8 | 40.4 | 1.1 | 36.8 | 99.0 | 2.7 |
| | 12.6 | 29.1 | 1.2 | 24.1 | 98.1 | 3.4 |
| Temperature (°C) | 25 | 40.4 | 1.1 | 36.8 | 99.0 | 2.7 |
| | 30 | 40.5 | 1.3 | 30 | 99.4 | 2.2 |
| | 35 | 39.1 | 0.8 | 46.6 | 95.6 | 4.9 |
| | 40 | 42.2 | 1.0 | 41.6 | 99.2 | 2.5 |
| | 45 | 45.8 | 1.1 | 43.2 | 99.1 | 2.6 |
| | 50 | 43.4 | 1.6 | 26.5 | 96.0 | 5.9 |
| **Extraction variables** | | **Antioxidant capacity (FRAP)** | | | | |
| | | $Y_\infty$ (mg trolox/g d. m.)[a] | $B$ (min)[b] | $R_O$[c] | VAR (%)[d] | MRE (%)[e] |
| Electric Power (%) | 40 | 43.4 | 2.7 | 15.8 | 96.9 | 4.8 |
| | 60 | 41.1 | 3.0 | 13.8 | 92.8 | 8.7 |
| | 80 | 50.7 | 1.7 | 30.0 | 96.9 | 5.3 |
| | 100 | 57.2 | 1.7 | 33.7 | 96.2 | 5.7 |
| Emitter surface ($cm^2$) | 1.5 | 49.9 | 0.2 | 318.0 | 99.5 | 1.8 |
| | 3.8 | 73.2 | 0.8 | 95.8 | 95.8 | 6.2 |
| | 12.6 | 57.2 | 1.7 | 33.7 | 96.2 | 5.7 |
| Temperature (°C) | 25 | 73.2 | 0.8 | 95.8 | 95.8 | 6.2 |
| | 30 | 77.0 | 1.6 | 48.9 | 97.6 | 4.2 |
| | 35 | 83.2 | 1.2 | 68.3 | 97.3 | 4.2 |
| | 40 | 84.2 | 1.2 | 67.8 | 98.4 | 3.3 |
| | 45 | 89.2 | 1.4 | 63.1 | 95.9 | 5.7 |
| | 50 | 81.7 | 1.2 | 66.0 | 94.6 | 6.0 |

[a] $Y_\infty$ represents the extraction yield at equilibrium as mg of gallic acid (GAE) or mg of trolox per g of dry mass of olive leaves.

[b] $B$ determines the extraction time needed to reach half of $Y_\infty$.

[c] $R_O$ shows the relation $Y_\infty/B$.

[d] VAR is the explained variance.

[e] MRE is the mean relative error.

### 3.1.2.2. Emitter surface

Experiments were carried out using 100 % of the total electric power supplied to the ultrasonic transducer and varying the ultrasonic emitter surface (1.5, 3.8 and 12.6 $cm^2$). This variable was evaluated since the ultrasonic probe used in this work allowed the use of different emitters by changing the probe tip.

Experimental results showed that the intermediate emitter surface tested (3.8 $cm^2$) provided higher TPC and AC in the extracts than the smaller (1.5 $cm^2$) or larger (12.6 $cm^2$) emitter surfaces (Fig. 3).

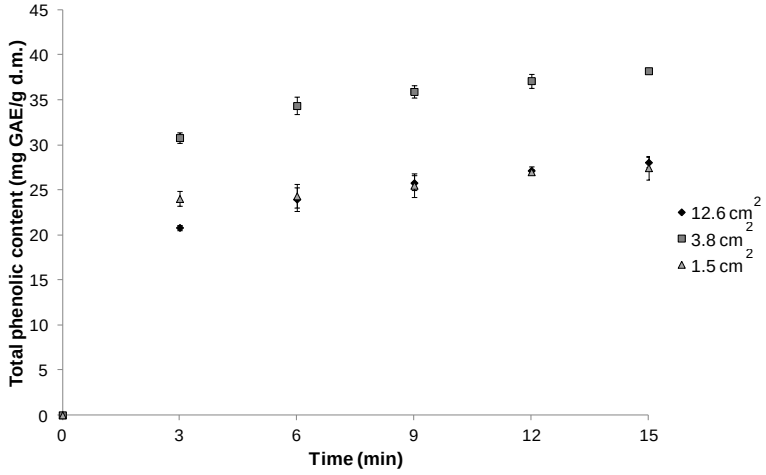


**Fig. 3.** Influence of transducer emitter surface on the evolution of the total phenolic content of olive leaf extracts obtained by ultrasound assisted extraction (100 % of the electric power supplied to the transducer and 25 °C extraction temperature).

This fact could be explained from the measurement of the effective acoustic power applied (Table 1). While probes of 1.5 and 12.6 $cm^2$ provided a power applied of 33.3 ± 0.5 and 28.4 ± 0.6 W, respectively, the emitter of 3.8 $cm^2$ increased the ultrasonic power transferred into the medium up to 51.47 ± 1.13 W (Table 1). The smallest emitter surface (1.5 $cm^2$) greatly concentrates the ultrasound energy, producing an intense cavitation but only in a very limited zone located around the tip, resulting in a non-homogeneous application in the medium. On the other hand, using the largest surface tip (12.6 $cm^2$) led to a more homogenous treatment but decreased the intensity of the ultrasonic power. Therefore, the best coupling between

the application system (probe) and the volume treated of the extraction medium was achieved with the intermediate emitter surface (3.8 $cm^2$), which was able to introduce the highest energy level per volume treated.

Modeling supported the previous results regarding the adequacy of the intermediate emitter surface, which provided the highest equilibrium of TPC and AC. Moreover, in the experiments carried out with the smallest emitter (1.5 $cm^2$), a high value of the initial extraction rate ($R_0$) was found. This fact could be linked to the snapshot cavitation generated by the intense cavitation of this emitter in a very limited volume.

### 3.1.2.3. Extraction temperature

Temperature could have an influence on ultrasound application since high temperatures can decrease surface tension, increase the vapor pressure and produce less cavitation energy conversion. In addition, it could also affect extraction composition since some bioactive compounds may be sensitive to heat exposure. Thereby, the extraction temperature is an important variable to be considered. In this work, the influence of temperature was studied in the range of 25 to 50 °C, by carrying out a set of experiments applying 100 % of the electric power and using a 3.8 $cm^2$ emitter surface, which allowed 51.47 ± 1.13 W to be applied to the medium.

The influence of the temperature on experimental kinetics was not very clear, as is observed in the evolution of both TPC and AC (Fig. 4). A statistical analysis showed that the influence of temperature was significant ($p<0.05$) on TPC, the content of which was significantly ($p<0.05$) higher at 45 °C. These results agreed with those previously found in the literature, since it is widely recognized that temperature enhances mass transfer by the improvement of the extraction rate. This fact can be explained by the effect temperature has on the vapor pressure, surface tension and viscosity of the liquid medium (Muthukumaran et al., 2006), which facilitates mass transfer. Moreover, the increase observed in the extraction yield may be linked to the increased ease with which solvent diffuses into cells and the enhancement of desorption and solubility at high temperatures (Esclápez et al., 2011). However, temperature had no significant ($p<0.05$) influence on the AC of extracts; the

experimental error and/or the natural variability of raw matter could contribute to mask the slight differences produced by the extraction temperature. In addition, the introduction of a given amount of ultrasound energy into the medium could also contribute to mask the effect of temperature. This fact has already been reported in literature, where there is controversy surrounding the influence of temperature in antioxidant extraction processes. Thus, Jerman et al. (2010) reported an increase in extraction efficiency at temperatures of up to 45 °C in olive fruit phenolic compounds. The same fact was observed by Zhang et al. (2009) in the range of 15-45 °C, where high temperatures reduced the extraction yield. However, Zhang et al. (2011) found that extraction yields rose as the temperature increased from 60 to 80 °C, while Rostagno et al. (2007) found that phenolics underwent an important degradation at temperatures of over 60 °C. Therefore, it seems that the temperature influence may be product-dependent, it being necessary to determine the proper extraction temperature for a specific commodity. The use of high temperatures, over the optimum, should be avoided due to the fact that they lead to solvent loss by volatilization, higher energy costs and more extraction impurities (Esclápez et al., 2011).

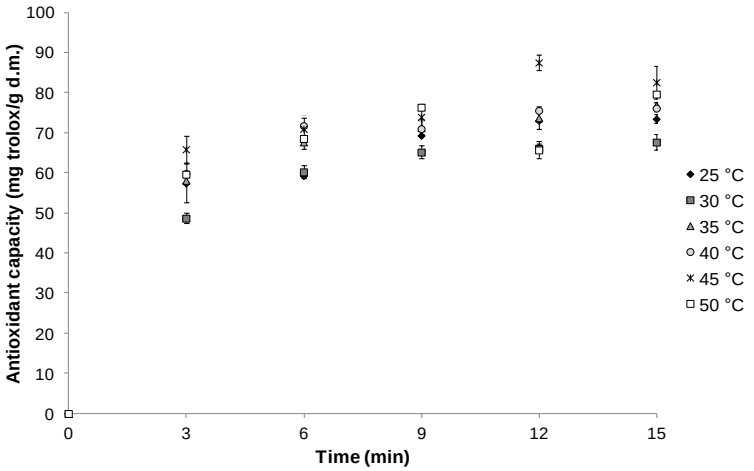


**Fig. 4.** Evolution of antioxidant capacity (FRAP) at different temperatures of ultrasound assisted extraction (100 % of the electric power supplied to the transducer, emitter surface 3.8 $cm^2$ and effective power 51.47 ± 1.13 W).

Naik's model parameters (Table 2) confirmed the scarce effect of temperature on extraction kinetics. As can be observed, the differences among the values identified at the temperatures tested were small. For example, the $Y_\infty$ ranged from 40.4 at 25 °C to 45.8 at 45 °C. The highest initial extraction ($R_0$) rate was achieved at 25 and 35 °C for AC and TPC, respectively, the identified values being very close to those found at 45 °C. Thus, taking into account both energy consumption and the slight improvement gained due to the increase in extraction temperature, the temperature of 25 °C was chosen as the most suitable for the ultrasound assisted extraction of polyphenols from olive leaves.

## 3.2. Ultrasound assisted extraction (USAE) versus conventional extraction

Once the best choice of process parameters for ultrasound application was identified: 51.47 ± 1.13 W (100 % of electric power), 3.8 $cm^2$ emitter and 25 °C; the feasibility of USAE was addressed. An overall study was conducted comparing USAE with conventional extraction processes, considering not only kinetic but also compositional issues.

### 3.2.1. Effect on extraction kinetics

The kinetic of the ultrasound assisted extraction (USAE) was compared with conventional extraction with agitation (CVE; 170 rpm) and conventional static extraction (STE).

Experimental results highlighted that solvent agitation significantly affected ($p<0.05$) extraction kinetics. As is shown in Fig. 5, the kinetic of TPC extraction was faster in CVE than in STE experiments. Obviously, the turbulence created by agitating the extracting medium reduced the external resistance to mass transfer, thereby, improving phenolic extraction. Nevertheless, CVE was significantly ($p<0.05$) slower than USAE. By applying ultrasound both TPC and AC were improved in extracts, causing phenolic compounds to migrate into the solvent faster. For example, after 3 min the AC in USAE was 119 and 332 % higher than in CVE and STE, respectively. Moreover, the TPC in USAE after 3 min was almost double that obtained after 15 min in CVE. Previous works have also reported an improvement in bioactive compounds

extraction brought about by the application of power ultrasound. Thus, Jiang-Bing et al. (2006) and Zhang et al. (2009) reported increases in the amount of extracted bioactive compounds of 16.5 and 60 %, respectively.

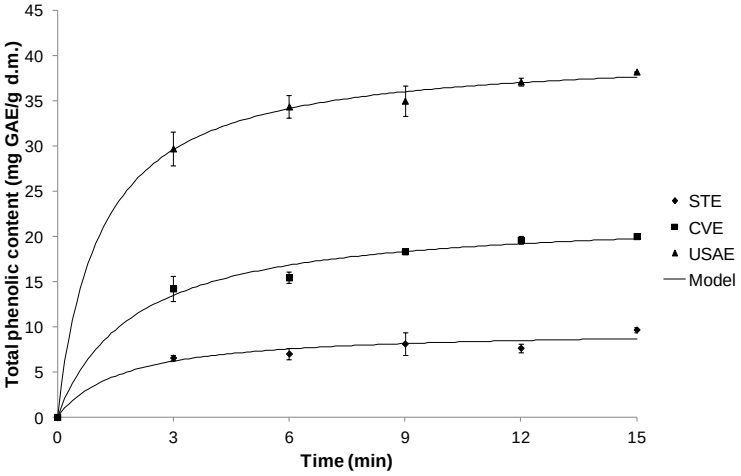


**Fig. 5.** Influence of extraction method on the total phenolic content. STE: static extraction (no agitation of extracting medium); CVE: conventional extraction (with agitation); USAE: ultrasound assisted extraction (100 % of the electric power supplied to the transducer; emitter surface 3.8 $cm^2$, effective power 51.47 ± 1.13 W and extraction temperature 25 °C).

In this study, the ultrasound application led to an immediate leaching of polyphenols into the solvent; thus, 84 % of TPC was extracted during the first 5 min of US treatment. Therefore, ultrasound effects accelerated the solubilization of accessible antioxidant compounds (washing effect) and contributed to the extraction of the non-accessible compounds. A review of the literature also brings opposite results to light, thus, Jerman et al. (2010) determined that the extraction efficiency of polyphenols from olive fruit was low for the first 4 min of ultrasound application, indicating that longer times were needed for wall disruption. This mild effect could be linked to the level of ultrasonic power applied, since these authors carried out the experiments in an ultrasonic bath, which actually supplies lower ultrasonic intensities than probe systems like the one used in the current study.

On the other hand, in USAE experiments, the increase in the TPC and AC of the extracts was almost negligible after 15 min of extraction. This fact suggests that long sonication times were not effective. During extraction times of over 15 min, the TPC and AC were kept constant, which also indicates that continuous ultrasound application seems to have no effect on bioactive compounds. These results agreed with Rodrigues et al. (2008), who indicated that 15 min of sonication time were enough to extract phenols from coconut. The effect of ultrasound could be mainly linked to the phenomenon of cavitation and the generation of microstreaming, alternative pressures or interfacial instabilities. The implosion of cavitation bubbles generates macro-turbulence, high-velocity inter-particle collision and perturbation in the micro-porous particles of the biomass accelerating the eddy diffusion and internal diffusion, thereby, increasing mass transfer (Jian-Bing et al., 2006). Moreover, the asymmetric implosion of bubbles near vegetable particles generates micro-jets (Mason & Lorimer, 2002) that hit cellular surfaces disrupting them and allowing their contents to be extracted.

Naik's model fitted the extraction kinetics for both CVE and USAE experiments well, such as is observed in Fig. 5. The initial extraction rate identified for USAE experiments, $R_0$, was three times higher than the one identified for CVE ones (37.3 and 11.6 mg GAE/min•g d.m., respectively) indicating the significant effect of ultrasound on the extraction rate. As far as equilibrium is concerned, the identified value of $Y_\infty$ was 41 ± 2 mg GAE/g d.m. for USAE and 22 ± 1 mg GAE/g d.m. for CVE. The $Y_\infty$ value identified for CVE experiments should be considered a modeling artifact since the experimental conditions are not a valid means of identifying the equilibrium point. This is due to the fact that, at the longest time tested (15 min), the system is a long way from equilibrium, which under these conditions was reached after approximately 24 h. Therefore, the results obtained showed just how effective ultrasound application is at extracting antioxidants from olive leaves, thus reducing extraction times. This fact could be very interesting for industrial purposes, since ultrasound assisted extraction would make it possible to improve process rates and, consequently, reduce processing times and costs.

### 3.2.2. Influence on extract composition and antioxidant potential

In order to complete the study into the feasibility of ultrasound assisted extraction, it was necessary to evaluate not only the extraction rate but also the quality of the obtained extracts. For that purpose, a different batch of olive leaves was collected and processed as already explained in section 2.1. The extracts were obtained by USAE after 15 min and CVE after 24 h and characterized (Table 3). The TPC of extracts obtained by CVE and USAE was similar (66 mg GAE/g d. m.). As for AC, FRAP and TEAC methods gave slightly different results. While no significant ($p<0.05$) differences were observed between USAE and CVE extracts when using TEAC, the use of FRAP implied a significant ($p<0.05$) increase (10 %) in AC when USAE was applied. This fact could be explained by the fact that these methods are based on different chemical principles, which involves a different sensitivity towards evaluating changes in extract composition linked to antioxidant capacity.

**Table 3.** Characterization of olive leaf extracts obtained by conventional (CVE, 24 h, 170 rpm) and ultrasound assisted extraction (USAE, 15 min, 51.47 W).

| | | CVE | USAE |
|---|---|---|---|
| Oleuropein (mg/g d. m.) | | $74 \pm 2^{a}$ | $65 \pm 2^{b}$ |
| Verbascoside (mg/g d. m.) | | $18.7 \pm 0.3^{a}$ | $18.5 \pm 0.6^{a}$ |
| Luteolin -7-O-glucoside (mg/g d. m.) | | $9.7 \pm 0.4^{a}$ | $11 \pm 4^{a}$ |
| Total phenolic content (mg GAE/g d. m.) | | $66 \pm 3^{a}$ | $66 \pm 8^{a}$ |
| Antioxidant capacity (mg trolox/g d. m.) | FRAP | $102 \pm 3^{a}$ | $112 \pm 6^{b}$ |
| | TEAC | $6.2 \pm 0.3^{a}$ | $7.2 \pm 1.2^{a}$ |

Note: The subscripts a and b show homogeneous groups established from LSD (Least Significance Difference) intervals ($p<0.05$).

The extracts obtained from USAE and CVE extraction were also analyzed by chromatography, which allowed the main phenolic compounds present in olive leaf extracts to be identified (Table 4). Chromatograms from USAE and CVE extracts were very similar, as is observed in Fig. 6. Thus, ultrasound application did not promote the formation of new phenolic compounds or induce phenolic degradation. The main polyphenols identified in this study: oleuropein, verbascoside and luteolin-7-O-glucoside have been already reported in previous studies of olive leaf extracts

(Benavente-García et al., 2000; Japón-Luján & Luque de Castro, 2006). However, other known phenols, such as tyrosol and hydroxytyrosol, which are characteristic of olive fruit and leaf, were not found in either CVE or USAE extracts. It is likely that these differences could be explained by the olive cultivar and collecting season.

**Table 4.** Identification of the main phenolic compounds present in olive leaf extracts.

| Peak no. | Phenolic compound | Molecular mass (g/mol) | Retention time (min) |
|---|---|---|---|
| 1 | Cafeoil | 354.31 | 4.70 |
| 2 | Apigenin-6,8-diglucoside | 594.52 | 9.41 |
| 3 | Verbascoside | 624.6 | 13.85 |
| 4 | Luteolin-7-O-rutinoside | 578.52 | 14.57 |
| 5 | Luteolin-7-O-glucoside | 448.38 | 15.27 |
| | Luteolin-7-O-glucoside (isomer) | 448.38 | 18.50 |
| 6 | Oleuropein glucoside | 702 | 16.45 |
| 7 | Apigenin rutinoside | 578.53 | 17.11 |
| 8 | Apigenin-7-O-glucoside | 432.37 | 18.24 |
| 9 | Oleuropein | 540.52 | 19.02 |
| 10 | Luteolin | 286.24 | 25.50 |

In this study, only the main polyphenols were quantified (oleuropein, verbascoside and luteolin-7-O-glucoside) using standard compounds. No significant ($p<0.05$) difference was found between the verbascoside and luteolin-7-O-glucoside content of USAE and CVE extracts. In the case of oleuropein, however, USAE extracts exhibited a 12 % significantly ($p<0.05$) lower content than CVE ones. Jerman et al. (2010), who studied ultrasound assisted extraction of olive fruit phenolic compounds, found that the extraction method had a significant ($p<0.05$) influence on the content of all the compounds quantified in this study. In all likelihood, these authors did not compare extracts obtained at equilibrium, as the result is masked by a kinetic effect linked to ultrasound application.

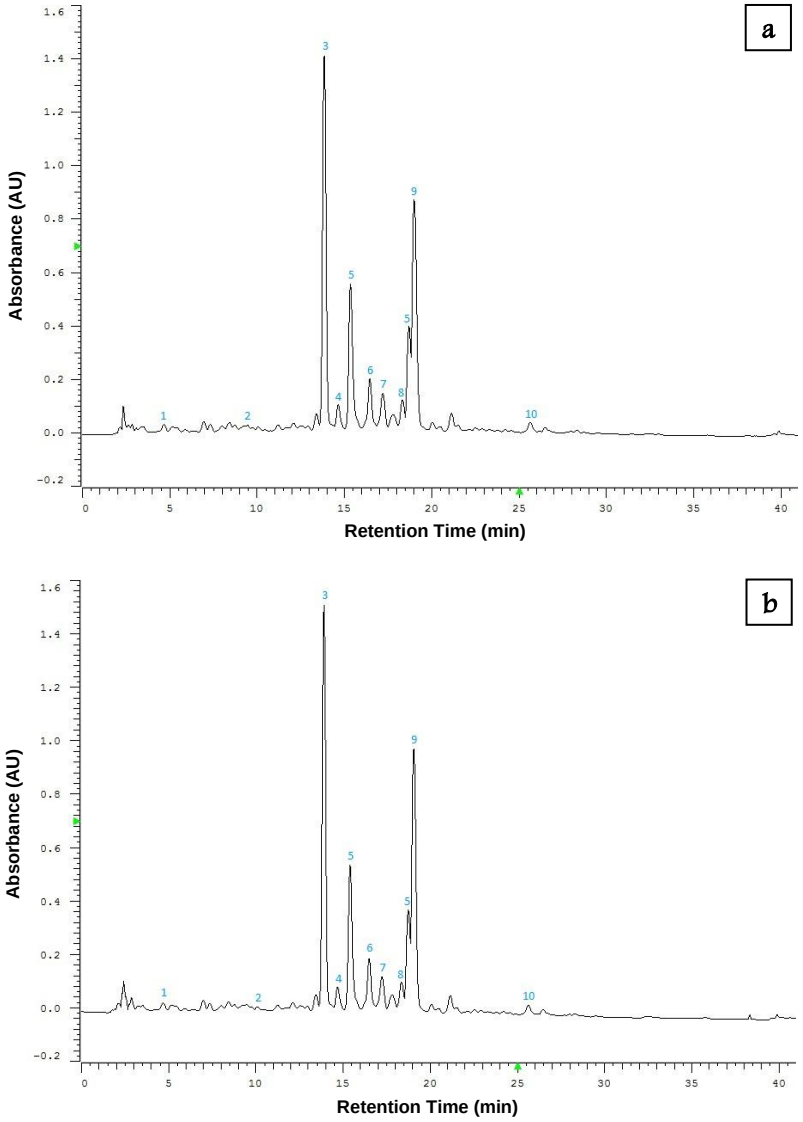


**Fig. 6.** HPLC chromatograms at 280 nm of olive leaf extracts obtained at 25 °C by CVE (a; extraction time 24 h) and USAE (b; 100 % of the electric power supplied to the transducer, emitter surface 3.8 $cm^2$, effective power 51.47 ± 1.13 W and extraction time 15 min).

As regards the extraction yields reached in this study, the polyphenol content was higher than that published by other authors using other extraction methods. As an example, the oleuropein content was 222 % and 347 % higher than that determined by Japón-Luján & Luque de Castro (2006) in olive leaves and

Jerman et al. (2010) in olive fruits, respectively. Thus, extracts with a higher content of oleuropein (65-74 mg/g d. m.), verbascoside (18.5-18.7 mg/g d. m.) and luteolin-7-O-glucoside (9.7-11 mg/g d. m.) were obtained. Although there are many factors which can affect the extract composition, such as the cultivar or sampling season, both extraction methods used in this study can be considered adequate and efficient procedures. Moreover, it is necessary to highlight that ultrasound application reduced the extraction time from the 24 h needed in the conventional method to 15 min, maintaining the phenolic composition and antioxidant potential of the extracts. In this sense, the application of ultrasound would be an interesting alternative method to conventional procedures, since it greatly increased the extraction rate and was able to generate extracts rich in bioactive compounds.

## 4. Conclusions

The application of ultrasound energy could be considered an interesting alternative as a means of intensifying the extraction process of phenolic compounds from olive leaves. The ultrasound effect was mostly dependent on the effective ultrasonic power applied to the medium, and was influenced not only by the amount of electric power supplied but also by how well the emitter surface and extracting medium coupled. Thereby, it was highlighted that the greatest improvement of polyphenolic extraction was achieved by supplying 100 % of the total electric power to the ultrasonic device and using the intermediate emitter surface tested (3.8 $cm^2$) for an extracting medium of 200 mL. Moreover, temperature was found to have no clear effect on extraction kinetics. Therefore, compared with conventional techniques, ultrasound assisted extraction can be considered a more efficient procedure, being able to provide olive leaf extracts with a similar content of bioactive compounds, such as oleuropein, verbascoside and luteolin-7-O-glucoside, but markedly shortening the extraction time, from 24 h to 15 min.

The ultrasonic assisted extraction is still a challenge on an industrial scale. Therefore, further research is necessary in order to develop efficient ultrasonic transducers and thus improve the extraction processes. These facts would allow the

processing costs to be minimized, giving rise to a new more competitive market in which the bioactive properties would remain intact.

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# CHAPTER 3

## *Extract stability*

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# *Influence of olive leaf processing on the bioaccessibility of bioactive polyphenols*

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# Influence of olive leaf processing on the bioaccessibility of bioactive polyphenols


## ABSTRACT

Olive leaves are rich in bioactive compounds which are beneficial for humans. The objective of this work was to assess the influence of the processing conditions (drying and extraction) of olive leaves on the extract's bioaccessibility. Thus, extracts obtained from dried olive leaves (hot air drying at 70 and 120 °C or freeze drying) by means of conventional or ultrasound assisted extraction were subjected to *in vitro* digestion. Antioxidant capacity, total phenolic content and HPLC-DAD/MS-MS analysis were carried out during digestion.

The dehydration treatment used for the olive leaves did not have a meaningful influence on bioaccessibility. The digestion process significantly ($p<0.05$) affected the composition of the extracts. Oleuropein and verbascoside were quite resistant to gastric digestion but were largely degraded in the intestinal phase. Nevertheless, luteolin-7-O-glucoside was the most stable polyphenol during the *in vitro* simulation (43 % bioaccessibility). Therefore, this compound may be taken into consideration in further studies that focus on the bioactivity of olive leaf extracts.


***Keywords***: *Olea europaea*, drying, *in vitro* digestion, antioxidant potential, byproduct.

## 1. Introduction

Although olives (*Olea europaea* L.) are cultivated in several parts of the world, the Mediterranean region is the major crop production area, accounting for about 98 % of the world's olive cultivation.[1] Olive hydrophilic extracts contain a large number of phenolic compounds.[2] The most important source of these polyphenolic compounds is the olive fruit; however, they are also present in the leaves.[3] The most abundant polyphenols identified in olive leaf extracts have been oleuropein, hydroxytyrosol, verbascoside, apigenin-7-glucoside and luteolin-7-glucoside.[4] These compounds confer bioactive properties on the olive leaf extracts, such as antioxidant,[4] antimicrobial,[5] and antitumor capacity.[6] Moreover, they are able to reduce the risk of coronary heart disease.[7]

Not only do olive leaves appear as a byproduct during oil processing in the olive oil industries (10 % of the total weight of the olives), they are also a residue of olive tree pruning.[8] Despite the aforementioned bioactive potential of olive leaves, the large volumes generated of this byproduct have been traditionally used as animal feed.[9] The exploitation of olive leaves as a natural resource rich in antioxidant phenolic compounds would involve the valorization of this byproduct. However, it is essential to determine if the leaf processing (drying and extraction methods) used to obtain the extracts can affect not only the antioxidant potential but also the stability necessary for later applications. Hot air and freeze drying are common dehydration techniques used in the food, chemical and pharmaceutical industries. Nevertheless, although both methods reduce the water content of the raw material improving the polyphenol extraction, the extracts obtained exhibit different compositions,[10] and their degree of stability could also vary. Moreover, the stability of extracts obtained by conventional maceration could also be different to those obtained by the use of new, emerging extraction technologies, such as ultrasound assisted or supercritical $CO_2$ extraction. In the case of ultrasound (US), although its ability to speed the extraction process has already been stated, it is also well known that it induces the formation of free radicals,[11] which even at low concentrations could affect extract behavior.

The aforementioned facts, together with the scarce amount of knowledge about the stability and behavior of olive leaf phenolic compounds under digestion conditions, opens up a new line of research in the area of antioxidant extracts and their possible applications as natural remedies. Thus, to use the olive leaf polyphenols for the benefit of human health, it is important not only to extract and quantify these bioactive compounds but also to study their bioaccessibility. This term is defined as the amount of an ingested compound that is available for absorption in the gut after digestion.[12] Nowadays, there are two possible methods for the evaluation of the behavior of bioactive compounds during the digestive processes: *in vivo* and *in vitro*. On the one hand, *in vivo* methods simulate the digestion better, but are complex, expensive, lengthy,[13] and the control of the process variables is complicated. On the other hand, *in vitro* digestion models simulate the release of compounds from the food matrix[14] and the gastrointestinal conditions, are relatively inexpensive, technically simple,[13,15] and allow for the accurate testing of several process variables. Moreover, considering that there exist some host related factors, such as intestinal factors, gender, age, disorders or physiological conditions which could influence bioaccessibility,[16] *in vitro* methods seem to be a suitable alternative to obtain proper simulation data. In fact, they are considered a proper alternative to animal experimentation by the European Directive on the protection of animals (2010/63/UE).[17]

Even so, the bioaccessibility study is a challenge because, apart from human factors, there are external, food or processing factors that could affect bioactive compounds. Thus, there is ample evidence that the physical state of the matrix plays a key role in the release, mass transfer, accessibility, and biochemical stability of many food components.[18] Hence, nutrients located in natural cellular compartments or within assemblies produced during processing need to be released during digestion so they can be absorbed in the gut.[19] Therefore, removing the handicap of structural barriers could facilitate the extraction of the components and, consequently, it would improve their bioaccessibility. In this sense, obtaining extracts rich in bioactive compounds could be a suitable alternative.

The objective of this work was to assess the influence of the processing conditions (drying and extraction) of olive leaves on the bioaccessibility of the extracts. The study aims to evaluate not only the compositional but also the kinetic changes that take place in the extracts during the *in vitro* gastrointestinal digestion.

## 2. Materials and methods

### 2.1. Olive leaf extracts

Olive leaf extracts were obtained from leaves (*O. europaea* var. Serrana) dried by following two different methods described in previous works:[10] hot air drying at 70 °C (HAD-70) and 120 °C (HAD-120) and freeze drying using conventional freezing at -28 °C (FD).

The extraction of olive leaf phenolic compounds was carried out by using conventional[10] and ultrasound (US) assisted extraction.[20] Briefly, extractions were performed at 25 °C using milled olive leaves (Blixer 2, Robot Coupe USA, Inc., Jackson, MS, USA) and an 80:20 (v/v) ethanol-water solution as solvent (extracting medium). The ratio between the weight of olive leaves and the solvent volume used was different depending on the method: 0.125 and 0.031 g/mL for conventional and ultrasound assisted extraction, respectively. In the ultrasonic experiments, the ultrasonic emitter, with a surface of 3.8 $cm^2$, was immersed 1 cm into the solution, and 100 % electric power of the system (400 W) was supplied to the transducer. The extracts obtained were centrifuged for 10 min at 5000 rpm (Medifriger BL-S, J.P. Selecta, Barcelona, Spain), filtered (nylon filters of 0.45 μm) and stored in opaque vials at 4 °C until used. Thus, combining the drying (FD, HAD-70 and HAD-120) and the extraction methods (conventional or US assisted) a total of 4 different extracts were obtained: FD, HAD-70, HAD-120 and HAD-120+US. At least 3 extraction replicates were made for each different processing condition.

## 2.2. Polyphenolic standard solutions

Standard solutions of the main polyphenols found in the olive leaf extracts were prepared in water. Concentrations of the standard solutions were similar to the concentration (mg compounds/mL extract) of the polyphenols in the olive leaf extracts. The standard polyphenols used are described in the following sections.

## 2.3. *In vitro* digestion

*In vitro* digestion was simulated using a slightly modified version of previously described methods.[21,22] 10 g of sample (olive leaf extract, polyphenolic standards solution or blank) were diluted with distilled water (1:8, w/v) and acidified to pH 2.0 using 6 N HCl under vigorous stirring. The acidified sample was stirred for 15 min. Then, the pH value was checked and eventually corrected if necessary with 6 N HCl. The extract sample was then mixed with 3 mL of a solution (160 mg/mL) of pepsin from pig gastric mucosa (Sigma Chem.Co., 3.8 units/mg protein) in 0.1 N HCl. Subsequently, distilled water was added to reach a final volume of 100 mL. The mixture was stirred for 2 h at 37 °C. After gastric digestion, the pH of the digesta was increased to pH 5 with 0.9 M $NaHCO_3$, and 22.54 mL of pancreatin–bile solution (pancreatin 4 mg/mL, bile 25 mg/mL in 0.1 M $NaHCO_3$) were added. Then, to complete the intestinal digestion, the pH was increased to 7.0 with 0.1 M $NaHCO_3$, and the mixture was stirred for 2 h at 37 °C.

The digestion simulation was carried out in sealed containers protected from light and placed in an orbital incubator (Rotabit, J. P Selecta, Barcelona, Spain) at 120 rpm. To monitor the changes undergone by extracts during *in vitro* digestion, samples of 2 mL were taken at preset times (0, 1, 2, 3 and 4 h). All samples were immediately cooled and filtered (nylon filters of 0.45 μm), stored in opaque vials at 4 °C, and analyzed within 24 h. At least 3 digestion replicates were made for each different olive leaf extract.

## 2.4. Total phenolic content (TPC) measurement

The phenolic content was determined by the Folin-Ciocalteu method.[23] Briefly, 100 μL of sample or blank were mixed with 200 μL of Folin-Ciocalteu's phenol reagent (Sigma-Aldrich, Madrid, Spain) and 2 mL of distilled water. After 3 min at 25 °C, 1 mL of $Na_2CO_3$ (Panreac, Barcelona, Spain) solution ($Na_2CO_3$-water 20:80, w/v) was added to the mixture. The reaction was kept in the dark at room temperature for 1 h. Finally, the absorbance was read at 765 nm using a spectrophotometer (Helios Gamma, Thermo Spectronic, Cambridge, UK). The measurements were carried out in triplicate, unless otherwise stated. The standard curve was previously prepared using solutions of a known concentration of gallic acid hydrate (Sigma-Aldrich, Madrid, Spain) in water and ethanol-water (80:20, v/v). Results were expressed as mg of gallic acid (GAE) per g of dry weight of olive leaves (g d.w.) or in the case of the standards as mg of gallic acid (GAE) per g of compound.

## 2.5. Antioxidant capacity (AC) measurement

### 2.5.1. Ferric-reducing ability power (FRAP)

The FRAP method, which is a simple method used to estimate the reduction of a ferric-tripyridyltriazine complex, was applied[24] with some modifications. Briefly, 900 μL of freshly prepared FRAP reagent were mixed with 30 μL of distilled water and 30 μL of test sample or blank and kept at 37 °C for 30 min. The FRAP reagent contained 2.5 mL of a 10 mM TPTZ (Fluka, Steinheim, Germany) solution in 40 mM HCl (Panreac, Barcelona, Spain) plus 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ (Panreac, Barcelona, Spain) and 2.5 mL of 0.3 M acetate buffer (Panreac, Barcelona, Spain), pH 3.6.[25] Readings at the maximum absorption level (595 nm) were taken using a spectrophotometer (Helios Gamma, Thermo Spectronic, Cambridge, UK). Four replicates were made for each measurement. The antioxidant capacity was evaluated through a calibration curve, which was previously determined using water and ethanol solutions (ethanol-water 80:20, v/v) of known Trolox (Sigma-Aldrich, Madrid, Spain) concentrations and expressed as mg Trolox per g of dry weight of olive leaves (g d.w.) or in the case of the standards as mg Trolox per g of compound.

### 2.5.2. Trolox equivalent antioxidant capacity (TEAC)

The TEAC method, which measures the reduction of the radical cation of ABTS by antioxidants, was performed.[26] Briefly, ABTS radical cation ($ABTS^{+\bullet}$) was produced by reacting ABTS (Sigma-Aldrich, Madrid, Spain) stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stay in the dark at room temperature for 12-24 h before use. The $ABTS^{+\bullet}$ solution was diluted with distilled water until an absorbance value of 0.714 ± 0.02 at 734 nm was reached. For the photometric assay, the absorbance of 200 µL of the $ABTS^{+\bullet}$ solution or blank was measured (Spectrostar Omega, BMG Labtech, Offenburg, Germany). Afterwards, 20 µL of the antioxidant extract or blank (water or ethanol-water 80:20, v/v) were added and, after 29 min at 734 nm, the final absorbance was measured (Spectrostar Omega, BMG Labtech, Offenburg, Germany). The antioxidant capacity was determined from the difference of absorbance, using a Trolox calibration curve (Sigma-Aldrich, Madrid, Spain). Four replicates were made for each extract. The antioxidant capacity results were expressed as mg of Trolox per g of dry weight of olive leaves (g d.w.) or in the case of the standards as mg Trolox per g of compound.

### 2.6. Identification and quantification of polyphenols by HPLC-DAD/MS-MS

In order to identify and quantify the main polyphenols, olive leaf extracts were analyzed using an HPLC instrument (Agilent LC 1100 series; Agilent Technologies, Inc., Palo Alto, CA, USA) controlled by the Chemstation software. The HPLC instrument was coupled to an Esquire 3000+ (Bruker Daltonics, GmbH, Bremen, Germany) mass spectrometer equipped with an ESI source and ion-trap mass analyzer, and controlled by Esquire control and data analysis software. A Merck Lichrospher 100RP-18 (5 µm, 250 x 4 mm) column was used for analytical purposes.

Separation was carried out through a linear gradient method using 2.5 % acetic acid (A) and acetonitrile (B), starting the sequence with 10 % B and programming the gradient to obtain 20 % B at 10 min, 40 % B at 35 min, 100 % B at 40 min, 100 % B at 45 min, 10 % B at 46 min and 10 % B at 50 min. For the LC-MS pump to perform accurately, 10 % of organic solvent was pre-mixed in the water phase. The flow-rate was 1 mL/min and the chromatograms were monitored at 240, 280 and 330 nm.

Mass spectrometry operating conditions were optimized to achieve maximum sensitivity values. The ESI source was operated in negative mode to generate $[M-H]^-$ ions using the following conditions: desolvation temperature at 365 °C and vaporizer temperature at 400 °C; dry gas (nitrogen) and nebulizer were set at 12 L/min and 4.83 bar, respectively. The MS data were acquired as full scan mass spectra at 50–1100 m/z by using 200 ms for collection of the ions in the trap.

The main compounds were identified by HPLC-DAD analysis, comparing the retention time, UV spectra and MS/MS data of the peaks in the samples with those of authentic standards or data reported in the literature. Only the main olive leaf polyphenols were quantified using commercial standards: oleuropein (Extrasynthese, Genay Cedex, France), luteolin-7-O-glucoside (Phytolab, Vestenbergsgreuth, Germany) and apigenin (Nutrafur, Murcia, Spain). A verbascoside standard (96.85 %) purified from *Lippia citriodora* extract was kindly provided by Universidad Miguel Hernández (Elche, Spain). The quantitative evaluation of the compounds was performed with a calibration curve for each polyphenol, using ethanol (oleuropein), methanol (verbascoside and luteolin) or dimethyl sulfoxide (apigenin) solutions of known concentration. The polyphenol concentrations were expressed as mg polyphenol per g of dry weight of olive leaves (g d.w.) or, in the case of the standards, as mg compound per mL of initial solution.

Method accuracy for the determination of oleuropein, verbascoside and luteolin glucoside in samples was further assessed with recovery studies by spiking standard compounds into blank samples by triplicate. The linearity range of the responses was determined on seven concentration levels with three injections for each level. Calibration graphs for HPLC were recorded with sample amount ranging from 0.25 μg/mL to 0.25 mg/mL ($r^2$>0.9999). Quantitative evaluation of oleuropein, verbascoside luteolin glucoside was performed by means of a six-point regression curve ($r^2$>0.996, 0.997 and 0.986, respectively) in a concentration range between 0.25 μg/mL and 10 mg/mL, using oleuropein, verbascoside and luteolin glucoside as reference external standard and evaluated by DAD signal. The limit of detection (LOD) was 0.10 μg/mL and the limit of quantification (LOQ), 0.25 μg/mL.

## 2.7. Statistical analysis

Analysis of variance (ANOVA) was applied in order to determine the significant effects of *in vitro* digestion on extract composition taking a significance level of 95 %. The statistical analysis was performed using Statgraphics-Plus software 5.1 (Statistical Graphics, Rockville, MD, USA).

# 3. Results and discussion

## 3.1. Initial characterization of olive leaf extracts

Previous studies have reported that the composition of olive leaf extracts is greatly influenced by processing conditions, namely, the drying and extraction. In such a way, a conventional extraction method[10] was used with leaves hot air dried at 70 (HAD-70) and 120 °C (HAD-120) and freeze dried (FD). Moreover, HAD-120 leaves were subjected to ultrasound assisted extraction (HAD-120+US).[20]

As can be observed in Table 1, the TPC and AC of the extracts used were significantly ($p<0.05$) influenced by the olive leaf drying technique, which confirms the previously reported results.[10] It is relevant to highlight that the HAD-120 and FD extracts showed the highest and lowest antioxidant potentials, respectively. As regards the extraction method, ultrasonically assisted extraction provided extracts (HAD-120+US) with a bioactive content similar to that of the conventional procedure (HAD-120), as already reported.[20] Thus, although US did not involve a change in the initial polyphenolic composition of the extracts, the HAD-120+US extracts were included in the analysis due to the reported ability of US to promote the formation of free radicals.[27] These compounds, even at low concentrations, may induce compositional changes in the gastric/intestinal environment, a fact that has not been previously studied.

**Table 1.** Content (mg/g d.w.) of the main identified polyphenols and antioxidant activity of olive leaf extracts.

| Extract characterization | | Initial extracts [a] | | | |
|---|---|---|---|---|---|
| | | HAD-70 | HAD-120 | FD | HAD-120+US |
| TPC (mg GAE/g d.w.) | | 42 ± 4 [a] | 66 ± 3 [b] | 33 ± 3 [a] | 66 ± 9 [b] |
| AC (mg Trolox/g d.w.) | FRAP | 87 ± 6 [a] | 102.2 ± 1.7 [b] | 75 ± 4 [c] | 112 ± 4 [d] |
| | TEAC | 4.96 ± 0.17 [ab] | 6.3 ± 0.2 [bc] | 4.4 ± 0.9 [a] | 7.2 ± 1.4 [c] |
| Verbacoside | | 6.48 ± 0.15 [a] | 18.7 ± 0.3 [b] | 4.2 ± 0.4 [c] | 18.5 ± 0.8 [b] |
| Oleuropein | | 47 ± 3 [a] | 74.9 ± 0.4 [b] | 22.9 ± 0.4 [c] | 65 ± 3 [d] |
| Oleuropein glucoside [b] | | 3.7 ± 0.9 [a] | 16.4 ± 0.5 [b] | 3.6 ± 0.6 [a] | 13.9 ± 0.3 [c] |
| Luteolin glucoside | | 8.4 ± 0.5 [a] | 9.7 ± 0.4 [b] | 9.9 ± 0.7 [b] | 11 ± 4 [c] |
| Luteolin [c] | | 0.9 ± 0.2 [a] | 0.76 ± 0.07 [a] | 0.9 ± 0.3 [a] | 0.28 ± 0.03 [b] |
| Luteolin-7-O-rutinoside [c] | | 2.7 ± 0.5 [a] | 2.52 ± 0.13 [ab] | 2.1 ± 0.4 [bc] | 1.86 ± 0.19 [c] |
| Apigenin-7-O-glucoside [d] | | 0.67 ± 0.12 [ab] | 0.78 ± 0.02 [a] | 0.62 ± 0.06 [b] | 0.698 ± 0.014 [ab] |
| Apigenin 6,8-diglucoside [d] | | 0.48 ± 0.07 [a] | 0.40 ± 0.15 [ab] | 0.25 ± 0.03 [b] | 0.36 ± 0.06 [ab] |
| Apigenin rutinoside [d] | | 0.92 ± 0.15 [a] | 1.13 ± 0.03 [b] | 0.71 ± 0.08 [c] | 0.93 ± 0.02 [a] |

[a] Letters following entries show homogeneous groups in the same row established from least significance difference intervals ($p<0.05$).

[b] Content expressed as equivalents of oleuropein (mg/g d. w.).

[c] Content expressed as equivalents of luteolin-7-O-glucoside (mg/g d. w.).

[d] Content expressed as equivalents of apigenin (mg/g d. w.).

In order to gain insight into the differences in the antioxidant potential of olive leaf extracts, major polyphenolic compounds were identified by HPLC-DAD/MS–MS (Fig. 1 and Table 2). A similar phenolic profile was found in all the extracts, with minor differences. However, significant ($p<0.05$) differences were found (Table 1) for the concentration of the main polyphenols (verbascoside, oleuropein, luteolin glucoside). In overall terms, the highest contents of these compounds were correlated with the highest levels of TPC and AC.

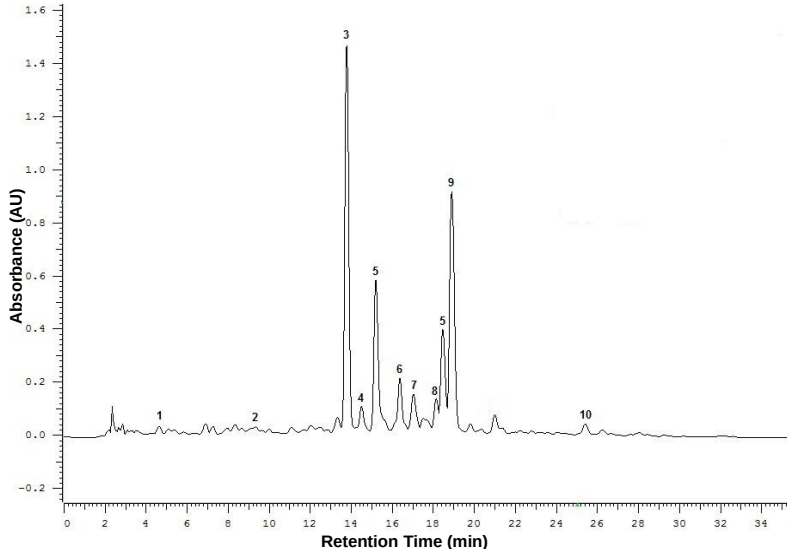


**Fig. 1.** HPLC chromatograms at 280 nm of extracts obtained from fresh olive leaves dried by hot air drying at 120 °C using conventional extraction (HAD-120).

**Table 2.** Relevant analytical data of compounds in olive leaf extracts by HPLC-ESI/MS-MS.

| Peak | Phenolic compound | Mol mass (g/mol) | Retention time (min) |
|---|---|---|---|
| 1 | Caffeoil | 354.31 | 4.70 |
| 2 | Apigenin-6,8-diglucoside | 594.52 | 9.41 |
| 3 | Verbascoside | 624.6 | 13.85 |
| 4 | Luteolin-7-O-rutinoside | 578.52 | 14.57 |
| 5 | Luteolin-7-O-glucoside | 448.38 | 15.27 |
| | Luteolin-7-O-glucoside(isomer) | 448.38 | 18.50 |
| 6 | Oleuropein glucoside | 702 | 16.45 |
| 7 | Apigenin rutinoside | 578.53 | 17.11 |
| 8 | Apigenin-7-O-glucoside | 432.37 | 18.24 |
| 9 | Oleuropein | 540.52 | 19.02 |
| 10 | Luteolin | 286.24 | 25.50 |

## 3.2. Influence of *in vitro* gastrointestinal digestion on antioxidant potential

In order to simulate the stability of the main identified bioactive polyphenols, *in vitro* digestions of the olive leaf extracts and polyphenolic pure standards (oleuropein, verbascoside and luteolin glucoside) were carried out. During digestion, samples were taken at preset times (0, 1, 2, 3 and 4 h) and the TPC and AC determined.

A significant ($p<0.05$) decrease in the TPC was observed for all the extracts within the first hour of the gastric phase (Fig. 2a), thereafter remaining practically constant for the rest of the intestinal digestion. This result indicates that a portion of the bioactive compounds may be degraded in the first steps of the digestion[28] due to the combined effect of the pH changes and the enzymatic activity, but a significant portion still reached the duodenum. Thus, after the gastric digestion (2 h), the TPC of HAD-120 extracts decreased by 31.4 %, whereas the reduction in HAD-70 and FD extracts was smaller (22.9 and 25.1 %, respectively). Grape bioaccessible polyphenols have been reported to behave differently during digestion.[29] Thus, after 2 h under gastric conditions, the TPC increased by 21.9 % in the liquid fraction. These differences could be ascribed to the nature of the matrix being digested. The bioaccessibility study of grape polyphenols[29] was conducted in solid matrices (grapes); therefore, a release of polyphenols from the solid parts of the grape must take place during digestion. However, in our study, polyphenols were solubilized into the solution and, hence, directly exposed to gastric conditions from the beginning.

The kinetics showed that the intestinal phase, from 2 to 4 h, hardly influenced the TPC, because only slight changes were found (Fig. 2a). The global balance of the TPC throughout the digestion showed a decrease of between 20.6 % (FD) and 39.3 % (HAD-120) when values are compared at 0 and 4 h. Although the HAD-120 extracts exhibited the highest TPC at the beginning of the digestion, they also showed a more significant decrease, indicating that these extracts were less resistant to gastrointestinal conditions. In contrast, FD and HAD-70 processed extracts were less affected by the digestion process.

On the other hand, when conventional and US extraction methods were compared, the US extracts exhibited a smaller reduction of TPC at the end of the *in*

*vitro* digestion (29 % for HAD-120+US and 39 % for HAD-120). Consequently, the extraction method would have a significant influence ($p<0.05$) on the TPC bioaccessibility; ultrasound application would be a good alternative to reduce olive leaf polyphenol extraction time[20] and ensure a better preservation of phenols during digestion.

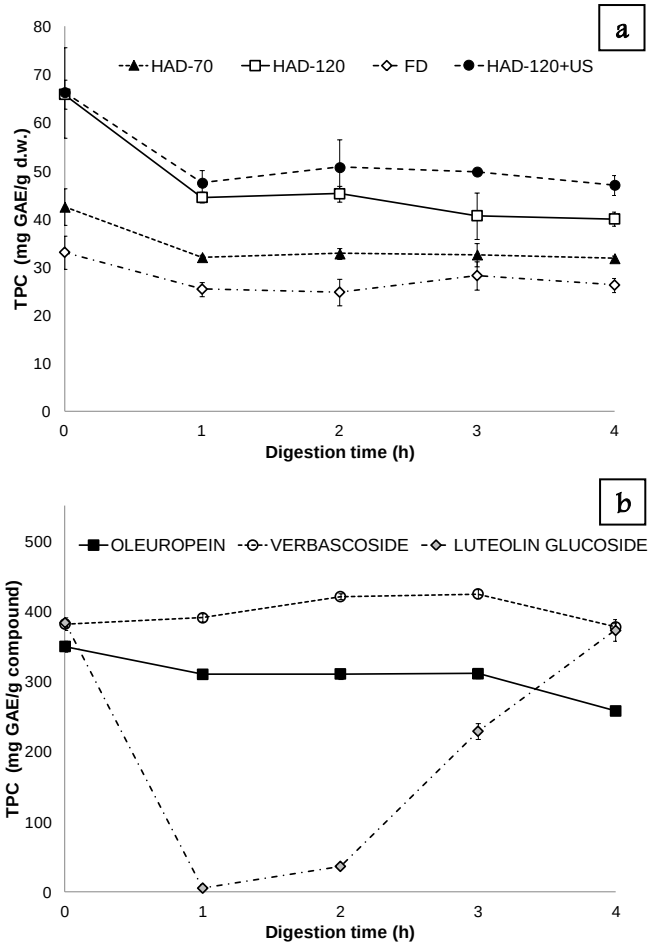


**Fig. 2.** Kinetics of total phenolic content (TPC) during the *in vitro* digestion of olive leaf extracts (a) and standard compounds (b). Means ± standard deviation are plotted.

To determine the contribution of the major compounds present in olive leaf to the TPC evolution under gastric digestion, their individual stability was studied (Fig. 2b) by subjecting selected polyphenolic standard solutions to digestion and monitoring the TPC. The behavior observed during the digestion of the standard

solutions was quite different compared to that of olive leaf extracts. Whereas oleuropein and verbascoside TPC values remained practically constant throughout the digestion process, the behavior of luteolin glucoside standard solution was unexpected; that is, it dropped to a value of 0 after 1 h of digestion and increased to initial values after 4 h of digestion. The only reasonable explanation for this fact is that luteolin glucoside shows a poor solubility or is complexed to proteins in the solutions for gastric digestion, so no TPC values were detected and its solubility recovered at the end of the intestinal digestion. This drop in the TPC of the luteolin glucoside standard solution could contribute to the TPC reduction of the olive leaf extracts in the first hour of digestion (Fig. 2).

The evolution of the AC of the extracts during digestion was determined by using two different methods, FRAP (Fig. 3) and TEAC (Fig. 4), which yielded different results. Whereas the TEAC kinetics of olive leaf extracts showed a behavior similar to that obtained for TPC (Fig. 2a and 4a), the AC evolution measured by the FRAP method behaved totally differently (Fig. 3a). The AC methods showed different trends, a fact that was also found in the case of *in vitro* tested wholegrain foods[30] and could be explained by the different chemical principles on which both AC methods are based.

The decrease in the TEAC values within the first hour of the extracts' digestion coincided with that observed for the TPC, and it may be related to the degradation of some compounds. We have reported that oleuropein is the major contributor to AC when measured by TEAC,[10] and it is also the most abundant compound in olive leaf extracts. The results of the evolution of the TEAC values for polyphenolic standard solutions under digestion (Fig. 4b) also agree with this hypothesis, because the oleuropein and verbascoside TEAC values dropped dramatically after 1 h of digestion. Otherwise, the TEAC values for luteolin glucoside recovered during intestinal digestion, coinciding with the behavior of the TPC and FRAP values during the same type of digestion (Fig. 2b and 3b), indicating that this compound may resist the *in vitro* digestion process and may be considered for the subsequent intestinal absorption process. This negative impact of the digestion process on the TEAC values of the extracts has also been observed in the *in vitro* digestion of polyphenols deriving

from extra virgin olive oils,[31] so the source of the compounds may not be the reason for this behavior.

However, although the FRAP values of olive leaf extracts remained almost constant during gastric digestion, despite the decrease in TPC and TEAC, a significant increase was observed during the intestinal digestion (Fig. 3a). It has been reported that the reaction rates for the radical scavenging capacity of some flavonoids increase in correlation with their anionic character due to their higher electron-donating capacity.

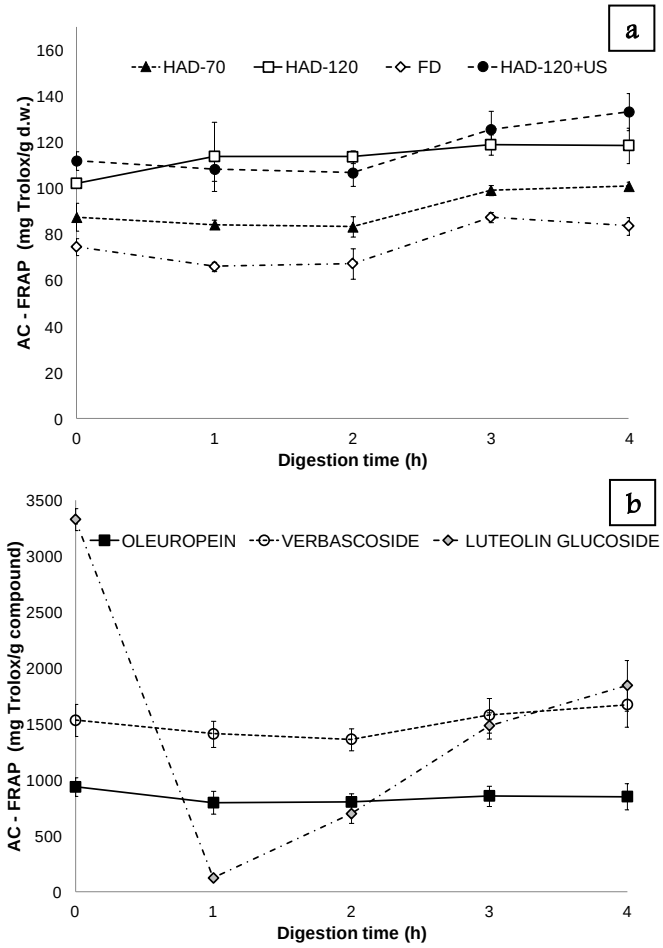


**Fig. 3.** Kinetics of antioxidant capacity (AC) measured by FRAP method during the *in vitro* digestion of olive leaf extracts (a) and standard solution compounds (b). Means ± standard deviation are plotted.

We postulate that at least luteolin may contribute to the greater capacity for reducing $Fe^{3+}$ ions during the intestinal digestion process (Fig. 3b, 3-4 h) due to the fact that less protonation occurs at neutral pH.[32,33] We have previously reported that verbascoside and oleuropein are the main polyphenols contributing to the measurement of the FRAP values in the olive leaf extract.[10] This result would support the steady FRAP values of the extracts throughout the digestion (Fig. 3a and 3b). However, although the luteolin glucoside in the extracts is much less concentrated, its contribution to the final stages of the digestion process would be significant.

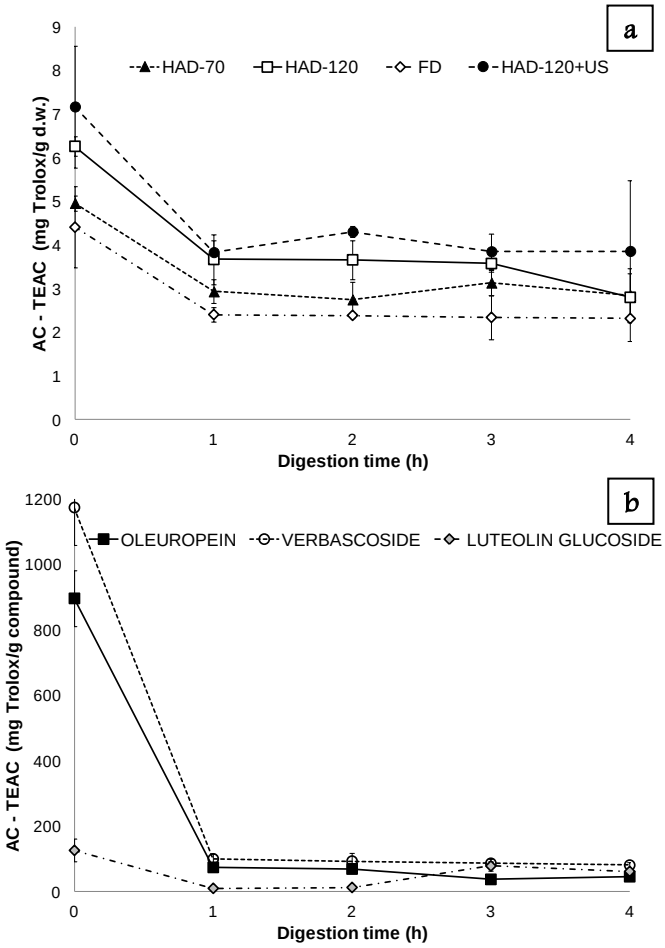


**Fig. 4.** Kinetics of antioxidant capacity (AC) measured by TEAC method during the *in vitro* digestion of olive leaf extracts (a) and standard compounds (b). Means ± standard deviation are plotted.

Moreover, the formation of polyphenol derivatives during the digestion with enhanced AC, as well as potential synergistic effects among individual olive phenols, could contribute to keep the AC[34] measured by FRAP despite the observed reduction in the TPC.

In general terms, processing conditions did not largely affect the AC kinetic of the extracts during the *in vitro* digestion. However, as in the case of the TPC, both AC methods confirmed that the US extracts withstood the gastrointestinal digestion slightly better than the conventional extracts.

Considering that AC and TPC are global measurements of the extracts' antioxidant potential and taking into account that the polyphenolic composition may vary throughout the digestion, a more thorough characterization of the extracts was proposed during the digestion process. Hence, the HPLC-DAD/MS-MS was chosen as the most reliable alternative to understand how individual olive leaf polyphenols evolved during *in vitro* digestion.

## 3.3. Degradation kinetics of the individual olive leaf polyphenols during *in vitro* digestion

With the aim of gaining insight into the stability of the major olive leaf polyphenols under gastric and intestinal conditions and to design strategies aimed at increasing their bioaccessibility in the gut, their contents were monitored during the *in vitro* digestion (0, 1, 2, 3 and 4 h) (Fig. 5).

The content of the studied phenolic compounds underwent a significant ($p<0.05$) decrease during the first hour of the gastric digestion. However, from 1 to 2 h the gastric conditions did not promote significant changes in the polyphenol concentrations and the content of every compound was almost constant. Oleuropein degradations ranged from 26 to 61 %, depending on the extract source (Fig. 5a). The most resistant oleuropein was the one extracted from HAD-120 leaves by means of US-assisted extraction (HAD-120+US). As far as the verbascoside is concerned (Fig. 5b), its degradation during the first hour under gastric conditions ranged from 22 to 31 %. Thus, it could be stated that verbascoside was less affected during gastric

incubation than oleuropein. The reduction in the luteolin glucoside content after 1 h of digestion was around 45 % for the extracts obtained by conventional extraction (Fig. 5c). However, a significantly ($p<0.05$) greater reduction (63 %) was found in the US extract. Dietary polyphenols have been reported to enjoy a good degree of stability during gastric digestion,[28,35,36] including all the major olive oil phenolics, such as tyrosol, hydroxytytrosol and related secoiridoids.[13] Our results show that verbascoside is a little more resistant to gastric digestion than oleuropein and luteolin glucoside.

The intestinal *in vitro* digestion hardly affected the luteolin-7-O-glucoside content (Fig. 5c). However, the main part of the verbascoside degradation during *in vitro* simulation happened under intestinal conditions (Fig. 5b) probably due to the fact that it is extremely susceptible at high pH.[37] As a consequence, the verbascoside practically disappeared, with its final content being close to 0 mg/g d.w. Although the oleuropein reduction was less marked than that of verbascoside, the intestinal phase affected oleuropein more than the gastric period (Fig. 5a). Thus, pancreatic enzymatic activity and alkaline pH contributed to a reduction in the bioaccessibility of oleuropein and verbascoside extracted from olive leaves, and both compounds almost disappeared from the medium. Some authors have also pointed to the possible interaction of pancreatic α-amylase and trypsin with phenolic compounds.[38] Therefore, the study of the degradation kinetics of polyphenols and the polyphenol-protein interactions would allow the bioaccessibility of these bioactive compounds to be improved through the design of pharmaceutical formulations aimed at increasing the amount of these compounds present at the site of intestinal absorption.

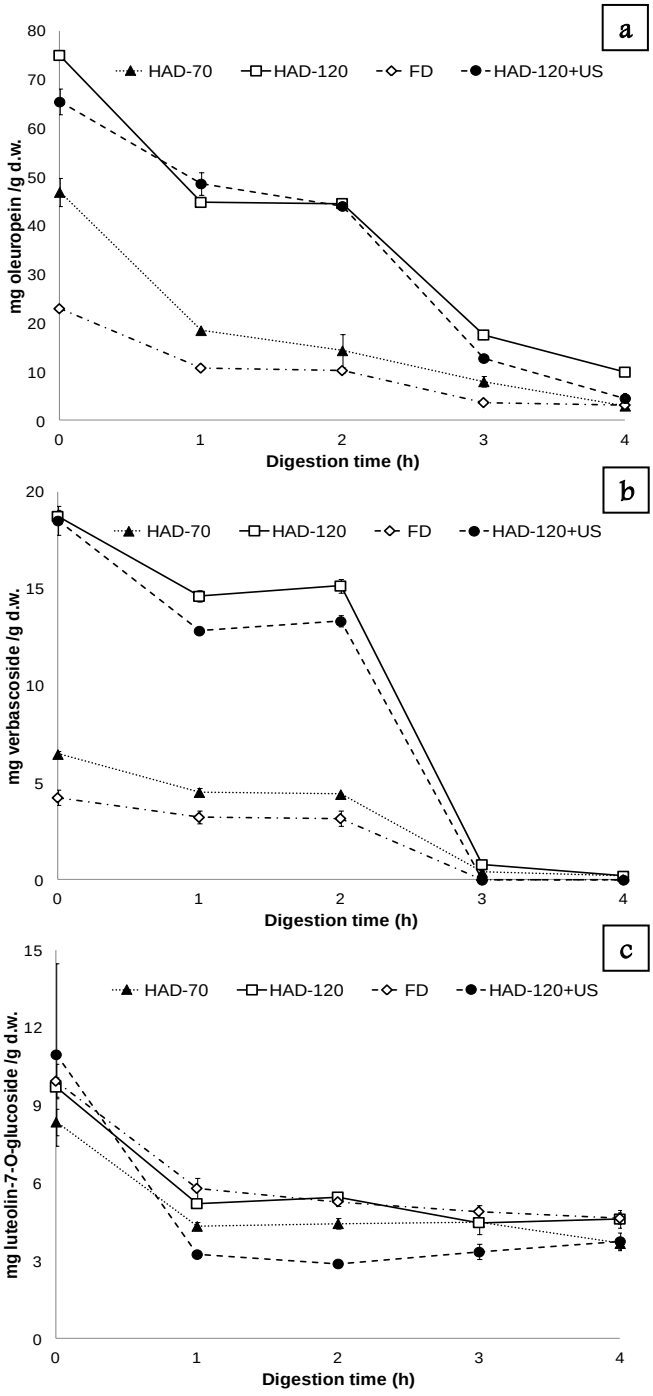


**Fig. 5.** Kinetics of oleuropein (a), verbascoside (b) and luteolin-7-O-glucoside (c) content during the in vitro digestion of olive leaf extracts. Means ± standard deviation are plotted.

Cooked amaranth leaves have been reported to exhibit β-carotene bioaccessibility values of 18 %.[39] Compared with this result, the percentage of bioaccessible luteolin-7-O-glucoside exhibited by olive leaf extracts was much higher, since 43 ± 6 % of its initial content was recovered after digestion. However, only 10 ± 4 % of oleuropein resisted the *in vitro* simulation, and most of the verbascoside practically disappeared in the extracts. Although oleuropein undergoes rapid hydrolysis under gastric conditions and it is rapidly degraded by the colonic microflora, leading to significant increases in the amount of tyrosol and hydroxytyrosol, [40,41] neither of these compounds was found in the HPLC-DAD/MS-MS analysis.

Another set of experiments was carried out to confirm if the stability of the compounds behaved in a similar manner when these were in the extract or in isolated form. Thus, oleuropein, verbascoside and luteolin glucoside standard solutions were subjected to complete digestions (Fig. 6). Oleuropein and verbascoside solutions were quite resistant to gastric conditions and behaved similarly to the digestions of the extracts (Fig. 6a and b). Thus, in both cases, the amount of these compounds at the end of the pancreatic digestion was almost negligible; therefore, their level of absorption must be even lower, as reported.[41,42] In contrast, isolated luteolin showed a completely different stability from that contained in the extract. Whereas the luteolin glucoside in olive leaf extracts remained fairly stable after the first hour of digestion, it was not identified by HPLC in the standard solution during gastric digestion and was detected again during intestinal digestion, as shown in Fig. 2b and 3b. As mentioned above, this effect could be due to the precipitation of this compound under acidic gastric conditions or its complexation with proteins under gastric conditions, a fact that may be enhanced when in pure form. Both digestions (Fig. 5c and 6c) demonstrate the pancreatic-biliar resistance of luteolin.

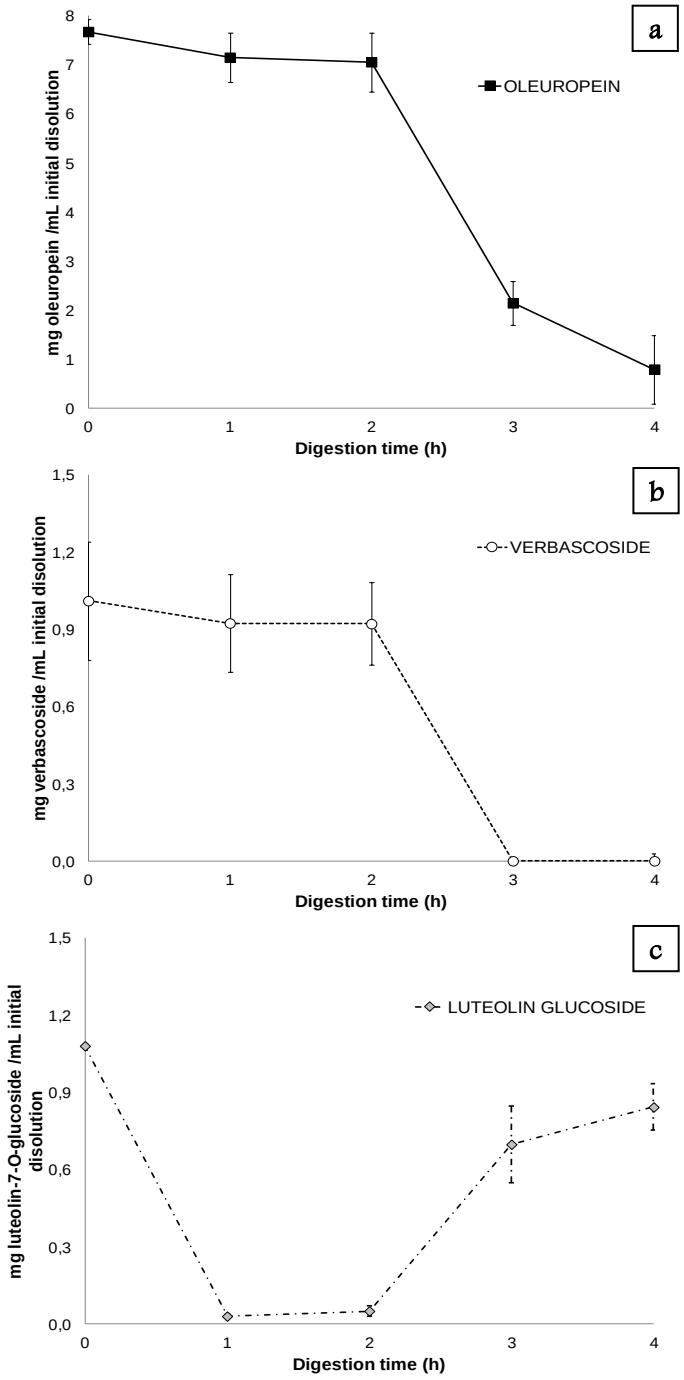


**Fig. 6.** Kinetics of oleuropein (a), verbascoside (b) and luteolin-7-O-glucoside (c) content during the *in vitro* digestion of its standard solutions. Means ± standard deviation are plotted.

In conclusion, the degradation kinetics of the major bioactive compounds derived from olive leaf extracts was not dependent on the type of extract or source. Hence, neither the drying treatment of olive leaves nor the extraction method (conventional or US application) had a significant ($p<0.05$) influence on oleuropein, verbacoside and luteolin-7-O-glucoside behavior during *in vitro* digestion. The most relevant finding of our study is that the amounts of oleuropein and verbascoside at the end of the digestion processes were almost negligible, mainly due to their instability during intestinal digestion. Interestingly, luteolin-7-O-glucoside was fairly resistant to digestion, and therefore must be considered as an interesting polyphenol for further absorption processes.

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## Abbreviations used

HAD, hot air drying; FD, freeze drying; TPC, total phenolic content; GAE, gallic acid equivalents; AC, antioxidant capacity; FRAP, ferric-reducing ability power; TPTZ, 2,4,6-Tri(2-pyridyl)-s-triazine; TEAC, trolox equivalent antioxidant capacity; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); HPLC-DAD, high performance liquid chromatography with diode array detection; MS-MS, tandem mass spectrometry; ESI, electrospray ionization; UV, ultraviolet; US, ultrasound.

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# *Drying and storage of olive leaf extracts. Influence on polyphenols stability*

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## Drying and storage of olive leaf extracts. Influence on polyphenols stability


### ABSTRACT

There is an increasing demand for natural antioxidants in food, cosmetics and pharmaceutical industries which has led to the search for not only natural extracts but also strategies with which to increase long-term storage stability. The aim of this work was to assess the influence of the drying and storage of olive leaf extracts on the bioactive potential and stability of polyphenols. Olive leaves were hot air dried (120 °C) and freeze dried. Then the extracts were obtained by maceration (ethanol–water, 80:20, v/v). Afterwards, a part of the extracts was dehydrated at 120 °C and vacuum dehydrated at 55 °C. The extracts obtained (liquids and powders) were stored at 4, 25 and 35 °C for 4 weeks. During this period, the extracts were characterized by determining the antioxidant capacity (AC), the total phenolic content (TPC) and the concentration of the major phenolic compounds.

The experimental results highlighted that drying the raw material not only influenced the initial extract composition but also the bioactive potential evolution during storage. Regardless of the method used, extract dehydration, reduced both AC and TPC by around 10 %. Finally, storage conditions (temperature and extract form) did not have a significant ($p<0.05$) effect on the extracts' antioxidant potential.


***Keywords:*** olive leaf, dehydration, storage, antioxidant capacity, total phenolic content, oleuropein.

## 1. Introduction

The health-related benefits of olive oil have mainly been ascribed to both the monounsaturated fatty acids and also to the presence of functional bioactive molecules, including tocopherols, carotenoids, phospholipids and phenolic compounds (Covas, 2008; Covas et al., 2006). Notwithstanding this, polyphenols with low molecular weight and potential health benefits, such as oleuropein and hydroxytyrosol, have also been found in olive leaves (Aouidi et al., 2012; Dekanski et al., 2011; Raederstorff, 2009). The phenolic compounds present in olive leaves exhibit bioactive properties; they are antioxidant, anti-hypertensive and anti-inflammatory as well as hypoglycaemic and hypocholesterolemic (Brahmi et al., 2012; Karakaya, 2009). Therefore, exploring the use of olive leaves and their extracts in the medical, cosmetic or food industry could be relevant. Among other things, the development of new products, such as natural drugs or functional foods, as well as the extension of the shelf life in foodstuffs could be some of their applications.

Generally, plant preparations are marketed in the form of liquid extracts, or as powders resulting from the drying of plant material or the liquid extracts themselves (Souza et al., 2008). On the one hand, it is well known that drying the raw material aids its preservation for longer periods of time and enhances the extraction of phenolic compounds. On the other hand, solid forms (powders) of plant extracts are being used increasingly due to the fact that they possess several advantages over fluid extracts: improved stability, cheaper transport and storage and the possibility of achieving higher concentrations (Moreira et al., 2009; Oliveira et al., 2006). However, it has been reported that drying can affect the activity and stability of bioactive compounds due to chemical and enzymatic degradation, losses caused by volatization and/or thermal decomposition (Dorta et al., 2012; Faustino et al., 2007).

Previous works illustrated that the hot air drying of olive leaves at high temperatures (120 °C) is an excellent pre-treatment, even better than freeze drying, prior to obtaining olive leaf extracts rich in bioactive compounds (Ahmad-Qasem et al., 2013a). Nevertheless, how raw material processing affects the further extract stability, as well as the impact of extract dehydration and storage conditions

(temperature and extract form: liquid or powder) on the evolution of antioxidant potential have not been explored. Spray drying is commonly used to dehydrate liquid extracts due to the fact that it presents several advantages, such as its operational flexibility and applicability to heat sensitive materials (Filková et al., 2007). However, as a means of minimizing the impact of temperature and/or exploring simpler and cheaper alternative methods of dehydrating, it is worth emphasizing the use of short-time drying at high temperatures and vacuum drying (Lewicki, 2006).

In consequence, the goal of this work was to assess the influence of the drying and storage of olive leaf extracts on the bioactive potential and polyphenols stability.

## 2. Materials and methods

### 2.1. Raw material

Olive leaves (*Olea europaea*, var. Serrana) were collected on a farm located in Segorbe (Castellón, Spain), packaged and stored at 4 °C until processed (less than 48 h). Following AOAC method nº 934.01, the initial moisture content was determined in a vacuum chamber at 70 °C until constant weight was reached (AOAC, 1997).

The olive leaves were dried by hot air (HAD) or freeze drying (FD). HAD experiments were conducted at 120 °C (HAD-120) for 12 min in a forced air laboratory drier (FD, Binder, Tuttlingen, Germany), using an initial mass load of 100 g and an air flow and air velocity of 0.094 $m^3$/s and 0.683 m/s, respectively. FD experiments (LIOALFA 6–50, Telstar, Madrid, Spain) were run over 24 h using a pressure of 1.4 $10^{-1}$ mbar, an initial mass load of 30 g, an initial temperature of -48 ± 2 °C and a tray temperature of 22 ± 2 °C. In both HAD and FD, the drying time was programmed to reach an initial weight loss of 42 ± 3 %. Dried olive leaves were packaged in plastic bags and stored at 4 °C until extraction was performed.

## 2.2. Extraction process

In order to obtain olive leaf extracts, the leaves were milled (Blixer 2, Robot Coupe USA, Inc., Jackson, MS, USA) and sieved (Metallic mesh size 0.05 mm, Filtra Vibración, Barcelona, Spain) to select particles with a diameter of less than 0.05 mm. Extractions were carried out in sealed containers, protected from light and immersed in a thermostatic shaking water bath (SBS40, Stuart, Staffordshire, UK). In these containers, the ratio between the weight of the olive leaves and the solvent (ethanol–water, 80:20, v/v) volume used was 3.75 g/30 mL. During extraction, the mixture was stirred (170 rpm) at 22 ± 1 °C for 24 h. Afterwards, the extracts were centrifuged for 10 min at 5000 rpm (Medifriger BL-S, J.P. Selecta, Barcelona, Spain), filtered (nylon filters of 0.45 μm) and placed in opaque vials. The extractions were performed, at least, in triplicate.

## 2.3. Dehydration of olive leaf extracts

For the purposes of evaluating the influence of the extract form (liquid or dehydrated powder) on its antioxidant potential, a part of the obtained extracts was dried by means of two different methods: convective drying at 120 °C using forced air at atmospheric pressure (A-120) and vacuum drying (0.2 bar) at 55 °C (V-55). Regardless of the method used, the extracts were kept in the drying chamber (Vaciotem, P-Selecta, Barcelona, Spain) until reaching constant weight. Afterwards, the powder was collected and stored in hermetic opaque vials. Drying tests were conducted in triplicate.

## 2.4. Storage conditions

The extracts were stored in two different forms: liquid and powder. To this end, one part of the dehydrated extracts was kept as powder, whereas another part was re-constituted by using ethanol–water (80:20, v/v) in order to recover the initial volume (before dehydration). Liquid and powder extracts were stored at 4, 25 and 35 °C for 4 weeks. Thus, different possible storage temperatures (refrigeration, room temperature and hot environments) were explored. During this period, the samples were taken out every 7 days (0, 7, 14, 21 and 28 days) for extract characterization as

described in the following sections. In the case of the dried powders, they were previously diluted in the extraction solvent (ethanol–water, 80:20, v/v).

## 2.5. Total phenolic content (TPC)

The phenolic content was determined by means of the Folin-Ciocalteu method (Singleton et al., 1999). Briefly, 100 μL of sample or blank ethanol–water (80:20, v/v) were mixed with 200 μL of Folin-Ciocalteu's phenol reagent (Sigma-Aldrich, Madrid, Spain) and 2 mL of distilled water. After 3 min at 25 °C, 1 mL of $Na_2CO_3$ (Panreac, Barcelona, Spain) solution ($Na_2CO_3$-water 20:80, w/v) was added to the mixture. The reaction was kept in the dark at room temperature for 1 h. Finally, the absorbance was read at 765 nm using a spectrophotometer (Helios Gamma, Thermo Spectronic, Cambridge, UK). The measurements were carried out in triplicate. The standard curve was previously prepared using solutions of a known concentration of gallic acid hydrate (Sigma-Aldrich, Madrid, Spain) in ethanol–water (80:20, v/v). Results were expressed as follows: mg of gallic acid (GAE) per g of dried olive leaf (d.m.) or mg GAE per mL of olive leaf extract.

## 2.6. Antioxidant capacity (AC)

The antioxidant capacity was determined by using the ferric-reducing ability power (FRAP) method, which is a simple method used to estimate the reduction of a ferric-tripyridyltriazine complex. It was applied following the procedure described by Benzie and Strain (1996), with some modifications. Briefly, 900 μL of freshly prepared FRAP reagent were mixed with 30 μL of distilled water and 30 μL of test sample or ethanol–water (80:20, v/v) as appropriate reagent blank and kept at 37 °C for 30 min. The FRAP reagent contained 2.5 mL of a 10 mM TPTZ (Fluka, Steinheim, Germany) solution in 40 mM HCl (Panreac, Barcelona, Spain) plus 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ (Panreac, Barcelona, Spain) and 2.5 mL of 0.3 M acetate buffer (Panreac, Barcelona, Spain), pH 3.6 (Pulido et al., 2000). Readings at the maximum absorption level (595 nm) were taken using a spectrophotometer (Helios Gamma, Thermo Spectronic, Cambridge, UK). Four replicates were carried out for each measurement. The antioxidant capacity was evaluated through a calibration curve, which was

previously determined using ethanol–water (80:20, v/v) solutions of known Trolox (Sigma-Aldrich, Madrid, Spain) concentrations and expressed as: mg Trolox per g of dried olive leaf (d.m.) or mg Trolox per mL of olive leaf extract.

## 2.7. Identification and quantification of polyphenols by HPLC-DAD/MS-MS

In order to identify and quantify the main polyphenols present in the olive leaf extracts, these were analyzed using an HPLC instrument (Agilent LC 1100 series; Agilent Technologies, Inc., Palo Alto, CA, USA) controlled by the Chemstation software. The HPLC instrument was coupled to an Esquire 3000+ (Bruker Daltonics, GmbH, Germany) mass spectrometer equipped with an ESI source and ion-trap mass analyzer, and controlled by Esquire control and data analysis software. A Merck Lichrospher 100RP-18 (5 μm, 250 x 4 mm) column was used for analytical purposes.

Separation was carried out through a linear gradient method using 2.5 % acetic acid (A) and acetonitrile (B), starting the sequence with 10 % B and programming the gradient to obtain 20 % B at 10 min, 40 % B at 35 min, 100 % B at 40 min, 100 % B at 45 min, 10 % B at 46 min and 10 % B at 50 min. For the LC-MS pump to perform accurately, 10 % of organic solvent was pre-mixed in the water phase. The flow-rate was 1 mL/min and the chromatograms monitored at 240, 280 and 330 nm. Mass spectrometry operating conditions were selected in order to achieve maximum sensitivity values. The ESI source was operated in negative mode to generate $[M-H]^-$ ions under the following conditions: desolvation temperature at 365 °C and vaporizer temperature at 400 °C; dry gas (nitrogen) and nebulizer were set at 12 L/min and 4.83 bar, respectively. The MS data were acquired as full scan mass spectra at 50–1100 m/z by using 200 ms for the collection of the ions in the trap.

Phenolic compounds were identified by means of an HPLC-DAD analysis, comparing the retention time, UV spectra and MS/MS data of the peaks in the samples with those of authentic standards or data reported in the literature. Only the major compounds were quantified using commercial standards: oleuropein (Extrasynthese, Genay Cedex, France) and luteolin-7-O-glucoside (Phytolab, Vestenbergsgreuth, Germany). A purified extract (96.85 %) provided by Universidad Miguel Hernández (Elche, Spain) was used to quantify verbascoside. The quantitative evaluation of the

compounds was performed with a calibration curve for each polyphenol, using ethanol (oleuropein) or methanol (verbascoside and luteolin) solutions of known concentration. The polyphenol concentrations were expressed as mg polyphenol per g of dried olive leaf (d.m.) or mg polyphenol per mL of olive leaf extract.

## 3. Results and discussion

### 3.1. Impact of raw material drying on extract stability during storage

For the purposes of assessing whether raw material processing can affect the extract properties during storage, fresh (F), hot air dried at 120 °C (HAD-120) and freeze dried (FD) olive leaves were used to obtain three different extracts. To block the possible influence of other variables, extracts were stored at 4 °C, since low temperatures are able to reduce the enzymatic activity of polyphenol oxidase and peroxidase (Balois-Morales et al., 2007).

The initial characterization (day 0) of the different extracts ratified the results obtained in previous works (Ahmad-Qasem et al., 2013a). Hence, HAD-120 leaves provide the extracts with the highest AC and TPC (Fig. 1). As can be observed in Fig. 1a, F leaves gave rise to extracts which had practically the same AC over the whole storage period. On the contrary, extracts from HAD-120 and FD leaves had a constant AC during the first stages of storage, but underwent a significant ($p<0.05$) reduction from day 21. Thus, at the end of the period under study (28 days), the drying of the raw material promoted a decrease in the initial AC (day 0). The intensity of this reduction was dependent on the drying method: 30 and 63 % for HAD-120 and FD leaves, respectively.

As regards the evolution of TPC during storage (Fig. 1b), unlike the AC, no decrease was appreciated regardless of how the raw material was processed. This fact could be linked to the polyphenols' ability to react with one another and/or degrade, leading to phenolic compounds with a higher or lower antioxidant capacity (Ryan et al., 2003).

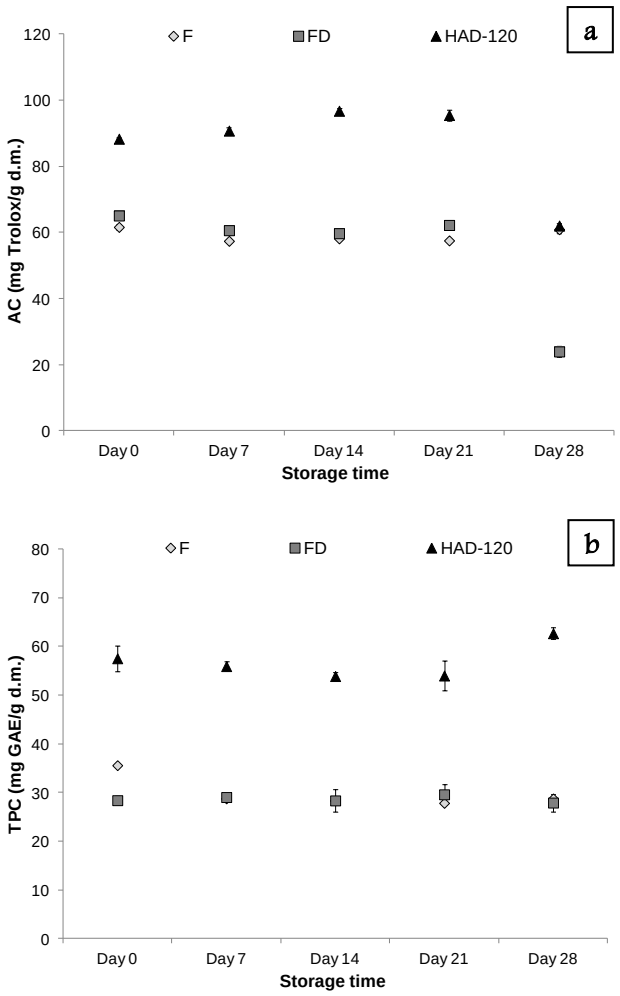


**Fig. 1.** Evolution of antioxidant capacity (a; AC) and total phenolic content (b; TPC) during storage at 4 °C of extracts obtained from fresh (F), freeze dried (FD) and hot air dried (HAD-120) olive leaves.

F, HAD-120 and FD leaves provide extracts with similar phenolic profiles, coinciding with the ones found in previous works (Ahmad-Qasem et al., 2013a, 2013b). Thus, the major polyphenols identified were oleuropein, verbascoside and luteolin and apigenin derivates. Nevertheless, the content of these polyphenols was significantly ($p<0.05$) different in the three extract types. The highest concentrations corresponded to extracts obtained from HAD-120 leaves (Fig. 2), which also exhibited the highest AC and TPC (Fig. 1). In this sense, the elevated content of

oleuropein (Fig. 2a) achieved with HAD-120 leaves should be noted (93.98 ± 1.07 mg oleuropein/mg d.m.). This was quite similar to that reported by Ahmad-Qasem et al. (2013a, 2013b), and 5 and 10 times higher than in extracts from FD and F leaves, respectively.

It should be noticed that the stability of polyphenols during storage was dependent on the phenolic compound studied and the raw material process used (fresh or dried leaves). The oleuropein content (Fig. 2a) increased from day 14 to the end of storage in both HAD-120 and FD samples, raising the initial content (day 0) by 10.3 and 55.1 %, respectively. Nevertheless, it remained almost constant in the case of F samples. The drying method also significantly ($p<0.05$) affected the verbascoside concentration reached at the end of storage (Fig. 2b). Thereby, compared to day 0, the content of verbascoside increased in HAD-120 extracts (7.7 %) whereas it slightly diminished in the FD ones (7.9 %). In the case of luteolin-7-O-glucoside (Fig. 2c), the drying of the raw material promoted the degradation of this compound throughout the storage by approximately 17 % of the initial content in both FD and HAD-120. On the contrary, in extracts obtained from F leaves, the luteolin-7-O-glucoside content was kept quite constant over the 28 days of storage. It must be highlighted that the content evolution of an individual polyphenol during storage could be conditioned by the rest of the phenolic compounds present in the extract (Mullen et al., 2006; Porrini and Riso, 2008), which would explain the different trends found for major polyphenols in F, HAD-120 and FD extracts.

Considering the different response of the phenolic compounds quantified, it seems that the concentrations of oleuropein and verbascoside would contribute to maintain the TPC of extracts during storage (Fig. 1b). Nevertheless, the reduction in AC observed at the end of storage could be ascribed not only to the degradation of luteolin-7-O-glucoside and other minority compounds but also to the abovementioned transformation of polyphenols into others with lower AC. As an example, hydroxytyrosol, a product of the degradation of oleuropein and other compounds and, at the same time, a precursor of oleuropein, has a higher antioxidant capacity (Benavente-García et al., 2000). Thus, despite the fact that hydroxytyrosol was not detected at the moment of performing the HPLC-DAD/MS-MS analysis, it

could be considered that the formation of oleuropein (Fig. 2a) from hydroxytyrosol took place in the extracts, reducing the AC (Fig. 1a).

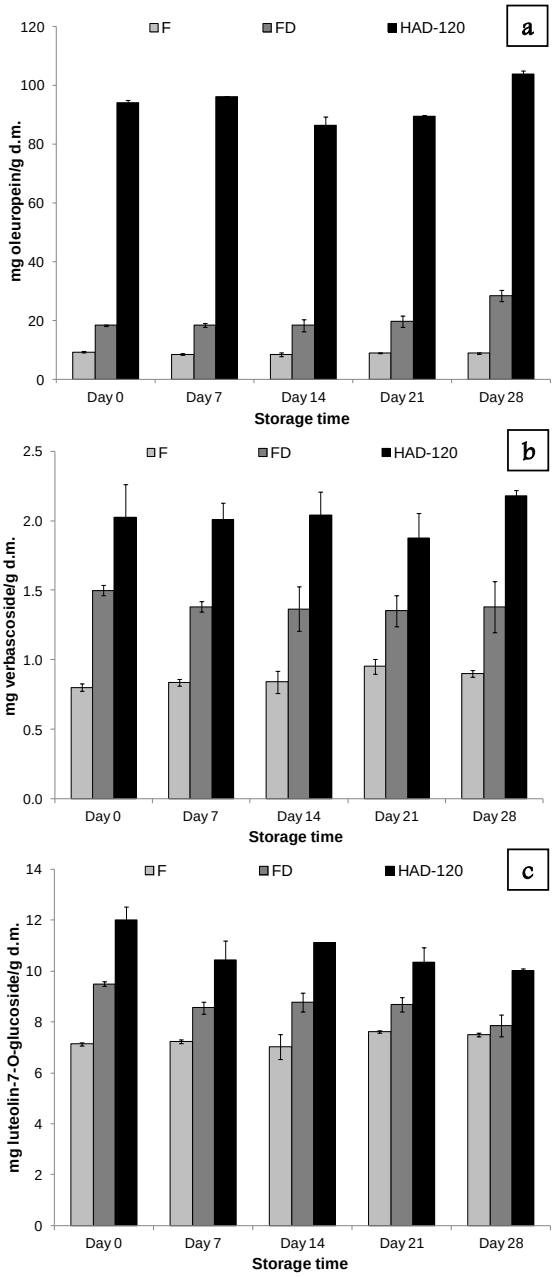


**Fig. 2.** Evolution of oleuropein (a), verbascoside (b) and luteolin-7-O-glucoside (c) content during storage at 4 °C of extracts obtained from fresh (F), freeze dried (FD) and hot air dried (HAD-120) olive leaves.

## 3.2. Influence of extract dehydration on the antioxidant potential

In order to evaluate how the drying of the extract affects its antioxidant potential, the extracts obtained from olive leaves dried at 120 °C (HAD-120) were dehydrated following two different methods: drying at atmospheric pressure and 120 °C (A-120) and vacuum drying at 55 °C (V-55). Afterwards, the extracts were re-diluted to recover the initial volume (before dehydration), and the AC, the TPC and the content of the main phenolic compounds identified were analyzed (oleuropein, verbascoside and luteolin-7-O-glucoside).

Dehydration had a significant ($p<0.05$) effect on the antioxidant potential of the extracts (Table 1). Thus, regardless of the method used, dehydration reduced both the AC and TPC of the extracts by around 10 %.

**Table 1.** Characterization of the initial extract obtained from olive leaves dried at 120 °C (HAD-120) and the extract after its dehydration at 120 °C (A-120) and at 55 °C by applying vacuum (V-55).

| | Initial | Dehydrated at 120 °C | Dehydrated at 55 °C+V |
|---|---|---|---|
| AC (mg Trolox/mL extract) | 13.36 ± 0.09 $^{a}$ | 12.08 ± 0.17 $^{b}$ | 12.4 ± 0.5 $^{b}$ |
| TPC (mg GAE/mL extract) | 8.20 ± 0.03 $^{a}$ | 7.60 ± 0.05 $^{b}$ | 7.33 ± 0.04 $^{b}$ |
| Oleuropein $^{*}$ | 13 ± 1 $^{a}$ | 10.9 ± 0.3 $^{b}$ | 13.7 ± 0.8 $^{a}$ |
| Verbascoside $^{*}$ | 0.27 ± 0.03 $^{a}$ | 0.2600 ± 0.0114 $^{a}$ | 0.291 ± 0.014 $^{a}$ |
| Luteolin-7-O-glucoside $^{*}$ | 1.328 ± 0.006 $^{a}$ | 1.17 ± 0.03 $^{b}$ | 1.32 ± 0.08 $^{a}$ |

* Content expressed as mg compound/mL extract.

$^{a-d}$ Show homogeneous groups in the same row established from LSD (Least Significance Difference) intervals ($p<0.05$).

This mild reduction has been reported when other natural extracts have been dried by means of different techniques. Thus, Fang and Bhandari (2011) observed that the spray drying of bayberry juice reduced the AC and the TPC of the initial extracts by 6 % and 4 %, respectively. In the same way, Benelli et al. (2013) were able to dry aromatic plant extracts by spouted bed, reducing the TPC of the original extracts by only 8.5 %. The decrease in the initial antioxidant potential as a result of

extract dehydration could be ascribed to the fact that polyphenols are less protected in extracts than during the drying of raw material. Hence, phenolic compounds extracted from vegetable materials would be more exposed to the oxygen, which would favor the oxidation of bioactive compounds (Nicoli et al., 1999). Moreover, high temperatures could promote the degradation and/or volatilization of some heat sensitive compounds (Dorta et al., 2012).

Despite the fact that V-55 dehydration reduced the AC and TPC, this method caused no significant ($p<0.05$) changes in the concentration of the major phenolic compounds quantified in the extracts (Table 1). Consequently, oleuropein, verbascoside and luteolin-7-O-glucoside contents were quite similar to those determined in the initial extract (obtained from HAD-120 leaves). On the contrary, the A-120 drying significantly ($p<0.05$) lessened the oleuropein and luteolin-7-O-glucoside concentration, by 16.2 and 11.9 %, respectively. The negative effect of high temperature on the luteolin content was consistent with the results reported by Ungar et al. (2003) for other flavonoids, such as genistein and daidzein. Moreover, in this work, the luteolin-7-O-glucoside degradation during extract dehydration could also be favored by the abovementioned contact with oxygen.

In any case, when drying olive leaf extracts, the differences between the two dehydration methods tested (V-55 and A-120) could be considered minimum. As stated before, only some variations were found in the oleuropein and luteolin-7-O-glucoside content. Nevertheless, there was a great influence of the method used on the dehydration time, it being about 4.5 ± 0.1 h for A-120 and 19.5 ± 0.1 h for V-55. Therefore, for the purposes of the industrial dehydration of olive leaf extracts, it would be more advisable to conduct the process at 120 °C due to the shortening of processing times, which has direct implications on productivity. Moreover, A-120 would facilitate continuous processing and avoid the use of vacuum systems, which makes industrial facilities more affordable.

## 3.3. Influence of time and storage conditions on the antioxidant potential of dehydrated extracts

In order to determine the effect of storage conditions on the phenolic composition and antioxidant capacity of olive leaf dehydrated extracts (V-55 and A-120), these were stored at different temperatures (4, 25 and 35 °C) and in two different forms for 28 days. A batch of samples was directly stored as powder, whereas another batch was stored as liquid after its re-dilution.

The experimental results highlighted that neither the extract form (liquid or powder) nor the storage temperature significantly ($p<0.05$) affected the AC (Fig. 3).

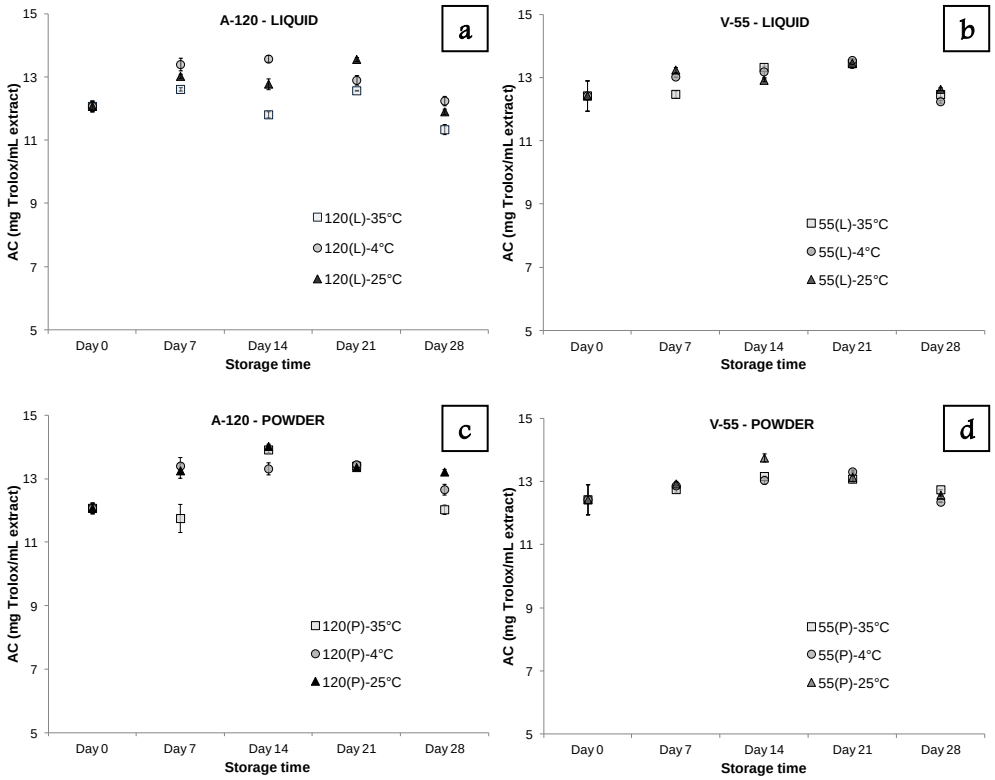


**Fig. 3.** Evolution of antioxidant capacity (AC) at 4, 25 and 35 °C of olive leaf extracts dehydrated at 120 °C (A-120) and stored as liquid (a), vacuum dehydrated at 55 °C (V-55) and stored as liquid (b), dehydrated at 120 °C (A-120) and stored as powder (c) and vacuum dehydrated at 55 °C (V-55) and stored as powder (d).

Nevertheless, in overall terms, it was possible to appreciate a slight increase in AC until day 21 of storage. After this time, a reduction took place, giving rise to final AC values (day 28) close to the initial ones (day 0). This drop during the last week of storage was coherent with the abovementioned one for the non-dehydrated extracts obtained from dried olive leaves and stored at 4 °C (Fig. 1a). Despite the lack of a clear influence of storage conditions, some particularities are worthy of consideration. The extracts dehydrated at 120 °C (A-120) and stored as powder (Fig. 3c) showed a final AC which was slightly higher than liquid extracts (Fig. 3a), especially at 4 and 25 °C where the increases in the initial AC (day 0) were 4.9 and 9.5 %, respectively.

TPC evolution (Fig. 4) during the storage was, as in the case of the AC, quite similar to non-dehydrated extracts (Fig. 1b). Hence, TPC remained practically constant. As far as the different storage conditions (temperature and form of extract) are concerned, they hardly affected the TPC of A-120 extracts (Fig. 4a and c). However, in V-55 powder extracts (Fig. 4d) the storage temperature had a significant ($p<0.05$) effect. Thus, the TPC decreased by 32.4 % at the end of the storage (day 28) at 35 °C (55(P)-35 °C), whereas it remained almost constant at 4 and 25 °C.

The results obtained in AC and TFC differed from those reported by Flores et al. (2014). These authors studied the storage of spray dried blueberry pomace extract for 16-42 days at different temperatures, appreciating not only an increase in the TPC of the extracts but also in their AC. Olive leaf extracts did not exhibit antioxidant potential enhancement during storage (Fig. 3 and 4). Nevertheless, from the results obtained, it could be affirmed that, both liquid (with high water activity) and powder extracts were able to preserve the initial antioxidant potential at the temperatures evaluated. This fact was also unlike that found in other works, where the increase in the storage temperature and water activity of spray dried extracts favored the degradation of the phenolic compounds and the reduction in the antioxidant capacity (Fang and Bhandari, 2011; Vongsak et al., 2013).

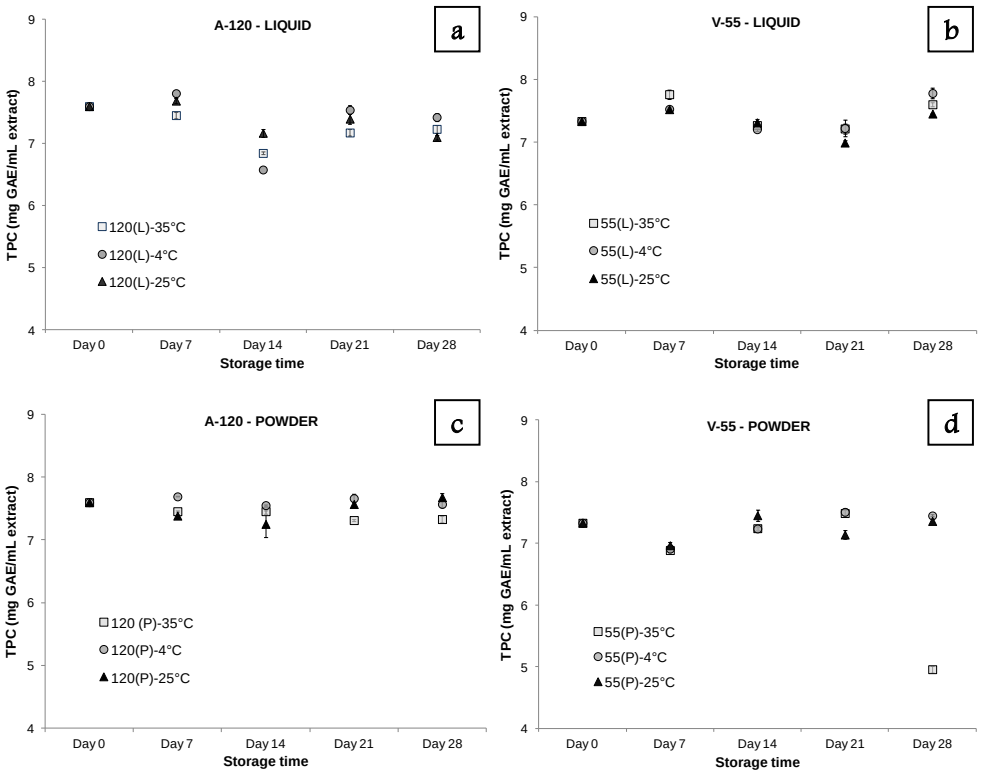


**Fig. 4.** Evolution of total phenolic content (TPC) at 4, 25 and 35 °C of olive leaf extracts dehydrated at 120 °C (A-120) and stored as liquid (a), vacuum dehydrated at 55 °C (V-55) and stored as liquid (b), dehydrated at 120 °C (A-120) and stored as powder (c) and vacuum dehydrated at 55 °C (V-55) and stored as powder (d).

Storage conditions did not significantly ($p<0.05$) influence the content of the major polyphenolic compounds quantified in the extracts. Thus, only the content at the end of the storage period (day 28) was depicted in Fig. 5. None of the temperature-extract form combinations involved a remarkable reduction in the final content of oleuropein, verbascoside and luteolin-7-O-glucoside (Table 1 and Fig. 5). Nevertheless, the extract forms (powder or liquid) especially affected the V-55 extracts stored at 4 °C. In this case, there were lower concentrations of all the main polyphenols measured in powder than in liquid extracts. The differences were around 11.9, 20.6 and 15.4 % for oleuropein (Fig. 5b), verbascoside (Fig. 5d) and luteolin-7-O-glucoside (Fig. 5f), respectively.

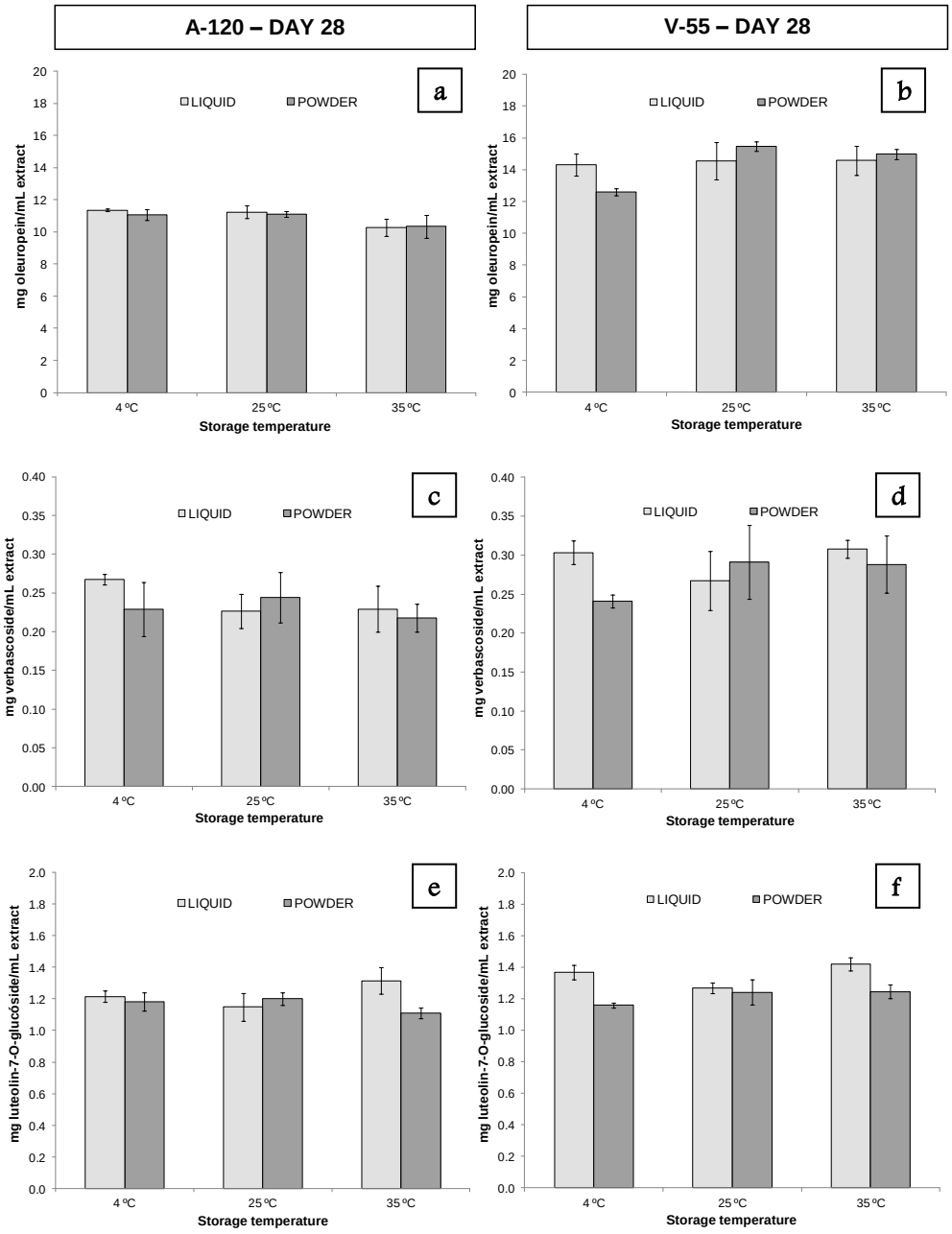


**Fig. 5.** Content of oleuropein (a and b), verbascoside (c and d) and luteolin-7-O-glucoside (e and f) in olive leaf extracts dehydrated at atmospheric pressure and 120 °C (A-120) or applying vacuum at 55 °C (V-55) after storage under different conditions for 28 days.

To sum up, the results obtained indicated that previously dehydrated olive leaf extracts remained stable during the studied period (28 days), at temperatures ranging from 4 to 35 °C and in both powder and re-diluted form.

## 4. Conclusions

The drying of olive leaves determined not only the initial bioactive content, the antioxidant capacity and the concentration of the main polyphenols in the extracts but also their evolution during storage. As regards extract dehydration, this operation only implied a reduction in the TPC and AC of around 10 %. Moreover, in terms of phenolic characterization, no remarkable differences were found between the dehydration treatments tested (drying at 120 °C and vacuum drying at 55 °C).The storage study revealed that, at least for 4 weeks, the antioxidant potential of olive leaf extracts could be considered as stable, regardless of the extract form (liquid or powder) and the storage temperature tested (4-35 °C).

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## Abbreviations used

F, fresh; HAD, hot air drying; HAD-120, hot air drying at 120 °C; FD, freeze drying; A-120, dehydration of extracts at 120 °C using forced air at atmospheric pressure; V-55, dehydration of extracts at 55 °C applying vacuum (0.2 bar); TPC,

total phenolic content; GAE, gallic acid equivalents; AC, antioxidant capacity; FRAP, ferric-reducing ability power; TPTZ, 2,4,6-tri(2-pyridyl)-s-triazine; HPLC-DAD, high performance liquid chromatography with diode array detection; MS-MS, tandem mass spectrometry; ESI, electrospray ionization; LC-MS, liquid chromatography-mass spectrometry; UV, ultraviolet; 120(L), extracts dehydrated at 120 °C and stored as liquid; 120(P), extracts dehydrated at 120 °C and stored as powder; 55(L), extracts vacuum-dehydrated at 55 °C and stored as liquid; 55(P), extracts vacuum-dehydrated at 55 °C and stored as powder.

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# CHAPTER 4

## *Prospective applications*

Food and Bioprocess Technology, 2015, 8, 120-133.

# *Influence of drying on the retention of olive leaf polyphenols infused into dried apple*

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# Influence of drying on the retention of olive leaf polyphenols infused into dried apple


## ABSTRACT

Olive leaf extracts are rich in polyphenolic compounds. Their inclusion by impregnation in food solid matrices could improve the nutritional value and antioxidant capacity of dietary products, such as apple. Drying the food matrix is interesting not only because it speeds up the infusion but also because of its effect on the final stabilization of impregnated food. In this work, the influence of drying method on the retention of infused olive leaf polyphenols in a solid matrix (apple) was addressed. For this purpose, apple cubes (10 mm side) were initially dehydrated by freeze drying or hot air drying at 60 °C and then impregnated with the olive leaf extract. After the polyphenolic infusion, samples were dried for the final stabilization by means of three different methods: freeze drying and hot air drying at 60 °C with and without ultrasound application. The retention of infused polyphenols in apple samples was evaluated by determining the total phenolic content and antioxidant capacity and quantifying the main olive leaf polyphenols by HPLC-DAD/MS–MS. The drying kinetics and the loss of apple solids during impregnation were modeled by using diffusion equations and the Weibull model, respectively. The role of fresh apple drying on the retention of infused olive leaf polyphenols was more significant than the further drying of the impregnated apple. Thus, hot air drying of fresh apple provided the highest antioxidant capacity (47.1 ± 2.6 mg Trolox/g d.m.) and oleuropein contents in the final dried apple of up to 1928 mg/100 g d.m. were found.


***Keywords***: dehydration, impregnation, modeling, antioxidant potential, HPLC-DAD/MS-MS.

## 1. Introduction

Due to new lifestyles, a large group of the population lacks a generous intake of basic foods (Schieber et al., 2001), such as fruit and vegetables and, therefore, of their nutritional and bioactive compounds. In consequence, the requirements of modern-day society and the demands of the market have promoted the innovation and development of new products. Nowadays, there is a growing demand for snacks that not only provide convenience and taste but also provide nutritional and health benefits (Jack et al., 1997; Zandstra et al., 2001). Thus, the impregnation of vegetable solid matrices with bioactive compounds has gained importance in recent years.

Apple is one of the most widely consumed fruits (fresh and dehydrated). Its tissue, composed of parenchyma cells, interspersed with air and liquid gaps (Khan & Vincent, 1990), facilitates the infusion of solutions, which is particularly noticeable if the water is previously removed, e.g. by drying. The most commonly used impregnation mediums have been water, sweet solutions or fruit juices. However, the increasing attention paid to the role played by natural active ingredients and their beneficial effects on human health, such as antioxidants (Fernandes et al., 2011), has opened up a new research topic in the field of the impregnation of food products. In this sense, although the infusion of ascorbic acid solutions (Blanda et al., 2008a) and osmotic solutions enriched with grape phenolic compounds (Ferrando et al., 2011; Rózek et al., 2010) into apples has been the subject of previous studies, none of them have addressed the infusion of pure plant extracts. Olive leaf extracts could be an interesting material with which to impregnate food products since they are rich in phenolic compounds, such as oleuropein, verbascoside and luteolin glucoside (Ahmad-Qasem et al., 2013a, 2013b), with noticeable bioactive properties (Karakaya, 2009; Menéndez et al., 2013).

Osmotic treatments (Ferrando et al., 2011; Rózek et al., 2010) and vacuum impregnation (Blanda et al., 2008a) are the techniques which are most commonly used as a means of including compounds of interest in solid matrices. In solid-liquid treatments, mass transfer depends not only on the properties of the solution and the working pressure but also on the structure of the solid matrix (Spiess & Behsnilian,

1998). Thus, in the rehydration operation of the previously dried matrix, the degree of rehydration is linked to the level of cellular and structural disruption (Cunningham et al., 2008). Therefore, the drying operation greatly influences the infusion rate and capacity. Moreover, once the solid matrix is impregnated, a further dehydration stage is necessary in order to improve its shelf life and reduce storage costs. To some extent, this final drying stage could also affect the infused compounds, and therefore it should be carefully designed.

On the one hand, forced air drying, using hot air, is the most commonly used drying technique due to its simplicity and the fact that it is relatively low cost. As is well known, air drying induces physical and chemical changes, such as shrinkage, porosity decrease, textural changes, loss of nutritional value and color modifications (Lewicki & Jakubczyk, 2004; Maskan, 2001). On the other hand, freeze drying provides products with the highest nutritional quality (Mujumdar & Law, 2010) and a minimal reduction of volume (Jankovié, 1993). However, the high cost of implementation and operation of freeze drying limits its use to only high quality products. Recently, in order to provide new alternatives to conventional dehydration methods, new emerging technologies have been developed, such as power ultrasound assisted drying or low-temperature dehydration (García-Pérez et al., 2012). The feasibility of ultrasound technology has been demonstrated for intensifying not only drying operations but also other relevant food processes. Indeed, literature reports ultrasonically enhanced extraction of natural compounds (Esclápez et al., 2011), inactivation of microorganisms with supercritical carbon dioxide (Ortuño et al., 2014), filtration, sterilization/pasteurization and emulsification (Chemat et al., 2011).

The development of novel processing techniques to obtain healthier and safer food products is one of the major challenges facing the food industry in the new century (Barros, 2011). Thus, the effective incorporation of natural bioactive compounds, such as olive leaf polyphenols, into food matrices would be an interesting achievement. For that purpose, it is necessary to evaluate the different processing steps accurately. Thus, the goal of this work was to assess the influence of the drying method on the retention of olive leaf polyphenols impregnated into previously dried

apple. Both the initial drying of the raw apple and the further drying of the impregnated apple with polyphenols were addressed.

## 2. Materials and methods

### 2.1. Raw materials

Olive leaves (*Olea europaea*, var. Serrana) were collected on a farm located in Segorbe (Castellón, Spain), packaged and stored at 4 °C until drying (less than 48 h). The initial moisture content was determined according to the Association of Official Analytical Chemists (AOAC) method no. 934.01. For that purpose, samples were kept in a vacuum chamber at 70 °C until constant weight was reached (AOAC, 1997).

The olive leaves were hot air dried at 120 °C for 12 min in a forced air laboratory drier (FD, Binder, Tuttlingen, Germany) using an initial mass load of 150 g, an air flow of 0.094 $m^3/s$ and an air velocity of 0.683 m/s. The dehydration process was finalized when the samples lost 40 ± 1 % of the initial weight. After drying, olive leaves were packaged in plastic bags and stored at 4 °C until the extraction operation.

In order to obtain olive leaf extracts, the leaves were milled (Blixer 2, Robot Coupe USA, Inc., Jackson, MS, USA). The obtained powder was sieved (Metallic mesh size 0.05 mm, Filtra Vibración, Barcelona, Spain) to select particles with a diameter of less than 0.05 mm. The extractions were carried out in sealed containers, protected from light and immersed in a thermostatic shaking water bath (SBS40, Stuart, Staffordshire, UK). The ratio between the weight of the olive leaves and the solvent (water) volume used was 10 g/150 mL. During extraction, the mixture was stirred (170 rpm) at 22 ± 1 °C for 24 h. Afterwards, the extracts were centrifuged for 10 min at 5000 rpm (Medifriger BL-S, J.P. Selecta, Barcelona, Spain), filtered (nylon filters of 0.45 μm) and stored in opaque vials or bottles at 4 °C until used for apple impregnation.

Cubes of 10 mm side were obtained from the apple flesh (*Malus domestica* cv. Granny Smith) by using a cutting machine (CL50 Ultra, Robot Coupe USA, Inc.,

Jackson, MS, USA) and immediately processed. Following AOAC method no. 934.06, the initial moisture content was determined by drying in a vacuum chamber at 70 °C until reaching constant weight (AOAC, 1997).

## 2.2. Apple drying experiments

For the purposes of obtaining the solid matrix to be impregnated, fresh apple cubes were dehydrated by means of two different methods: freeze drying (FD) and hot air drying (HAD). Once the samples were impregnated, further dehydration was carried out by freeze drying (FD) and hot air drying with (HAD-US) or without power ultrasound (HAD) application. A scheme of the experiments carried out and the nomenclature used is shown in Fig. 1.

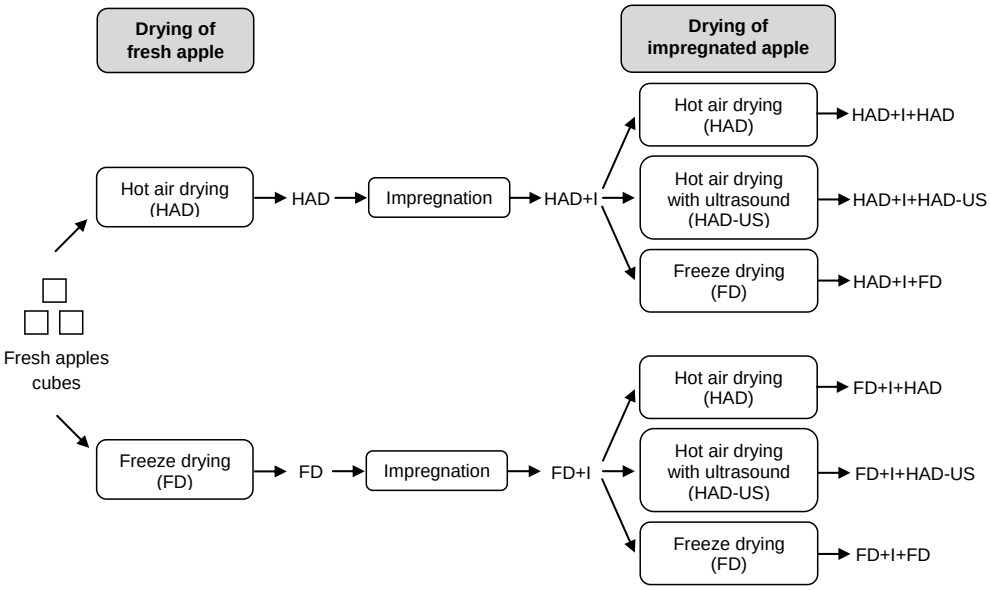


**Fig. 1.** Sequence of different treatments undergone by apple samples.

The FD experiments were conducted at an initial temperature of -48 ± 2 °C, keeping the shelf temperature at 22 ± 2 °C and the pressure at $1.4x10^{-1}$ mbar (LIOALFA 6-50, Telstar, Madrid, Spain). The initial mass load used was 120 g.

For the HAD and HAD-US experiments, apple samples were dried in an ultrasonically assisted convective drier already described in the literature (García-Pérez et al., 2006). The equipment consists of a pilot-scale convective drier

with an aluminum cylindrical ultrasonic radiator working as the drying chamber and driven by a piezoelectric transducer (21.8 kHz). The drier operates completely automatically: air temperature and velocity are controlled using a PID algorithm, and samples are weighed at preset times by combining two pneumatic systems and a PLC (CQM41, Omron, Japan). The HAD experiments were carried out at 60 °C, keeping a constant air velocity of 2 $ms^{-1}$ and using an initial mass load of 120 g. The experiments assisted by power ultrasound (HAD-US) were conducted under the same experimental conditions as the HAD experiments but by applying an acoustic power of approximately 20 $kW/m^3$, which is defined as the electric power supplied to the ultrasonic transducer divided by the chamber volume.

At least three drying tests were carried out for each condition tested, and they were extended until the samples lost 85 ± 1 % of the initial weight of fresh apple and 91.3 ± 0.3 % in the case of impregnated apple.

## 2.3. Impregnation experiments

For the infusion of olive leaf phenolic compounds into the dry apple, 4 g of dried apple cubes were immersed in 250 mL of olive leaf extract at 25 °C using a flask protected from light. The polyphenolic infusion kinetic was monitored by weighing the samples at preset times. For that purpose, apple cubes were blotted with tissue paper to remove the excess superficial extract before being weighed. It was considered that equilibrium was reached when the difference between two consecutive weights was less than 0.02 g. Experiments were conducted in triplicate.

## 2.4. Solids loss during apple impregnation

A new set of experiments was carried out to evaluate the loss of apple solid compounds that takes place throughout the impregnation. For that purpose, 4 g of dry apple (HAD or FD) were rehydrated in 250 mL of distilled water at 25 °C for different times. Then, samples were blotted with tissue paper to remove the excess superficially adhered water, and afterwards, the moisture content (no. 934.06; AOAC, 1997) was determined. Three replicates were made for each rehydration time. The solid content throughout the rehydration was estimated from the difference between

the rehydrated sample weight and its moisture content. The loss of solids was assumed to be the same as the one produced during the impregnation with the olive leaf extract.

The Weibull empirical model (Cunha et al., 1998) was used for the prediction of the solid content during impregnation (Eq. (1)):

$$C(t) = C_e + (C_o - C_e)\exp\left(-\left(\frac{t}{\beta}\right)^{\alpha}\right) \quad (1)$$

where $C(t)$ (g/g of apple) represents the solid content after an impregnation time $t$, subscripts $o$ and $e$ represent the initial condition and equilibrium, respectively, $\alpha$ the dimensionless parameter assimilated to the behavior index of the product during impregnation, and $\beta$ ($min^{-1}$) is the kinetic parameter inversely related ($1/\beta$) with the process rate. The identification of the model parameters ($\alpha$, $\beta$ and $C_e$) was carried out by minimizing the sum of the squared differences between the experimental and calculated solid content of the samples by using the Solver tool from Excel™ (Microsoft Corporation, Seattle, USA).

## 2.5. Sample preparation for phenolic content and antioxidant capacity determination

For the purposes of assessing the antioxidant potential, extraction experiments were carried out on the dried and dried-impregnated-dried apple samples in order to release the phenolic compounds in aqueous extracts. The extraction conditions were similar to those used for obtaining the olive leaf extracts. 10 g of milled apple sample were placed in sealed containers protected from light with 150 mL of distilled water at 170 rpm and 22 ± 1 °C for 24 h. Afterwards, the extracts were centrifuged (10 min at 5000 rpm) and filtered (nylon filters of 0.45 μm).

## 2.6. TPC measurement

The phenolic content was determined by the Folin-Ciocalteu method (Singleton et al., 1999). Briefly, 100 μL of sample were mixed with 200 μL of Folin-Ciocalteu's phenol reagent (Sigma-Aldrich, Madrid, Spain) and 2 mL of distilled

water. After 3 min at 25 °C, 1 mL of $Na_2CO_3$ (Panreac, Barcelona, Spain) solution ($Na_2CO_3$-water 20:80, w/v) was added to the mixture. The reaction was kept in the dark at room temperature for 1 h. Finally, the absorbance was read at 765 nm using a spectrophotometer (Helios Gamma, Thermo Spectronic, Cambridge, UK). The measurements were carried out in triplicate. The standard curve was previously prepared using solutions of a known concentration of gallic acid hydrate (Sigma-Aldrich, Madrid, Spain) in distilled water. Results were expressed as follows: mg of gallic acid equivalents (GAE) per g of dried matter of apple (d.m.) or mg of GAE per mL of olive leaf extract, for apples and olive leaf extracts, respectively.

## 2.7. AC measurement

The antioxidant capacity of extracts was determined by using the ferric-reducing ability power (FRAP) method, which is a simple method used to estimate the reduction of a ferric-tripyridyltriazine complex. It was applied following the procedure described by Benzie & Strain (1996), with some modifications. Briefly, 900 μL of freshly prepared FRAP reagent were mixed with 30 μL of distilled water and 30 μL of test sample or water as appropriate reagent blank and kept at 37 °C for 30 min. The FRAP reagent contained 2.5 mL of a 10 mM TPTZ (2,4,6-tri(2-pyridyl)-s-triazine. Fluka, Steinheim, Germany) solution in 40 mM HCl (Panreac, Barcelona, Spain) plus 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ (Panreac, Barcelona, Spain) and 2.5 mL of 0.3 M acetate buffer (Panreac, Barcelona, Spain), pH 3.6 (Pulido et al., 2000). Readings at the maximum absorption level (595 nm) were taken using a spectrophotometer (Helios Gamma, Thermo Spectronic, Cambridge, UK). Four replicates were made for each measurement. The antioxidant capacity was evaluated through a calibration curve, which was previously determined using water solutions of known Trolox (Sigma-Aldrich, Madrid, Spain) concentrations and expressed as: mg Trolox per g of dried matter of apple (d.m.) or mg Trolox per mL of olive leaf extract, for apples and olive leaf extracts, respectively.

## 2.8. Identification and quantification of polyphenols by HPLC-DAD/MS-MS

In order to identify and quantify the main polyphenols present in olive leaf extracts and dried-impregnated-dried apples, these were analyzed using an HPLC instrument (Agilent LC 1100 series; Agilent Technologies, Inc., Palo Alto, CA, USA) controlled by the Chemstation software. The HPLC instrument was coupled to an Esquire 3000+ (Bruker Daltonics, GmbH, Germany) mass spectrometer equipped with an electrospray ionization (ESI) source and ion-trap mass analyzer, and controlled by Esquire control and data analysis software. A Merck Lichrospher 100RP-18 (5 μm, 250 x 4 mm) column was used for analytical purposes.

Separation was carried out through a linear gradient method using 2.5 % acetic acid (A) and acetonitrile (B), starting the sequence with 10 % B and programming the gradient to obtain 20 % B at 10 min, 40 % B at 35 min, 100 % B at 40 min, 100 % B at 45 min, 10 % B at 46 min and 10 % B at 50 min. For the LC-MS pump to perform accurately, 10 % of organic solvent was premixed in the water phase. The flow-rate was 1 mL/min and the chromatograms were monitored at 240, 280 and 330 nm. Mass spectrometry operating conditions were optimized in order to achieve maximum sensitivity values. The ESI source was operated in negative mode to generate $[M-H]^-$ ions under the following conditions: desolvation temperature at 365 °C and vaporizer temperature at 400 °C; dry gas (nitrogen) and nebulizer were set at 12 L/min and 4.83 bar, respectively. The MS data were acquired as full scan mass spectra at 50–1100 m/z by using 200 ms for the collection of the ions in the trap.

The main compounds were identified by HPLC with diode array detection (HPLC-DAD) analysis, comparing the retention time, UV spectra and MS/MS data of the peaks in the samples with those of authentic standards or data reported in the literature. Only the main olive leaf polyphenols were quantified using commercial standards: oleuropein (Extrasynthese, Genay Cedex, France), luteolin-7-O-glucoside (Phytolab, Vestenbergsgreuth, Germany) and apigenin (Nutrafur, Murcia, Spain). A purified extract (96.85 %) provided by Universidad Miguel Hernández (Elche, Spain) was used to quantify verbascoside. The quantitative evaluation of the compounds was

performed with a calibration curve for each polyphenol, using ethanol (oleuropein), methanol (verbascoside and luteolin) or dimethyl sulfoxide (apigenin) solutions of known concentration. The polyphenol concentrations were expressed as mg polyphenol per g of dried matter of apple (d.m.) or mg polyphenol per mL of olive leaf extract, for apples and olive leaf extracts, respectively.

## 2.9. Drying kinetics modeling

A diffusion model (Eq. (2)) was used to mathematically describe the drying kinetics of impregnated samples (Simal et al., 2005).

$$W(t) = W_e + (W_e - W_0)\left[\sum_{n=0}^{\infty} \frac{8}{(2n+1)^2 \pi^2} e^{\left(-\frac{D_w (2n+1)^2 \pi^2 t}{4L^2}\right)}\right]^3 \quad (2)$$

where $W$ is the average moisture content (kg w/kg d.w.), $L$ the half-length of the cube side (m), $t$ is the time (s), $D_w$ is the effective moisture diffusivity ($m^2/s$) and subscripts $0$ and $e$ represent the initial and equilibrium state, respectively.

$D_w$ was estimated by fitting a diffusion model to experimental kinetics. Thus, the generalized reduced gradient (GRG) optimization method, available in Microsoft Excel™ spreadsheet (Microsoft Corporation, Seattle, WA, USA) was used, defining the objective function to be minimized as the sum of the squared difference between experimental and calculated average moisture content. The percentage of explained variance (% VAR, Eq. (3)) was calculated in order to determine the goodness of the fit to the experimental data.

$$\% \text{ VAR} = \left[1 - \frac{S_{xy}^2}{S_y^2}\right] 100 \quad (3)$$

where $S^2_{xy}$ is the variance of the estimation and $S^2_y$ is the variance of the sample.

## 3. Results and discussion

### 3.1. Characterization of dried apple samples and olive leaf extract

In order to obtain different solid matrices for impregnation, apple cubes were dried by means of two different methods, hot air (HAD) and freeze drying (FD). The moisture of fresh apple (85.3 ± 0.9 g w/100 g) was reduced to a final moisture content of 3.1 ± 0.2 g w/100 g, which represents a reduction of 96 % in the initial water mass. Thereby, stable dehydrated products with water activity of under 0.31 ± 0.03 were obtained.

The antioxidant potential of the dried apple was estimated from the determination of total phenolic content (TPC) and antioxidant capacity (AC), as described in the Materials and Methods section. HAD apples showed a TPC (1.16 ± 0.03 mg GAE/g d.m.) and AC (3.87 ± 0.08 mg Trolox/g d.m.) that were significantly ($p<0.05$) higher than the one measured in FD (TPC of 0.45 ± 0.09 mg GAE/g d.m. and AC of 1.07 ± 0.15 mg Trolox/g d.m.). Previous works have reported different results for apple. Thus, Vega-Gálvez et al. (2012) suggested that total phenolics decreased as the drying temperature rose (40-80 °C), while Joshi et al. (2011) did not find any meaningful differences between the drying methods tested (freeze, air, oven and vacuum drying).

With regard to the olive leaf extracts, the average TPC and AC values were 2.0 ± 0.6 mg GAE/mL and 5.9 ± 0.5 mg Trolox/mL, respectively, as can be observed in Table 1. These figures are slightly lower than the ones published in previous works (Ahmad-Qasem et al., 2013a, 2013b), which could probably be ascribed to the different solvent used in this work (water instead of hydroalcoholic solutions) and the harvest period of the olive leaves. However, the profile of bioactive compounds identified was similar to the ones previously found (Ahmad-Qasem et al., 2013a, 2013b), oleuropein, verbascoside, and luteolin and apigenin derivates being the main polyphenols.

**Table 1.** Olive leaf extracts characterization: antioxidant potential and phenolic composition.

| Olive leaf extract characterization | |
|---|---|
| TPC (mg GAE/mL) | 2.0 ± 0.6 |
| AC (mg Trolox/mL) | 5.9 ± 0.5 |
| Oleuropein (mg/mL) | 3.8 ± 0.3 |
| Oleuropein glucoside [a] | 0.060 ± 0.007 |
| Verbascoside (mg/mL) | 0.25 ± 0.02 |
| Luteolin glucoside (mg/mL) | 0.44 ± 0.03 |
| Luteolin diglucoside [b] | 0.037 ± 0.012 |
| Luteolin-7-O-rutinoside [b] | 0.07 ± 0.03 |
| Apigenin-6,8-diglucoside [c] | 0.023 ± 0.002 |
| Apigenin-7- rutinoside [c] | 0.036 ± 0.004 |

Average values ± standard deviation.

[a] Content expressed as equivalents of oleuropein (mg/mL).

[b] Content expressed as equivalents of luteolin-7-O-glucoside (mg/mL).

[c] Content expressed as equivalents of apigenin (mg/mL).

## 3.2. Impregnation of dried apple with the olive leaf extract

FD and HAD apples were impregnated with the olive leaf extract. During this process, two opposite mass transfer processes took place: on the one hand, the infusion of the extract compounds into the solid matrix and, on the other hand, the lixiviation of some solid compounds of the matrix to the liquid medium. The latter was observed from the increase in the soluble solid content in the olive leaf extracts (from 1.6 ± 0.2 to 2.6 ± 0.3 °Brix). As a consequence, the quantification of total solids loss in the apples during impregnation was studied, and the kinetics of solids loss in water was determined (Fig. 2) and assumed to be roughly the same as in the olive leaf extract. Once the dry apple was soaked in water, it underwent a sudden rehydration, which caused a meaningful decrease in the solid content. Thus, in FD samples, the solid content dropped from 0.97 to 0.25 g/g apple in less than 10 s, while in HAD, the solid content decreased to 0.50 g/g apple in approximately 60 s. The release of the solids from the apple matrix is coupled to the water gain, but it is only noticeable

once the sample is almost fully rehydrated. This latter stage was accurately described using the Weibull model (Fig. 2), which provided explained variances of over 0.97 for both FD and HAD. The major differences between FD and HAD were found in the rate of solids loss, since it was much faster in FD (Fig. 2). However, both FD and HAD reached a similar solid content in the equilibrium (0.059 ± 0.005 g/g of apple). It is important to highlight that the impregnated apple could be considered as practically a sugar-free product.

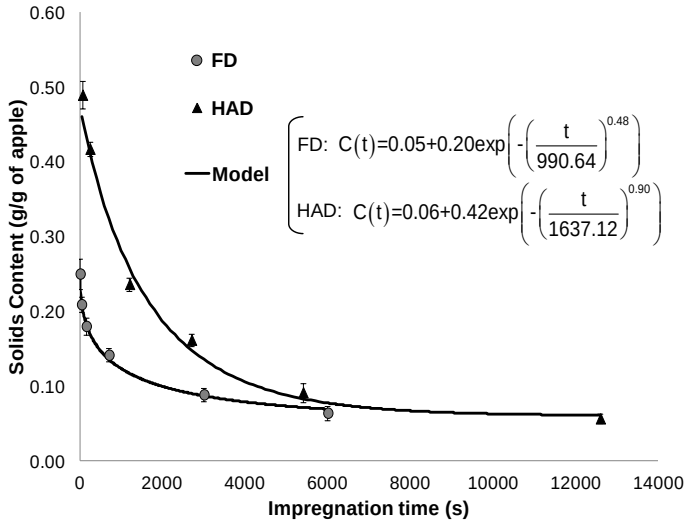


**Fig. 2.** Solid content in FD and HAD apples during soaking in water.

Fig. 3 depicts the global mass change (ΔM) for apples during impregnation. As observed, the drying method of fresh apples had a significant ($p<0.05$) influence on the further impregnation rate (Fig. 3). Thus, the infusion of olive leaf extract in FD was faster than in HAD. Thus, after 50 min of treatment, FD samples achieved practically a constant ΔM, whereas HAD required 2 h 30 min. The faster infusion of olive leaf extract (Fig. 3), as well as the solids loss (Fig. 2), into the FD apple could be related to the cellular disruption suffered by the vegetable material as a result of freezing (Van Buggenhout et al., 2006) and the formation of a high porosity matrix. These facts are also evidenced in the final mass gain, which was slightly larger in FD samples.

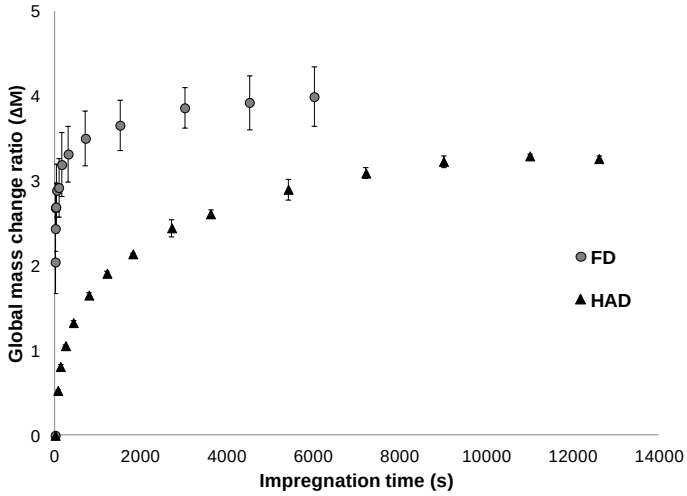


**Fig. 3.** Global mass change ratio (ΔM) of FD and HAD samples during impregnation with olive leaf extract.

The evolution of AC in the apple during impregnation may be estimated (Fig. 4) from the global mass balance (Fig. 3), the solids loss kinetics (Fig. 2), and by considering the AC of the extract entering the particle.

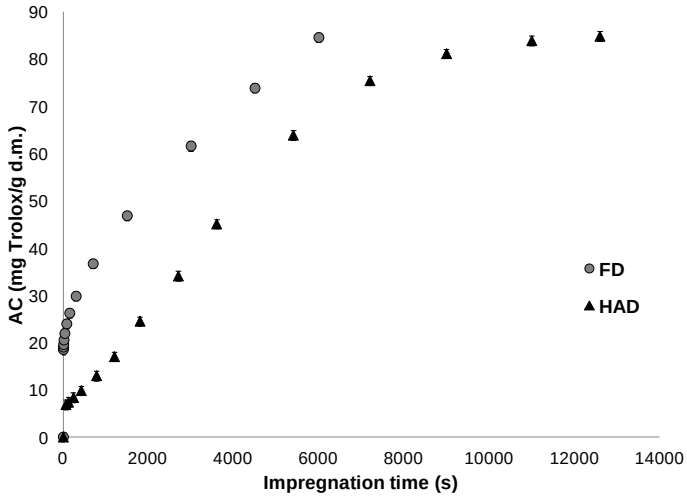


**Fig. 4.** Kinetics of polyphenolic infusion into freeze (FD) and hot air dried (HAD) apples. Means ± standard deviation of antioxidant capacity (AC) are plotted.

Fresh apple drying did not significantly ($p<0.05$) affect the final estimated AC (Fig. 4). Thus, an average AC of 84.7 ± 0.2 mg Trolox/g d.m. was found for both FD

and HAD apples. Notwithstanding, in order to reach the same AC, HAD needed almost twice as long as FD. Therefore, the technique of freeze-drying could be considered a reliable means of speeding up the impregnation of dried apple with the olive leaf extract.

The impregnated apple is an unstable matrix due to its high water content (close to 94 %). As a consequence, further dehydration is necessary in order to reduce the storage costs and increase shelf life. How the further drying affects not only the dehydration rate but also the antioxidant potential are relevant aspects to be considered and are addressed in the following sections.

## 3.3. Drying kinetics of impregnated apple

Impregnated apples (FD+I and HAD+I) were dehydrated by freeze drying (FD) or hot air drying with or without power ultrasound (HAD-US and HAD) application. The drying kinetics were determined (Fig. 5) due to the water removal of the impregnated apple that constitutes an additional cost, both in terms of energy and time consumption. The kinetic study could not be completed in FD samples due to the fact that the freeze drier operates in batch (24 h).

The explained variances reached with the proposed diffusion model were low, ranging from 88 to 91 % (Table 2). This fact suggests that diffusion was not the only controlling mass transport mechanism, probably because of the high rate of the impregnated water moving freely through the solid to the surface, lending a significant role to convection. Even the differences in drying kinetics were not marked (Fig. 5); a significantly ($p<0.05$) higher effective moisture diffusivity was found in FD+I+HAD ($12.9 \pm 0.7 \times 10^{-10}$ $m^2/s$) than in HAD+I+HAD ($11.7 \pm 0.5 \times 10^{-10}$ $m^2/s$) (Table 2). This fact was probably due to the more porous matrix promoted by FD, which aids the further removal of the impregnated water.

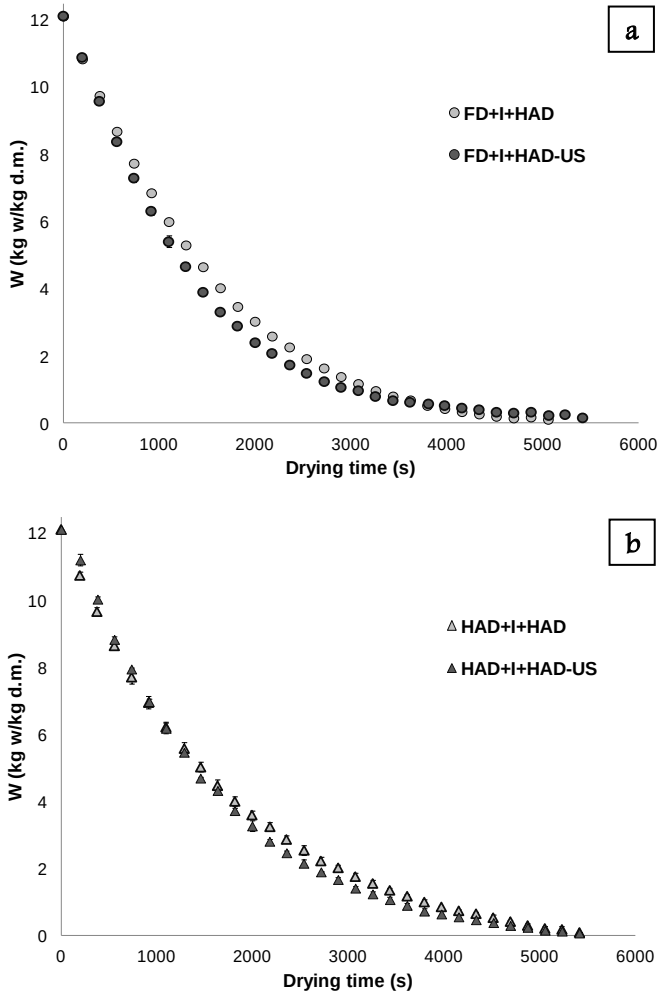


**Fig. 5.** Hot air drying kinetics with (HAD-US) or without ultrasound assistance (HAD) of apples impregnated with olive leaf extract (a: FD+I; b: HAD+I). Means ± standard deviation of moisture (kg w/kg d.m.) are plotted.

As to ultrasound application during drying, the effective diffusivity identified for HAD+I+HAD-US was only 5.1 % higher than that obtained for HAD+I+HAD (Table 2). In the case of FD+I samples, the increase in $D_w$ when ultrasound was applied was of 14.7 %. In both cases, the improvement was less significant than that reported for the ultrasonic drying of fresh vegetables and fruits (García-Pérez et al., 2012; Ozuna et al., 2014). Therefore, the use of ultrasound for the purposes of improving the drying

of impregnated apples seems not to be very promising as a means of increasing productivity and reducing energy consumption.

**Table 2.** Effective moisture diffusivity ($m^2/s$) and percentage of explained variance (VAR) identified from the modeling of the drying of impregnated apples.

| | $D_w$(x$10^{10}$ $m^2/s$) | VAR (%) |
|---|---|---|
| FD+I+HAD | 12.9 ± 1.8 [b] | 88.0 |
| FD+I+HAD-US | 14.8 ± 1.6 [a] | 89.8 |
| HAD+I+HAD | 11.7 ± 1.4 [d] | 90.5 |
| HAD+I+HAD-US | 12.3 ± 1.7 [c] | 88.2 |

Average values ± confidence intervals (at 99 % significance level).

[a-d] Homogeneous groups in the same row established from least significance difference (LSD) intervals ($p<0.05$).

## 3.4. Effect of drying of the impregnated apple on antioxidant potential

Once the impregnated apple was dried, the obtained final product had much higher antioxidant potential values than those found in the dehydrated raw apples (Fig. 6 and 7). However, the type of drying operation had a noticeable effect on the final antioxidant potential achieved, as observed in Fig. 6 and 7.

Firstly, the drying of the fresh apple greatly affected the antioxidant potential of dried-impregnated-dried apple (Fig. 6 and 7). Thereby, FD samples achieved significantly ($p<0.05$) lower TPC (Fig. 6a) and AC (Fig. 7a) than HAD (Fig. 6b and 7b). The average final TPC and AC for HAD apples (HAD+I+HAD, HAD+I+HAD-US and HAD+I+FD) was 2-3 times higher than for FD (FD+I+HAD, FD+I+HAD-US and FD+I+FD). In consequence, although FD is considered a suitable method for drying fresh vegetables and obtaining enriched products with high antioxidant potential (Mahn et al., 2012), in this work, HAD (a simpler and more affordable technique) provided a dry product with higher antioxidant potential.

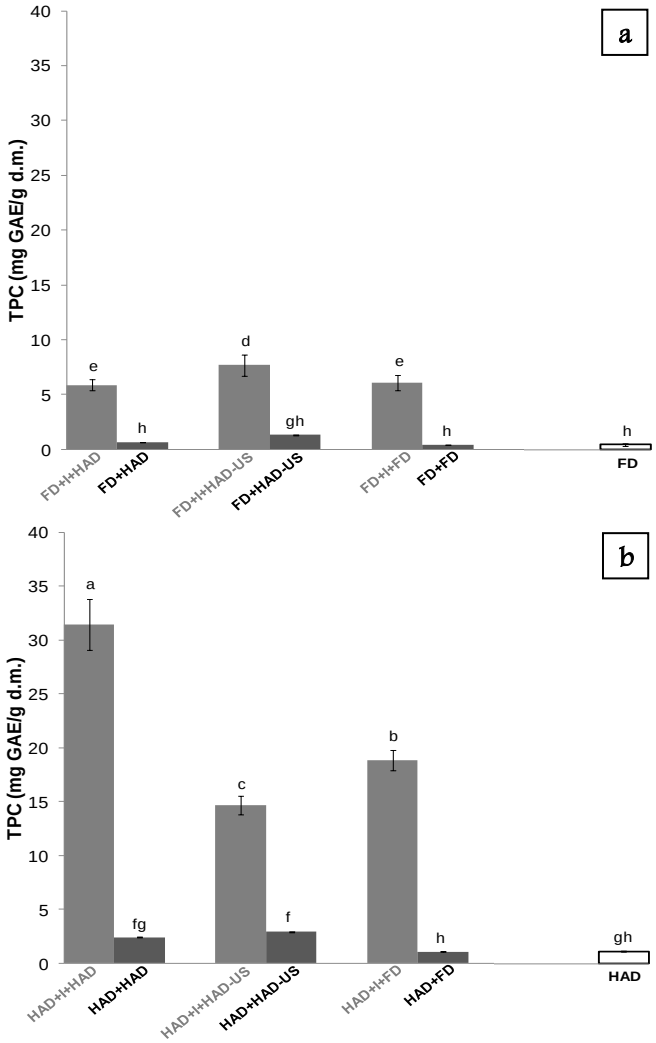


**Fig. 6.** Influence of the different treatments on the total phenolic content (TPC) of freeze dried (a) or hot air dried (b) apples. Means ± standard deviation are plotted. Superscript letters show homogeneous groups established from least significance difference (LSD) intervals ($p<0.05$). I, impregnated; HAD, hot air dried; HAD-US, ultrasound assisted hot air dried; FD, freeze dried.

As far as we are aware, these results have not previously been reported and could be explained by considering, among other facts, the residual enzyme activity present in the unfrozen rubbery-state water fraction of frozen samples, as well as how freezing affects the solid matrix. Thus, in impregnated FD apples, both enzymatic

and hydrolytic reactions could take place (Blanda et al., 2008b), reducing the antioxidant potential achieved with the olive leaf extract infusion.

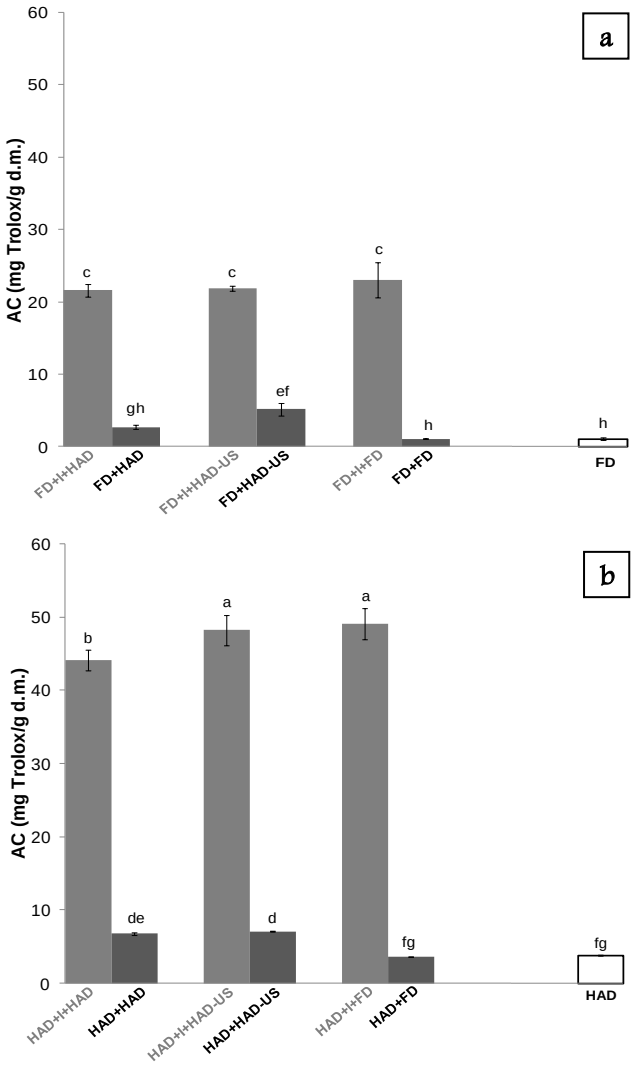


**Fig. 7.** Influence of the different treatments on the antioxidant capacity (AC) of freeze dried (a) or hot air dried (b) apples. Means ± standard deviation are plotted. Superscript letters show homogeneous groups established from least significance difference (LSD) intervals ($p<0.05$). I, impregnated; HAD, hot air dried; HAD-US, ultrasound assisted hot air dried; FD, freeze dried.

With regard to the influence of freezing on the solid matrix, the injury to the cell integrity caused would facilitate the release of intracellular components, thus,

polyphenols, polyphenol oxidase and oxygen may be placed in contact (Ferreira et al., 2002) during impregnation favoring the abovementioned residual enzymatic activity. In addition, the growth of ice crystals pushes, compresses and breaks cells , greatly degrading the native structure (Voda et al., 2012) and creating an open, weak structure (Sham et al., 2001). This suggests that polyphenols are more exposed to dehydration conditions, due to their weak interaction with the poorly consolidated solid matrix of FD samples.

Although the influence of the further drying of the impregnated apple was much less noticeable on the retention of infused polyphenols (Fig. 6 and 7) than the drying of the fresh apple, some facts could be highlighted. Thus, the TPC of HAD+I+HAD (Fig. 6) was 115 and 67 % higher than that of HAD+I+HAD-US and HAD+I+FD, respectively. However, HAD+I+HAD showed a similar AC to HAD+I+HAD-US and HAD+I+FD (Fig. 7a), which suggests that the method used to dry the impregnated apples did have an effect, but to a lesser extent. The ultrasound assisted drying of FD impregnated samples (FD+I+HAD-US) slightly increased ($p<0.05$) the TPC as compared to those dried using other techniques (Fig. 6a), but no positive effects were observed in AC (Fig. 7a).

Therefore, once the impregnated apples were dried, the products obtained presented a much higher antioxidant potential than those found in the dehydrated raw apple (Fig. 6 and 7). As a consequence, the method proposed in this work, combining drying-impregnation-drying steps, could be considered a convenient apple-processing alternative in order to obtain a stable product, low in sugar and enriched with olive leaf bioactive polyphenols with high antioxidant activity. These results agreed with other works found in the literature where the combination of impregnation and drying is considered suitable for developing dried fruit products with bioactive effect (Betoret et al., 2003; Noorbakhsh et al., 2013).

Finally, additional experiments were conducted for the purposes of investigating how the further drying affects the antioxidant potential of the apple itself. Thus, FD and HAD samples were again subjected to FD, HAD and HAD-US experiments. Obviously, it cannot be considered a dehydration step due to the fact that the initial

water content was only 0.032 kg w/kg d.m. The experimental results (Fig. 6 and 7) showed that the additional HAD step (both with and without ultrasound application) significantly ($p<0.05$) increased the TPC and AC for both FD and HAD samples. Thus, HAD+HAD apples showed significantly ($p<0.05$) higher TPC and AC (109 and 74 %, respectively) than HAD ones. This fact could be linked to the formation of Maillard-derived melanoidins, responsible for color changes during HAD, since these molecules have already been linked to the potential antioxidant enhancement of dried products as a result of the formation of novel compounds with antioxidant activity (Manzocco et al., 2001). However, the additional FD step did not imply any increase in the TPC and AC for either HAD and FD apples, as may be observed if FD+FD and HAD+FD are compared to FD and HAD (Fig. 6 and 7), respectively.

## 3.5. Effect of drying of the impregnated apple on phenolic composition

In order to gain insight into the influence of the different drying techniques on the retention of infused polyphenols, the phenolic compounds were identified and quantified by HPLC-DAD/MS-MS.

The main polyphenols identified and quantified in the olive leaf extract (Table 1) were also found in the dried-impregnated-dried apple samples (Table 3). In agreement with the antioxidant potential results, the polyphenol retention was mostly affected by how the fresh apple was dehydrated. Dried HAD+I apples had a significantly ($p<0.05$) higher content of the main polyphenols than the FD+I ones (Table 3). These differences were particularly noticeable in the case of the oleuropein, its HAD+I content being up to 3 orders of magnitude higher than in FD+I. Oleuropein was not even detected in FD+I+HAD-US apples.

As far as the drying method applied to the dehydration of impregnated samples was concerned, no meaningful effect was found. Indeed, no significant ($p<0.05$) differences were found between FD+I+HAD, FD+I+HAD-US and FD+I+FD. In the case of HAD+I, the drying method had a significant ($p<0.05$) influence on the concentration of some compounds, such as oleuropein, oleuropein glucoside and luteolin glucoside.

**Table 3.** Main polyphenols retained in the apple matrix after impregnation (I) with olive leaf extract and different drying treatments: freeze drying (FD), hot air drying (HAD), hot air drying assisted by power ultrasound (HAD-US).

| | FD+I+HAD | FD+I+HAD-US | FD+I+FD | HAD+I+HAD | HAD+I+HAD-US | HAD+I+FD |
|---|---|---|---|---|---|---|
| Oleuropein (mg/100 g d.m.) | 11 ± 4 [g] | nd | 6.7 ± 0.5 [g] | 1152 ± 82 [f] | 1710 ± 225 [e] | 1928 ± 111 [d] |
| Oleuropein glucoside [a] | 197 ± 10 [c] | 304 ± 11 [d] | 232 ± 20 [e] | 238 ± 74 [e] | 285 ± 84 [d] | 338 ± 17 [d] |
| Verbascoside (mg/100 g d.m.) | nd | nd | nd | 11 ± 2 [e] | 26 ± 4 [d] | 25 ± 2 [d] |
| Luteolin glucoside (mg/100 g d.m.) | 52 ± 9 [e] | 56 ± 12 [e] | 52 ± 13 [e] | 80 ± 25 [de] | 109 ± 38 [d] | 108 ± 15 [d] |
| Luteolin diglucoside [b] | nd | nd | nd | nd | 15 ± 5 [d] | 7 ± 2 [e] |
| Luteolin-7-O-rutinoside [b] | nd | nd | nd | nd | nd | nd |
| Apigenin-6,8-diglucoside [c] | 8.9 ± 1.4 [e] | 7.4 ± 0.6 [e] | 8.1 ± 0.4 [e] | 10.2 ± 0.4 [e] | 14 ± 3 [d] | 14 ± 3 [d] |
| Apigenin-7- rutinoside [c] | 5.0 ± 0.8 [f] | 10 ± 2 [e] | 5.7 ± 0.03 [f] | 9.8 ± 1.4 [e] | 17 ± 3 [d] | 11.4 ± 0.6 [e] |

Average values ± standard deviation.

nd not detected.

[a] Content expressed as equivalents of oleuropein (mg/100 g d.m.).

[b] Content expressed as equivalents of luteolin-7-O-glucoside (mg/100 g d.m.).

[c] Content expressed as equivalents of apigenin (mg/100 g d.m.).

[d-g] Homogeneous groups in the same row established from least significance difference (LSD) intervals ($p<0.05$).

Thus, HAD+I+FD apples showed the highest concentrations of the main compounds: oleuropein (1928 ± 111 mg/100 g d.m.) and oleuropein glucoside (338 ± 17 mg/100 g d.m.). The same effect was observed when quercetin derivatives were analyzed in dried impregnated apple, resulting HAD of samples in a greater quercetin degradation than FD (Schulze et al., 2014). Therefore, FD seemed to be a convenient method with which to dehydrate the HAD+I samples, which appears contradictory if compared with the already mentioned negative effect on the drying of fresh apple. This fact could be explained by considering different aspects. On the one hand, the freezing step did not favor the release of oxidative enzymes due to they were previously inactivated by HAD (drying of fresh apple). On the other hand, the low temperature applied during FD caused less degradation of the bioactive compounds in HAD+I+FD apples than in HAD+I+HAD and HAD+I+HAD-US.

## 4. Conclusions

The method proposed in this work, combining drying-impregnation-drying steps, could be considered as a convenient apple processing alternative as a means of obtaining a stable product of high antioxidant potential and low-sugar content enriched with olive leaf polyphenols. However, the retention of infused polyphenols was greatly dependent on how the drying steps were performed. In this regard, the fresh apple drying process influenced the retention of infused olive leaf polyphenols more than the further drying process of the impregnated apple. Firstly, the infusion rate was improved by freezing prior to drying; thus, freeze dried apples impregnated faster than hot air dried ones. Secondly, hot air dried apples were found to retain a greater quantity of the olive leave polyphenols than those that were freeze dried. An oleuropein content of up to 1928 mg/100 g d.m. was achieved in the dried-impregnated-dried apple. Finally, further research should be carried out in order to elucidate the biochemical mechanisms involved.

## Acknowledgments

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## Abbreviations used

FD, freeze drying; HAD, hot air drying; HAD-US, hot air drying assisted by power ultrasound; TPC, total phenolic content; GAE, gallic acid equivalents; AC, antioxidant capacity; FRAP, ferric-reducing ability power; HPLC-DAD, high performance liquid chromatography with diode array detection; MS-MS, tandem mass spectrometry; ESI, electrospray ionization; LC-MS, liquid chromatography-mass spectrometry; UV, ultraviolet; $D_w$, effective moisture diffusivity; FD+I, freeze dried apples impregnated with olive leaf extract; HAD+I, hot air dried apples impregnated with olive leaf extract; FD+I+HAD, freeze dried apples impregnated with olive leaf extract and further hot air dried; HAD+I+HAD, hot air dried apples impregnated with olive leaf extract and further hot air dried; HAD+I+HAD-US, hot air dried apples impregnated with olive leaf extract and further ultrasonically-assisted hot air dried; FD+I+HAD-US, freeze dried apples impregnated with olive leaf extract and further ultrasonically-assisted hot air dried; HAD+I+FD, hot air dried apples impregnated with olive leaf extract and further freeze dried; FD+I+FD, freeze dried apples impregnated with olive leaf extract and further freeze dried; FD+FD, freeze dried apples and further subjected again to freeze drying conditions; HAD+HAD, hot air dried apples and further subjected again to hot air drying; HAD+FD, hot air dried apples and further subjected to freeze drying.

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# *The role of microstructure and enzymatic activity on the phenolic content of impregnated dried apple*

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# The role of microstructure and enzymatic activity on the phenolic content of impregnated dried apple


## ABSTRACT

Infusion of antioxidants into dried vegetable products is a new strategy in food processing. However, it can be managed by the impact of how vegetable matrixes are processed due to its influence on microstructure and enzymatic activity. In this work, raw and blanched apple cubes were air dried or freeze dried by using different freezing methods: conventional (-28 °C), blast freezing (-30 °C) and liquid $N_2$ (-196 °C). Dried samples were vacuum impregnated with tea extract and, afterwards, dehydrated for their stabilization by air drying. Total phenolic content (TPC), antioxidant capacity (AC), polyphenol oxidase and peroxidase activity and microstructure were analyzed. Regardless pre-treatments, the impregnation and the further drying improved the antioxidant potential. The highest bioactive content was found in blanched and freeze dried samples by using $N_2$ prior to the phenolic infusion. These samples showed the most porous microstructure free of degradative enzymes, providing stable products with high AC (78.5 ± 0.9 mg Trolox/g dried matter) and TPC (16.7 ± 0.2 mg GAE/g dried matter).


***Keywords***: blanching, freezing, drying, infusion, enzymatic activity, antioxidant potential.

## 1. Introduction

Apple polyphenols are important because of their contribution to sensory traits, being also recognized for their health promoting bioactive properties (Serra et al., 2010; Van der Sluis et al., 2002; Zhao et al., 2013). In addition, its structure with great number of air spaces makes apple a suitable vegetable material to be infused with bioactive solutions. These facts and the growing tendency to its consumption in the world, in the form of fresh fruit, juice or dried product, including snacks preparations, integral breakfast foods and other varieties (Biedrzycka & Amarowicz, 2008), make apple a suitable raw material to develop new foods with higher bioactive content. Recent studies have illustrated the production of this kind of foods by the infusion of olive leaf extracts (Ahmad-Qasem et al., 2015), grape phenolic compounds (Ferrando et al., 2011) or even probiotics (Röβle et al., 2010) into solid vegetable matrixes. For this purpose, not only the bioactive potential of the solution being infused is relevant but also how the raw solid material is processed before and after the infusion (Tripathi & Giri, 2014). In this way, blanching, freezing and drying are essential by their impact on the native structure and compounds, such as enzymes, polyphenols and cell wall components.

Blanching is a common pre-treatment for vegetable products. It not only induces the thermal inactivation of undesirable enzymes in vegetable tissue, including polyphenol oxidase (Ma et al., 1992), but also causes structural changes at a cellular level that result in a cell separation (Anderson et al., 1994) affecting the mass transfer phenomena during drying (González-Fésler et al., 2008).

In general terms, it is known that freezing rate determines the ice crystal size and the nucleation, which is extracellular or intracellular for slow and fast rates, respectively (Mazur, 1984). Thus, it is commonly accepted that fast freezing better preserves native structure due to the production of a large number of small ice crystals that cause less migration of water and less breakage of cell walls, and consequently less texture deterioration. However, if the process is too fast it can provoke breakage at the product level (Brown, 1977; Delgado & Rubiolo, 2005; Marti & Aguilera, 1991). Therefore, depending on the freezing method the material

will show different structural properties, which should be relevant for the further infusion.

The removal of water by prior drying of the raw material could facilitate the infusion of the extracts into vegetable matrixes. Nevertheless, drying could also negatively affect not only the nutritional quality but also the microstructure, being this dependent on drying conditions and technique employed. Among the most relevant structural modifications, cell shrinkage should be considered because it causes the major modification in the global structure of the product (Lewicki & Pawlak, 2003) and it is strongly linked to the further reconstitution. Moreover, from a technological point of view and aiming to long shelf-life foods, dehydration is the final step for the product stabilization (Sereno & Medeiros, 1990).

Vacuum impregnation has received increasing attention as potential process for the design of new enriched vegetable products. It makes possible to introduce dissolved or suspended substances directly into the product porous structure, allowing fast compositional and structural changes (Chiralt et al., 1999). Although, as already mentioned, infusion capacity is mostly dependent on how the raw material was processed before.

Taking into account the aforementioned factors, the aim of this work was to evaluate how some processing steps (blanching, freezing and drying) affect the phenolic retention of infused extracts with high antioxidant potential into apple, paying special attention to the role of the microstructure and enzymatic activity.

## 2. Materials and methods

### 2.1. Raw material

A concentrated (40 °Brix) tea extract, afterwards pasteurized for 5 min at 75 °C, was used for the impregnation. Thus, it was diluted in water (1:50, v/v) to obtain the tea impregnation solution.

Cubes of 10 mm were obtained from the apple flesh (*Malus domestica* cv. Jonagold) by using a cutting machine (CL50 Ultra, Robot Coupe USA, Inc., Jackson,

MS, USA) and immediately processed. Half of the fresh samples were blanched by immersion in boiling water for 90 s. Then, the thermal treatment was stopped with cold water.

## 2.2. Apple drying

In order to obtain dehydrated samples to be impregnated, both fresh (non-blanched, NB) and blanched (B) apple cubes were dried by means of two different methods: freeze drying (FD) and hot air drying (HAD). Once the samples were impregnated, further dehydration was carried out by HAD. A scheme of the experimental design and the nomenclature employed is shown in Fig. 1.

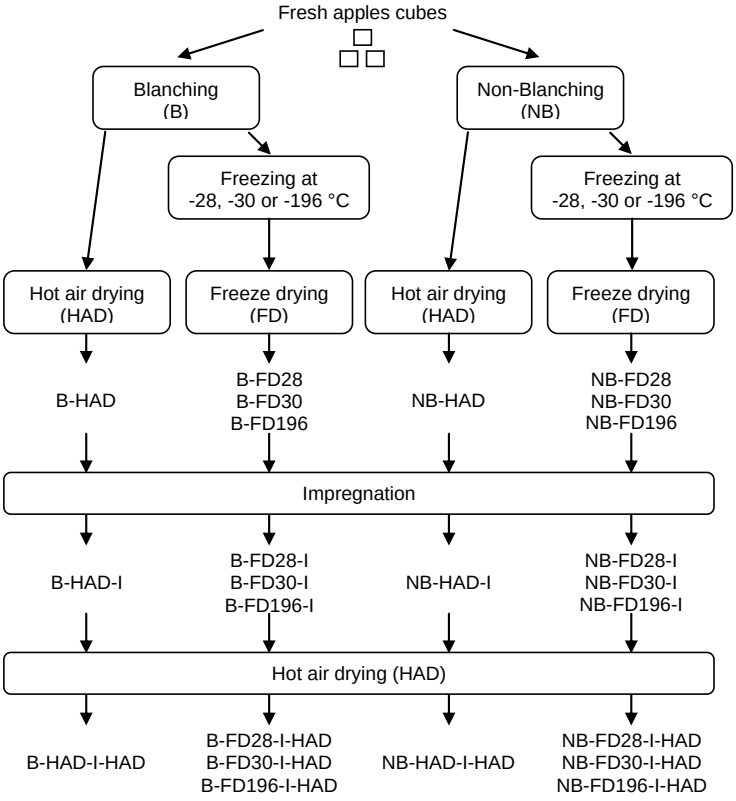


**Fig. 1.** Sequence of the different pre- and treatments undergone by apple samples.

In FD experiments, apple cubes were frozen at three different temperatures: -28 °C (conventional freezer), -30 °C (blast freezer) or -196 °C (liquid $N_2$). Finally, FD was stepped from -30 °C up to 50 °C at a constant pressure of 0.4 mbar

(Zirbus Technology, Bad Grund, Germany). For the HAD, apple samples were dried in a pilot-scale convective drier (Mitchell Dryers LTD, Carlisle, UK) at 60 °C.

In both FD and HAD, the initial mass load used was 3.5 kg. Drying was extended until the samples lost 89 ± 3 % of the weight for fresh and blanched apple, while impregnated apples lost 95.8 ± 0.3 %.

## 2.3. Impregnation

For impregnation, 6 g of dried apple cubes were immersed in 300 mL of the tea solution at 25 °C using a flask protected from light. The impregnation was carried out in two steps, a vacuum period of 14 h followed by 55 min at atmospheric pressure. Apple cubes were blotted with tissue paper to remove the excess of superficial tea solution before being weighed and processed. Experiments were conducted in triplicate.

## 2.4. Apple extracts for analysis

Apple samples (0.25-1 g) were mixed with distilled water (40 mL) and blended (Variable Speed Laboratory Blender, Waring Laboratory, USA) for 5 min. Afterwards, the extracts were filtered (nylon filters of 0.45 μm) and placed in opaque vials at 4 °C until analysis of the antioxidant potential. In the case of enzymatic activity determination, the extracts were filtered twice by using paper filters (MELB 1077, 185 mm) and a PD-10 desalting column (Amersham Pharmacia Biotech, NJ, USA). The desalted sample extracts were stored at 4 °C until being analyzed.

## 2.5. Total phenolic content measurement (TPC)

The phenolic content was determined by the Folin-Ciocalteu method (Singleton et al., 1999). Briefly, 100 μL of sample were mixed with 200 μL of Folin-Ciocalteu's phenol reagent (Sigma-Aldrich, Madrid, Spain) and 2 mL of distilled water. After 3 min at 25 °C, 1 mL of $Na_2CO_3$ (Panreac, Barcelona, Spain) solution ($Na_2CO_3$-water 20:80, w/v) was added to the mixture. The reaction was kept in the dark at room temperature for 1 h. Finally, the absorbance was read at 765 nm using a spectrophotometer (Helios Gamma, Thermo Spectronic, Cambridge, UK). The

measurements were carried out in triplicate. A calibration curve was previously prepared using solutions of a known concentration of gallic acid hydrate (Sigma-Aldrich, Madrid, Spain) in distilled water. Results were expressed as mg of gallic acid (GAE) per g of dried matter (d.m.).

## 2.6. Antioxidant capacity measurement (AC)

The antioxidant capacity was determined by using the ferric-reducing ability power (FRAP) method, which is a simple method used to estimate the reduction of a ferric-tripyridyltriazine complex. It was applied following the procedure described by Benzie & Strain (1996), with some modifications. Briefly, 900 μL of freshly prepared FRAP reagent were mixed with 30 μL of distilled water and 30 μL of test sample or water as appropriate reagent blank and kept at 37 °C for 30 min. The FRAP reagent contained 2.5 mL of a 10 mM TPTZ (Fluka, Steinheim, Germany) solution in 40 mM HCl (Panreac, Barcelona, Spain) plus 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ (Panreac, Barcelona, Spain) and 2.5 mL of 0.3 M acetate buffer (Panreac, Barcelona, Spain), pH 3.6 (Pulido et al., 2000). Readings at the maximum absorption level (595 nm) were taken using a spectrophotometer (UV-1800, Shimadzu, 's-Hertogenbosch, The Netherlands). Four replicates were made for each measurement. The antioxidant capacity was evaluated through a calibration curve, which was previously determined using water solutions of known Trolox (Sigma-Aldrich, Madrid, Spain) concentrations and expressed as mg Trolox per g of dried matter (d.m.).

## 2.7. Peroxidase (PO) activity

The PO activity was determined by monitoring the increase in the absorbance (UV-1601, Shimadzu, 's-Hertogenbosch, The Netherlands) at 414 nm and 25 °C with ABTS (Sigma-Aldrich, Madrid, Spain) as substrate. The reaction mixture consisted of 100 μL of ABTS 10 mM, 100 μL of Na-acetate buffer (Sigma-Aldrich, Madrid, Spain) 100 mM pH 5 and 790 μL of desalted sample extract. The reaction was started with the addition of 10 μL of 0.1 M $H_2O_2$, the optical density was recorded on-line for 10 min. The PO activity was expressed as units of enzymatic activity (UEA) per g of dried matter (d.m.). One UAE was defined as the amount of enzyme needed to

produce an increase of 0.001 optical density unit/min in a 1 cm cuvette under our standard assay conditions. Measurements were replicated three times.

## 2.8. Polyphenol oxidase (PPO) activity

The activity of PPO was measured by monitoring for 10 min the increase in the absorbance (UV-1601, Shimadzu, 's-Hertogenbosch, The Netherlands) at 400 nm and 25 °C with epicatechin (Sigma-Aldrich, Madrid, Spain) as substrate. The reaction mixture consisted of 500 μL of epicatechin 2 mM in MES buffer (Sigma-Aldrich, Madrid, Spain) pH 6 and 500 μL of desalted sample extract. The PPO activity was expressed as units of enzymatic activity (UEA) per g of dried matter (d.m.). One UAE was defined as the amount of enzyme needed to produce an increase of 0.001 optical density unit/min in a 1 cm cuvette under our standard assay conditions. Measurements were replicated three times.

## 2.9. Scanning electron microscopy (SEM)

A piece of dried apple was cut into two halves in such a way that a cross-section was obtained. In the case of dried apple, a very thin slice was cut off from the surface with a razor blade to obtain a high quality cross-sectional surface of the remaining piece of dry tissue. Obtaining the thin slice was not possible in the case of impregnated and dried apple so, the analysis focused only on the surface of the cross-section. In both cases, sample surface was sputter coated with platinum for better SEM imaging quality. The Pt coated sample was inserted into a scanning electron microscope (Jeol 6490LA, Tokyo, Japan) and both the peripheral and central areas were imaged at several magnifications: 25×, 50×, 100×, 250× and 500×.

## 2.10. Statistical analysis

Analysis of variance (ANOVA) were conducted (significance level of 95 %) in order to statistically identify the effect of the variables under study by using the Statgraphics-Plus software 5.1 (Statistical Graphics, Rockville, MD, USA).

## 3. Results and discussion

### 3.1. Influence of processing on the microstructure of dried apple

Changes at microstructural level were induced in apple by combining blanching, freezing and drying methods, as illustrated in Fig. 1, being structural modifications shown in Fig. 2.

Microstructural analysis showed that every pre-treatment greatly affected the microstructure of the dried apple. Therefore, it was possible to obtain samples with different structural properties (Fig. 2). In SEM micrographs, bright regions correspond to cell walls and membranes whereas intra and intercellular spaces appear as dark zones.

Prior blanching to drying (Fig. 2a, 2c, 2e and 2g) promoted remarkable changes on the microstructure of dried apple, being characterized by a more porous structure. The pectin substances are the main components of the middle lamella, a region which maintains cell to cell packing in fruit tissue (Johnston et al., 2002; Kunzek et al., 1999). During blanching, modifications of pectins and hemicelluloses may contribute to the collapse of the cell walls, resulting in cell separation and the increase of intercellular spaces (Chassagne-Berces et al., 2009). Moreover, blanching causes homogenization of sugars and other solutes over the tissue due to the disruption of membranes (Gonzalez & Barret, 2010).

The loss of membrane integrity facilitates free water permeation, giving no preference for extracellular nucleation during freezing (Voda et al., 2012). Thus, B-FD samples presented smaller ice crystals and more homogeneous distribution (Fig. 2c, 2e and 2g) than NB-FD samples (Fig. 2d, 2f and 2h). As noticed, this effect linked to blanching was more remarkable as the freezing rate increased. These results were consistent with the ones reported by Vergeldt et al. (2014), who stated that, in carrot, blanching before freezing at -150 °C resulted in smaller pores which were more homogeneously distributed whereas in freezing at -28 °C, the effect was less noticeable.

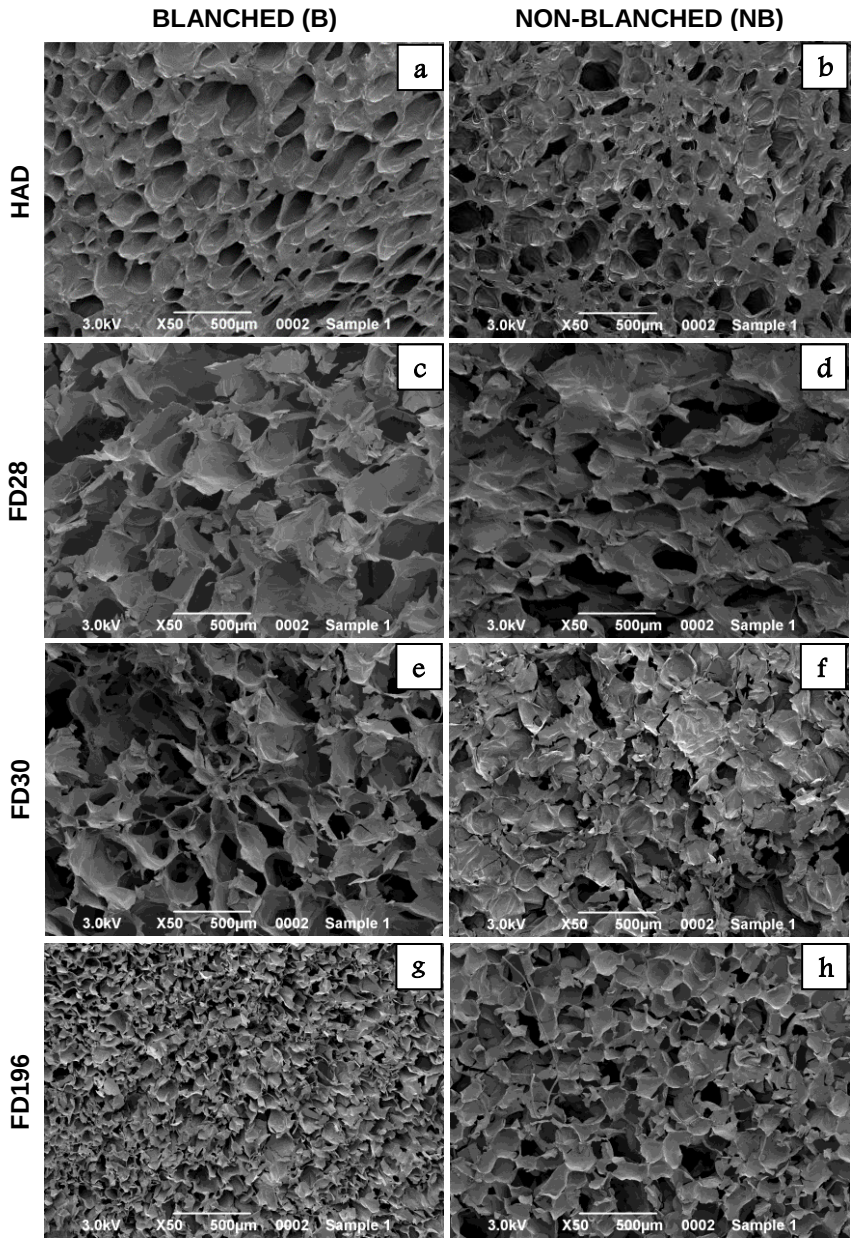


**Fig. 2.** Effects of blanching, freezing and drying on apple microstructure. B: blanching, NB: non-blanching, HAD: hot air drying, FD28: freezing at -28 °C in a conventional freezer and then freeze drying, FD30: freezing at -30 °C in a blast freezer and then freeze drying, and FD196: freezing at -196 °C in liquid $N_2$ and then freeze drying.

In the case of HAD apples, the enhancement of microstructure by blanching prior to drying was also observed (Fig. 2a and 2b). During drying, the structure and interactions with solid matrix affect diffusion of gases and liquids. Moreover, concentration gradients impose stresses on the material and diffusion can be accompanied by shrinkage and deformation (Lewicki, 2004). Nevertheless, these effects could be minimized by a previous blanching due to its abovementioned effects on cell structure. The more free water movement in blanched apples would facilitate the water leaving and would contribute to reduce the stress (Lewicki & Jakubczyk, 2004), giving rise to a less collapsed structure in B-HAD apple.

As regards freezing pre-treatment in FD apples, it also affected the structural integrity. In general terms, freeze-drying of apple caused structural modifications, such as cell wall collapse, texture breakage, membrane breakdown and more and larger intercellular spaces (Laurienzo et al., 2013). However, the structural modifications were mainly controlled by the ice crystal size, which is related to freezing rate. Thus, conventional freezing at -28 °C (Fig. 2c and 2d) induced the slow formation of bigger crystals, destroying the native cell structure and giving rise to the most open structure. Thus, structure of FD28 samples (Fig. 2c and 2d) was even more degraded than the one showed by HAD samples (Fig. 2a and 2b). On the contrary, FD196 apples (Fig. 2g and 2h) presented the highest levels of tortuosity, indicating better microstructure preservation, with less damage on cell walls and less cell collapse.

## 3.2. Effect of processing on enzymatic activity and antioxidant potential of dried apple

Aiming to characterize the dried material before carrying out the phenolic infusion, not only the microstructure of dried samples was analyzed but also other properties, such as the PPO and PO activities and the antioxidant potential (TPC and AC).

Blanching of fresh material affected the apple microstructure as aforementioned and, at the same time, had a significant ($p<0.05$) influence on the enzymatic activity. This pre-treatment completely inactivated the PO and PPO, providing dried materials free of active enzymes. In general terms, non-blanched apples showed higher PPO (Fig. 3b) than PO (Fig. 3a) activity, except the NB-HAD samples where the drying treatment seemed to be more effective in the denaturing of PPO enzymes.

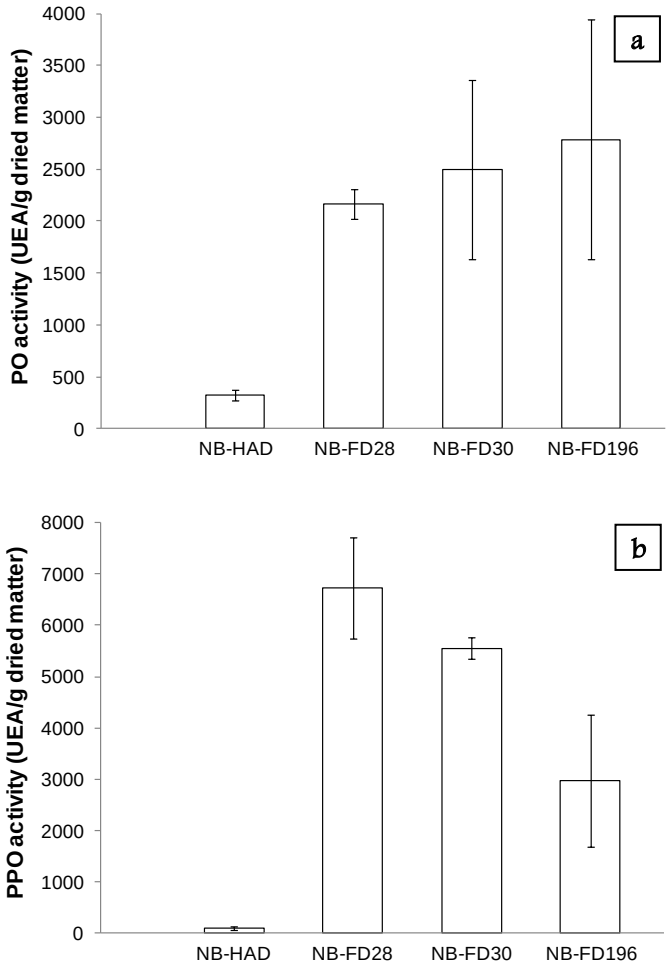


**Fig. 3.** Peroxidase (PO) and Polyphenol oxidase (PPO) activities of non-blanched (NB) dried apples. Means ± standard deviation are plotted. HAD: hot air drying, FD28: freezing at -28 °C in a conventional freezer and then freeze drying, FD30: freezing at -30 °C in a blast freezer and then freeze drying, and FD196: freezing at -196 °C in liquid $N_2$ and then freeze drying.

Drying temperature significantly ($p<0.05$) affected the enzymatic content, as also reported by Zhang et al. (2011). HAD at high temperature would positively contribute to inactivate the enzymes. Nevertheless, low temperatures applied during FD would preserve enzymes in latent state, recovering their activities when they are placed in contact with aqueous mediums. Thus, the NB-FD samples showed the highest PO (Fig. 3a) and PPO (Fig. 3b) activity. Even, the influence of freezing method was also appreciated since the faster the ice crystal formation the higher the PO (Fig. 3a) and the lower the PPO activity (Fig. 3b). However, it is important to highlight that this effect was significant ($p<0.05$) only in the case of PPO.

The cell damage suffered during drying and freezing not only promoted the further release of PO and PPO enzymes but also of other intracellular compounds, such as apple polyphenols. Thereby, processing could manage the extractability of polyphenols, making them more or less available for extraction (Ferreira et al., 2002) and so, affecting the antioxidant potential of samples. Previous studies have reported that processing causes no change to antioxidant potential of fruit and vegetables or enhances it due to the improvement of antioxidant properties of naturally occurring compounds or formation of novel compounds (Manzocco et al., 2001). Nevertheless in this study, this fact was not observed and all processing conditions reduced the TPC of fresh material (Fig. 4a). The degradation of the TPC was consistent with other works where the impact of apple drying on the phenolic content was studied (Rodríguez et al., 2014; Vega-Gálvez et al., 2012). Regarding the AC (Fig. 4b), the effect was different depending on the previous processing of apple. On the one hand, its reduction was significant ($p<0.05$) for HAD samples regardless the pre-treatment (B or NB), probably due to the high sensitivity of apple polyhenols to high temperatures. On the other hand, the FD samples previously blanched were the only ones able to keep the AC (Fig. 4b) despite the TPC decrease. This fact could be linked to the capacity of phenolic compounds to interact among them to provide new polyphenols with higher AC. In NB-FD samples, the reduction of both TPC and AC should be consequence of the residual enzymatic activity (Fig. 3a and 3b). Regarding the freezing method, no influence was observed in the antioxidant potential of FD samples.

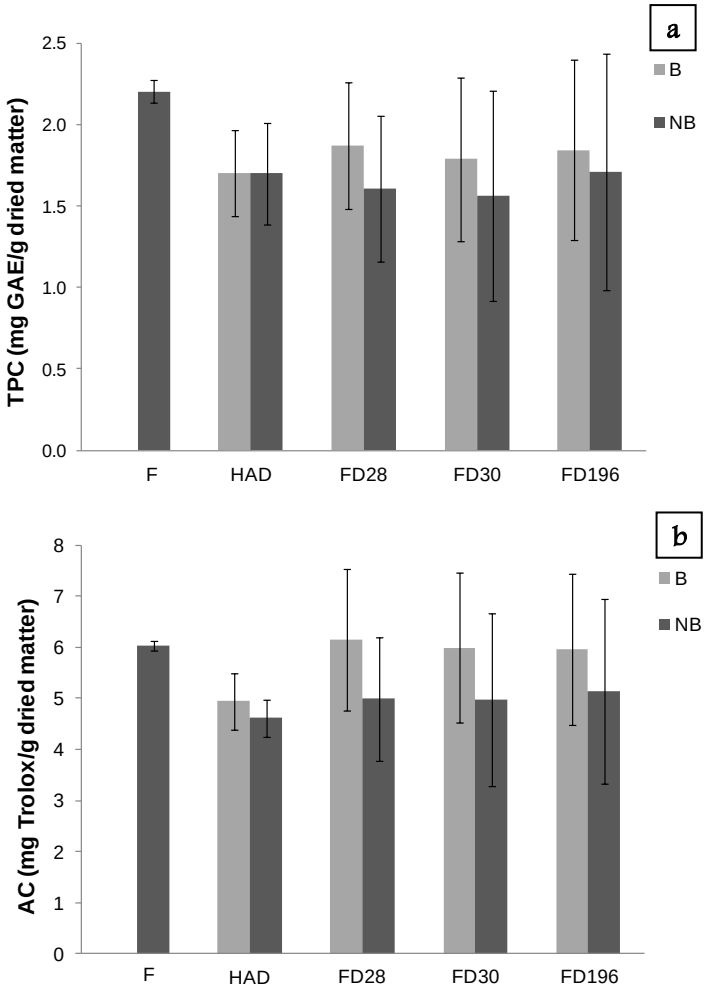


**Fig. 4.** Influence of processing on (a) the total phenolic content (TPC) and (b) antioxidant capacity (AC) of dried apple. Means ± standard deviation are plotted. B: blanching, NB: non-blanching, F: fresh, HAD: hot air drying, FD28: freezing at -28 °C in a conventional freezer and then freeze drying, FD30: freezing at -30 °C in a blast freezer and then freeze drying, and FD196: freezing at -196 °C in liquid $N_2$ and then freeze drying.

## 3.3. Phenolic infusion into dried apple and final stabilization by drying

Dried apple cubes were vacuum impregnated with tea extract rich in antioxidant compounds (Fig. 1). Afterwards, in order to obtain stable products, the drying of impregnated samples was performed and the TPC, AC and microstructure were analyzed.

The microstructural analysis highlighted that, regardless apple pre-treatments, the structure of the impregnated-dried samples was similar, this being characterized by a total tissue collapse (Fig. 5). This fact could be explained by the vacuum treatment during impregnation. Vacuum causes an expansion and a further release of the occluded internal gas (Gras et al., 2002). Then, the recovery and holding of the atmospheric pressure during the impregnation pushes the solvent (tea) into the spaces initially occupied by the gas keeping the sample volume. However, when the water is removed by the final drying, samples lose their integrity since there is neither air nor liquid to keep the structure, resulting in compact vegetable tissues. The undesirable structural changes as a consequence of the vacuum infusion have been also observed by other authors. Del Valle et al. (1998) attributed the structural changes suffered by apple cylinders to the vacuum application during the penetration of water into the samples. Although it has also been reported that the structural collapse could be, in certain way, controlled by the vacuum level (Bolin & Huxsoll, 1987).

The combination of drying-impregnation-drying provided stable products with much higher antioxidant potential (Fig. 6) than those found in the dehydrated raw apple (Fig. 4), which confirms the results obtained by Ahmad-Qasem et al. (2015). Blanching had a significant ($p<0.05$) effect in both TPC (Fig. 6a) and AC (Fig. 6b) when samples were dried by FD before the impregnation. FD samples improved the TPC and AC by prior blanching due to its influence on PPO and PO activity. This result highlighted the influence of the residual enzymatic activity on the antioxidant potential, a hypothesis already proposed in previous works (Ahmad-Qasem et al., 2015).

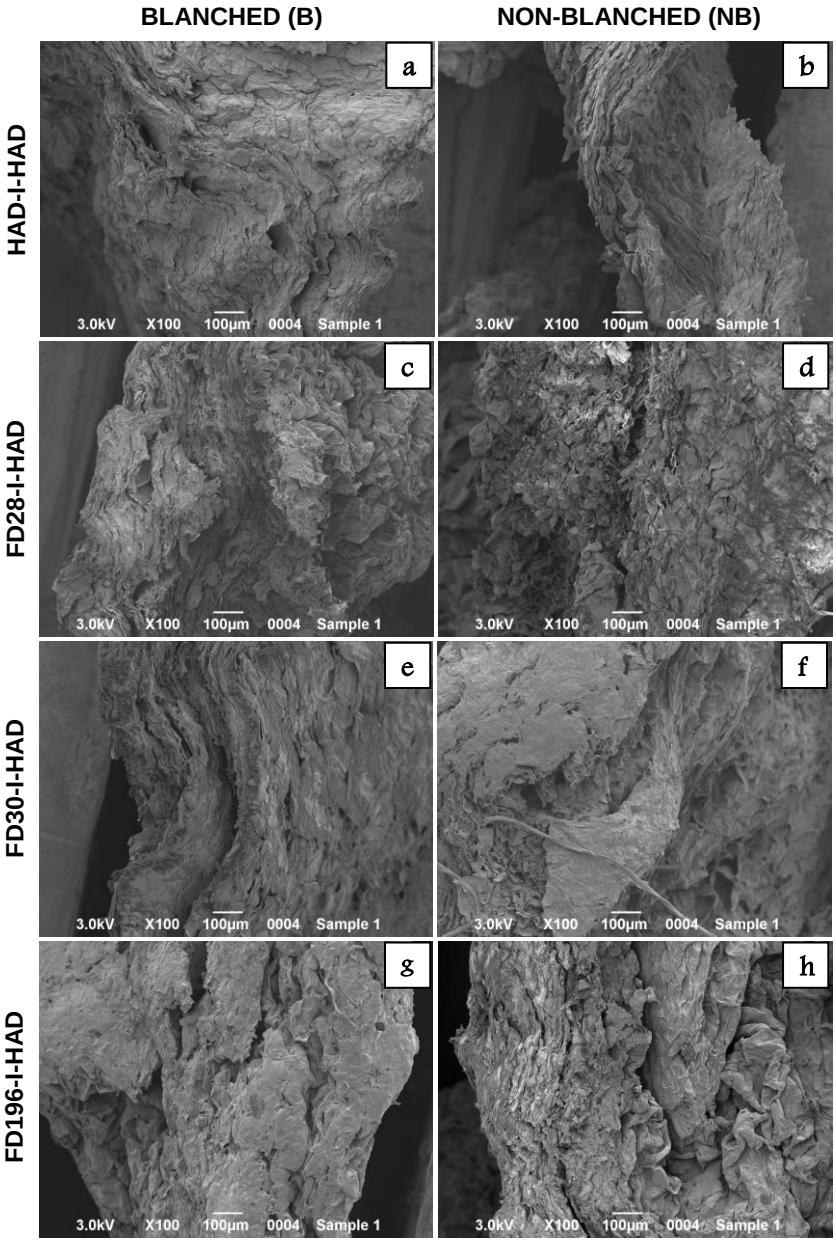


**Fig. 5.** Effects of processing on microstructure of dried apples previously pretreated and vacuum impregnated with tea extract. B: blanching, NB: non-blanching, I: impregnation, HAD: hot air drying, FD28: freezing at -28 °C in a conventional freezer and then freeze drying, FD30: freezing at -30 °C in a blast freezer and then freeze drying, and FD196: freezing at -196 °C in liquid $N_2$ and then freeze drying.

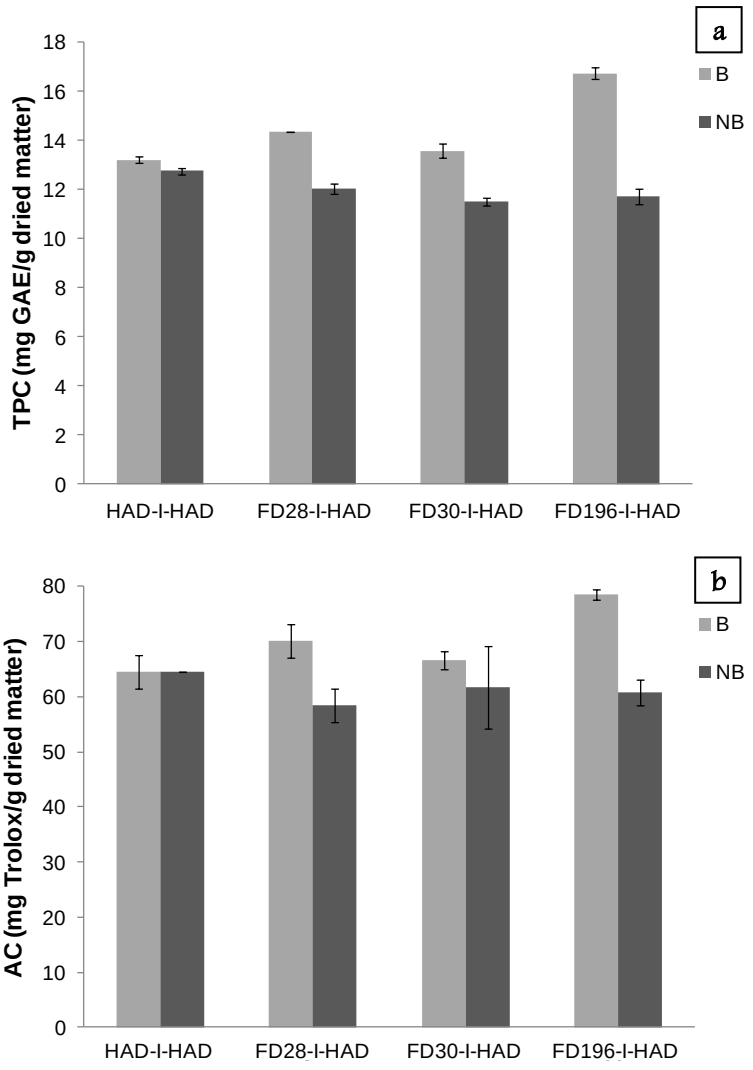


**Fig. 6.** Antioxidant potential (TPC and AC) of vacuum impregnated dried apples with tea extract. Means ± standard deviation are plotted. B: blanching, NB: non-blanching, I: impregnation, HAD: hot air drying, FD28: freezing at -28 °C in a conventional freezer and then freeze drying, FD30: freezing at -30 °C in a blast freezer and then freeze drying, and FD196: freezing at -196 °C in liquid $N_2$ and then freeze drying.

In addition, in blanched samples, it was possible to study the influence of microstructure on the phenolic infusion. Thus, the structure, determined by the freezing and drying method, affected significantly ($p<0.05$) TPC and AC when PPO and PO were denatured. The highest antioxidant potential

(TPC of 16.7 ± 0.2 mg GAE/g d.m. and AC of 78.5 ± 0.9 mg Trolox/g d.m.) was found in samples with the most porous structure (Fig. 2g), the FD196-I-HAD apples. This fact would confirm the hypothesis of a previous work (Ahmad-Qasem et al., 2015) where it was suggested that polyphenols infused in an open structure are more exposed to dehydration conditions due to their weak interaction with the poorly consolidated solid matrix of FD samples previously frozen by a conventional method (-28 °C).

For NB samples, no clear influences were observed due to the dual effect of the enzymatic activity and structure in FD samples. Ahmad-Qasem et al. (2015) postulated that HAD is better than FD to obtain final dried products with high TPC and AC (Fig. 6) due to it involves a combined thermal/drying treatment. The present study agreed with this result, although the differences between drying methods were smaller probably due to the different operating conditions of FD and the sensitivity of phenolic compounds (olive leaves or tea extract) to the processing conditions.

Regarding the drying applied after the impregnation of apple pieces, it inactivated the PPO and PO of NB-FD samples (Fig. 3). Thus, the enzymes were not found in any final dried product, providing materials completely stable.

## 4. Conclusions

Blanching, freezing and drying affected the microstructure, PPO and PO activity of dried apple, which are key factors to preserve the phenolic compounds infused into the solid matrix. Thus, latent oxidative enzymes in freeze dried materials contributed to the degradation of impregnated polyphenols. Meanwhile, a more porous and well consolidated structure protected the infused compounds by reducing their exposition to drying conditions. The combination of blanching and freezing with liquid $N_2$ prior to the freeze drying provided impregnated apples with the highest antioxidant potential.

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## Abbreviations used

B, blanching; NB, non-blanching; F, fresh apple; FD, freeze drying; HAD, hot air drying; FD28, freezing at -28 °C in a conventional freezer and then freeze drying; FD30, freezing at -30 °C in a blast freezer and then freeze drying; FD196, freezing at -196 °C in liquid $N_2$ and then freeze drying; I, impregnation; TPC, total phenolic content; GAE, gallic acid equivalents; AC, antioxidant capacity; FRAP, ferric-reducing ability power; TPTZ, 2,4,6-tri(2-pyridyl)-s-triazine; PO, peroxidase; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); UEA, units of enzymatic activity; PPO, polyphenol oxidase; MES, 2-(N-Morpholino)ethanesulfonic acid sodium salt; SEM, scanning electron microscopy.

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# 5. GENERAL DISCUSSION

Olive leaves are a potential source of phenolic compounds with several bioactive properties (Taamalli et al., 2012), which, even today, are not widely exploited on an industrial scale. A better use of these compounds should involve improving raw material processing and extraction technology, as well as studying the effects of processing on the subsequent stability of the extracts and searching for feasible applications.

Drying and freezing are two of the main preservation methods in agri-food industries. However, they could cause an impact in the raw materials which affects their subsequent processing. Thus, both methods could facilitate the extraction of target compounds from vegetable matrices. On the one hand, drying places great stress on cell wall constituents (Górnaś et al., 2014). Moreover, dried tissues become more brittle and easier to be milled. On the other hand, freezing implies the formation of ice crystals, which break the native structures of vegetable cells (Asami et al., 2003). Freeze drying, considered one of the drying methods par excellence in the preservation of product quality, entails not only a drying process but also a previous freezing operation. Thus, it could be assumed that freeze drying would be the most appropriate technique with which to improve the extraction of olive leaf phenolic compounds. Nevertheless, the experimental results obtained in this Thesis highlighted that, regardless of the freezing method, freeze drying did not have the expected positive effect on the polyphenol extraction. Thereby, besides reducing the drying time, hot air drying at 120 °C provided olive leaf extracts with a significantly ($p<0.05$) higher total phenolic content and antioxidant capacity than freeze dried leaves. Therefore, the drying method used in olive leaf processing had a crucial influence on the antioxidant potential of the extracts. Moreover, the use of an affordable technique (hot air drying) with which to achieve extracts with high content of polyphenols, such as oleuropein, could be considered a relevant finding, since this fact has not been previously reported in literature.

As previously mentioned, short drying times at high temperatures were the most appropriate drying conditions under which to obtain high extraction yields. This fact could be associated with the effect of the drying method on the enzymatic activity in

the raw material. Thus, the natural oxidative enzymes of olive leaves could remain latent in the freeze dried materials and they could recover their activity in the aqueous medium during the phenolic extraction (Hossain et al., 2010). On the contrary, the heat treatment applied during hot air drying may be enough to inactivate these oxidative enzymes of the plant tissue, avoiding subsequent activity. However, references in literature point to there being considerable controversy about the impact of high temperatures on the phenolic content of vegetable materials (Kim et al., 2006; Mejia-Meza et al., 2008). This fact suggests that, besides the drying method, the particular characteristics of each raw material may play a key role in the preservation or degradation of the bioactive compounds.

Another byproduct of the oil industry, olive pomace, and its use as a source of polyphenols, was also explored. As in the case of olive leaves, it was observed that the higher the drying temperature, the greater the antioxidant capacity of the extracts obtained. Moreover, extending the drying time at high temperature was also found to have a positive effect. Thus, the overheating of samples at 150 °C gave rise to an increase in the antioxidant potential. The fact that the antioxidant potential was improved by long drying times at high temperatures could be attributed to the generation and accumulation of Maillard-derived melanoidins with a varying degree of antioxidant activity (Que et al., 2008) and the conversion of some phenolic compounds into others with a higher antioxidant capacity (Benavente-García et al., 2000). In any case, despite the positive influence of high temperatures on both raw materials, olive leaves provided extracts with a much higher bioactive content than olive pomace.

Owing to there being several limitations of the conventional extraction methods, many new technologies are being developed to improve extraction technology, among others, power ultrasound. This is a simple, efficient and inexpensive new technology (Huang et al., 2009b) which was applied in this Thesis in order to intensify the extraction of olive leaf polyphenols. In this sense, a parametric study was carried out in order to identify how the different process variables affect the ultrasonic performance. The electric power applied to the ultrasonic transducer ended up being a relevant variable, since the more power supplied, the higher the

total phenolic content and antioxidant capacity of the extracts. It is important to highlight that in ultrasonically assisted extraction processes, among other phenomena, cavitation is what is mainly responsible for improving solvent penetration and favoring mass transfer (Wang et al., 2008). Thus, cavitation became more intense as the ultrasonic power applied in the medium increased. The ultrasonic emitter surface was also found to be a meaningful variable, since it influences the coupling between the emitter and the extracting medium. Therefore, combining the highest electric power supplied and the intermediate emitter surface (3.8 $cm^2$), a homogeneous treatment with intense cavitation was achieved. Finally, there was found to be no clear influence of temperature on the extraction process. It is likely that the mass transfer improvement produced by the increase in temperature (Esclápez et al., 2011) was masked by the effect of the mechanical energy introduced into the medium by ultrasonic waves. However, in an ultrasonically assisted extraction process it is important to accurately control the extraction temperature in order to avoid thermal effects linked to cavitation and any possible negative impact on thermolabile compounds. Therefore, the fine-tuning of ultrasound parameters was essential to achieve a high performance phenolic extraction. Compared to a conventional extraction method, ultrasound assisted extraction provided extracts with the same antioxidant potential, but reduced the processing time from 24 h to 15 min. Hence, power ultrasound could be presented as a reliable technology with which to intensify olive leaf polyphenol extraction.

By means of olive leaf drying, the bioactive content of extracts could be modified, and, by applying power ultrasound, it is possible to accelerate the extraction process. Nevertheless, could processing variables affect the stability, resistance or sensitivity of olive leaf extracts? In this context, the extracts' behavior was evaluated during both *in vitro* gastrointestinal digestion and storage.

In general terms, it could be stated that processing conditions did not greatly affect the stability of extracts during the *in vitro* digestion. In the different extracts studied, the total phenolic content was significantly ($p<0.05$) reduced during the first hour of the digestion, remaining stable during the rest. The degradation of the main phenolic compounds during the digestion was neither affected by how olive leaves

were dried nor how extraction was performed. As regards the particular stability of each compound, verbascoside was a little more resistant to gastric digestion than oleuropein and luteolin. However, the luteolin-7-O-glucoside content was hardly affected by the intestinal digestion, whereas oleuropein and verbascoside almost disappeared from the medium, as they were extremely sensitive to intestinal conditions. Therefore, luteolin-7-O-glucoside could be considered fairly resistant to digestion and an interesting polyphenol because of its high bioaccessibility. On the contrary, protective mechanisms against pancreatic enzymes and alkaline pH are essential in order to control the release of oleuropein and verbascoside and improve their bioaccessibility.

The stability of olive leaf extracts was also assessed by combining drying and storage conditions. As mentioned above, how olive leaves were dried greatly affected the antioxidant potential of the extracts obtained. However, it was still necessary to determine if the leaf drying itself and the drying method used could have any effect on the behavior of the liquid extracts during their storage at 4 °C. Thus, it was found that the drying of olive leaves promoted a significant ($p<0.05$) reduction in the antioxidant capacity of extracts at the end of the storage (day 21). Furthermore, from the quantification of the main polyphenols, it was observed that the stability during the storage of an individual phenolic compound could be conditioned by the other phenolic compounds present in the extract (Porrini & Riso, 2008). This would explain why the trend of an individual polyphenol differs depending on the overall extract composition (extracts obtained from fresh, hot air dried or freeze dried leaves). Therefore, the processing of the raw material affected not only the initial extract composition but also the evolution of individual compounds during the storage.

Considering that most of natural extracts are commercialized as powder, olive leaf extracts were dehydrated to test the impact of this stage on their bioactive content and stability. Notwithstanding this, powdered forms of the extracts could be re-diluted in order to be used in different industries. The dehydration of extracts by means of both hot air drying at 120 °C and vacuum drying at 55 °C preserved around 90 % of the antioxidant capacity and total phenolic content. These results were comparable to others obtained from the dehydration of other natural

antioxidant extracts by spray drying (Fang & Bhandari, 2011) or spouted bed drying (Benelli et al., 2013). Consequently, hot air drying at 120 °C would be an advisable method of conducting extract dehydration. Besides, due to its simplicity and high dehydration rate, drying at 120 °C would also have direct and positive implications on productivity. As for extract stability during storage, neither the storage temperature (4-35 °C) nor the form (powder or re-diluted) significantly ($p<0.05$) influenced the antioxidant potential. Hence, olive leaf extracts could be considered as stable for, at least, 28 days.

One of the most promising applications of olive leaf extracts in the food industry could be in the development of functional foodstuffs, mainly, due to the fact that these products are increasingly in demand (Çam et al., 2014; Ribas-Agustí et al., 2014). In consequence, the last part of this Thesis focused on the viability of obtaining a stable food matrix (apple) enriched with phenolic compounds from olive leaves. Thus, a three-stage process was designed by combining the drying of apple to favor the phenolic infusion, the impregnation of an olive leaf extract and a further drying step to stabilize the enriched apple (final product). This processing gave rise to a final, dried apple rich in olive leaf polyphenols and with much greater antioxidant potential than the initial dried apple. However, the retention of infused polyphenols was greatly dependent on how the drying steps were performed. In this regard, the fresh apple drying influenced the retention of infused olive leaf polyphenols more than the subsequent drying of the impregnated apple. It is well known that freeze drying provides dried material with a high impregnation rate, probably due to the cellular disruption suffered by the vegetable material as a result of freezing (Van Buggenhout et al., 2006) and the formation of a high-porosity matrix during drying. Hence, to reach the same antioxidant capacity, the impregnation time for hot air dried apples was almost twice as long as that for freeze dried apples. Notwithstanding this, when the antioxidant potential of the final product (dried-impregnated-dried) was determined, apples which had been initially hot air dried had a significantly ($p<0.05$) higher phenolic content than that observed in samples that were initially freeze dried. This could be explained by both the residual enzymatic activity present in the unfrozen rubbery state water fraction of frozen samples (Blanda et al., 2008)

and the cell damage caused by the freezing step. Thus, the cellular breakage in freeze dried apples (Laurienzo et al., 2013) would help to remove cellular barriers by facilitating the interaction between the oxidative enzymes and phenolic compounds of the impregnated extract, thereby reducing the antioxidant potential of infused apples. Moreover, as result of the growth of ice crystals during freezing, the native structure of freeze dried apples is the most open and degraded (Voda et al., 2012) which could also imply a greater exposure of the phenolic compounds to drying conditions during the final drying step. These possible causes were thoroughly studied by combining blanching, freezing (conventional, blast freezing and liquid $N_2$) and drying methods (hot air drying and freeze drying). Thus, from fresh apples, it was possible to obtain dried materials, which differed as to their enzymatic activity and structure, to be impregnated. It was confirmed that the antioxidant potential of impregnated dried samples depended on both the microstructure and the enzymatic activity of the initial dried material. Therefore, to obtain a final dried-impregnated-dried product with high antioxidant potential by using freeze dried apples for the impregnation, it would be necessary to blanch and freeze the fresh apple with liquid $N_2$. This would be an appropriate way to obtain a freeze dried product free of degradative enzymes and with a porous, well-consolidated matrix.

As regards the drying of the impregnated product, the method used did not exert a significant influence ($p<0.05$) on the antioxidant capacity or the total phenolic content of dried-impregnated-dried apples. Only the content of some individual olive leaf polyphenols, such as oleuropein, was affected by the method used in this final drying step. Thus, hot air dried apples, impregnated and subsequently freeze dried, exhibited the highest antioxidant potential. In this case, once the oxidative enzymes were inactivated by the hot air drying, the low temperature applied during freeze drying caused a lower degree of degradation of this bioactive compound. Notwithstanding this, in general terms, hot air drying could be considered a feasible stabilization technology for apple impregnated with olive leaf extract.

In conclusion, the exploitation of olive leaves for industrial purposes is of considerable potential. This olive oil byproduct presents a high antioxidant potential, which is largely affected by how it is processed, as this Thesis revealed. Processing conditions interfere in not only the composition of the extracts and the later stability of the extracts, but also the prospective applications, such as solid matrix impregnation.

# 6. CONCLUSIONS

According to the results obtained and the structure of the present Thesis, the main conclusions achieved are listed and grouped into four sections.

## 6.1. Influence of raw material processing on antioxidant potential of extracts obtained from olive byproducts

### Olive leaves

- The antioxidant potential (antioxidant capacity and phenolic content) of extracts was influenced by both freezing and drying leaves.
- Regardless of the freezing technique, freeze dried leaves provided extracts with lower antioxidant potential than those hot air dried.
- In hot air drying, the highest temperature tested (120 °C) was the most appropriate at which to dry olive leaves, increasing the antioxidant potential and oleuropein content of the extracts.
- Using high drying temperatures to improve the extraction of oleuropein has not been previously reported and could be a relevant finding for industrial purposes.

### Olive pomace

- A good description of the drying behavior of olive pomace was obtained using a compositional diffusion model considering different effective diffusivities in pit and pulp+peel fractions.
- Long drying times at the highest temperature tested (150 °C) increased the antioxidant potential.

## 6.2. Intensification of polyphenols extraction from olive leaves by means of power ultrasound

- Ultrasonic effectiveness depended on the effective power applied and the coupling between the emitter and the extracting medium.

- In the range studied, the extraction temperature had no clear effect either on the kinetics of the antioxidant capacity or the total phenolic content.

- Ultrasound assisted extraction can be considered a more efficient process than conventional extraction techniques for obtaining extracts with high antioxidant potential. Ultrasound application shortened the extraction time from 24 h (conventional extraction) to 15 min, achieving a similar content of bioactive compounds.

- Ultrasound application on an industrial scale requires an exhaustive tuning of processing parameters for each raw material as well as an economic study including all the costs implied in the extraction process.

## 6.3. Influence of processing stages on the stability of olive leaf extracts

- During the *in vitro* gastrointestinal digestion of olive leaf extracts, the degradation kinetics of the main bioactive compounds was not dependent on the processing stages applied to obtain these extracts. Thus, neither the drying of olive leaves nor the extraction method (conventional or ultrasound application) affected the extracts stability.

- The amount of oleuropein and verbascoside at the end of the digestion process was almost negligible.

- Luteolin-7-O-glucoside was fairly resistant to digestion, and therefore must be considered as a target polyphenol for further absorption processes.

- The drying of olive leaves influenced the stability of phenolic compounds during the storage of extracts.

- The dehydration of olive leaf extracts by simple and affordable methods, such as hot air drying at 120 °C and vacuum drying at 55 °C, only reduced the antioxidant potential by around 10 %.

- For 4 weeks, neither the extract form (liquid or powder) nor the storage temperature (4–35 °C) affected the bioactive content of extracts.

## 6.4. Prospective applications: Development of dried products (apple) enriched with phenolic compounds

- The combination of drying–impregnation–drying steps allowed a stable apple-based product enriched with olive leaf polyphenols to be obtained.

- The retention of infused polyphenols was more dependent on the fresh apple drying than on the further drying of the impregnated apple.

- Hot air dried apples retained a greater quantity of olive leaf polyphenols than those freeze dried.

- Blanching and freezing have to be considered as crucial pretreatments in the freeze drying of apple due to their impact on microstructure and enzymatic activity.

- The retention of phenolic compounds infused in a solid matrix (dried apples) greatly depended on both the microstructure and the enzymatic activity of the dried material used in the impregnation.

- The more porous and consolidated the structure of the dried material used for the phenolic infusion, the better the retention of phenolic compounds.

- The latent oxidative enzymatic activity of freeze dried materials degraded the phenolic compounds during and after the impregnation step.

## GENERAL CONCLUSION

In overall terms, olive leaves can be considered a potential source of natural phenolic compounds. Notwithstanding this, prior drying and freezing steps, applied during the raw material processing, are of paramount importance for obtaining natural extracts with a high content of bioactive compounds. Moreover, enhancing the extraction by applying power ultrasound was stated as a non-thermal way of shortening processing times. The stability of olive polyphenols during both storage and *in vitro* digestion was closely related to the individual component considered. Finally, the exploitation of olive leaf extracts for the purposes of enriching solid foodstuffs requires the use of porous solid matrices free of oxidative enzymes.

# 7. RECOMMENDATIONS

On the basis of the results obtained, the following aspects could be explored in further research works in order to complete the research topics addressed in this Thesis:

## Phenolic compounds sources

- To search for new alternative sources of phenolic compounds coming from byproducts generated in the food industry during both harvest of raw materials and manufacturing of foodstuffs.

## Raw material processing

- To study the combined effect of raw material pre-treatments, such as washing, blanching, freezing and drying, on the further extraction of bioactive compounds.
- To analyze the deep bed drying of olive pomace in order to validate the developed model and confirm the effect of temperature on the antioxidant potential of olive pomace.

## Bioactive compounds extraction from olive leaves

- To determine the influence of power ultrasound on the extraction kinetics of the individual polyphenols, identifying the optimal variables for each compound and elucidating the biochemical mechanisms involved in the interactions among them.
- To develop efficient ultrasonic transducers on an industrial scale in order to improve the extraction processes.
- To estimate the initial investment and operational costs of the implementation of power ultrasound assisted extraction on the industry.
- To test the viability of the use of other new technologies to enhance the extraction of bioactive compounds from vegetable byproducts, such as microwave or pulsed-electric fields.

## Olive leaf polyphenols bioaccessibility

- To carry out *in vivo* tests to verify the results obtained for the *in vitro* digestion of olive leaf extracts.
- To improve the bioaccessibily of olive leaf phenolic compounds by microencapsulation and spray drying of the extracts performing a parametric study of the main variables which can affect the process.

## Stabilization of antioxidant extracts

- To evaluate the use of new drying techniques, such as drying at low temperature with and without power ultrasound assistance, to obtain dehydrated extracts with high antioxidant potential.
- To design self-life studies with the aim of determining the effect of storage conditions on extracts properties during long periods of storage.

## Prospective applications of natural extracts in food industry

- To assess the impact of the addition of olive leaf extracts on the quality parameters of food formulations, such as dairy products or beverages.
- To promote the use of natural antioxidant compounds as food additives.

# 8. SCIENTIFIC CONTRIBUTION

## Research papers

Ahmad-Qasem, M. H., Barrajón-Catalán, E., Micol, V., Mulet, A., & García-Pérez, J. V. (2013). Influence of freezing and dehydration of olive leaves (var. Serrana) on extract composition and antioxidant potential. *Food Research International*, 50, 189-196.

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Ahmad-Qasem, M. H., Ahmad-Qasem, B. H., Barrajón-Catalán, E., Micol, V., Cárcel, J. A., & García-Pérez, J. V. Drying and storage of olive leaf extracts. Influence on polyphenols stability. *Industrial Crops and Products*, submitted.

## Contributions to congresses

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Ahmad-Qasem, M. H., Cánovas, J., Barrajón-Catalán, E., Carreres, J. E., Cárcel, J. A., & García-Pérez, J. V. (2011). Efecto de la digestión *in vitro* sobre la composición de los extractos polifenólicos obtenidos a partir de hoja de olivo (var. Serrana). VI Congreso Nacional de Ciencia y Tecnología de los Alimentos (CYTA 2011). Valencia (Spain).

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