University of the Aegean

School of the Environment

Department of Food Science and Nutrition

Recovery of antioxidant polyphenols from olive leaves (*Olea europaea* spp.) using a novel deep eutectic solvent made of bioorganic molecules

DOCTORAL THESIS

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Ανάκτηση αντιοξειδωτικών πολυφαινολών από φύλλα ελιάς (*Olea europaea* spp.) με χρήση καινοφανούς βαθέως εύτηκτου διαλύτη βασισμένου σε βιομόρια

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

του ΒΑΣΙΛΕΙΟΥ ΑΘΑΝΑΣΙΑΔΗ

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Υποβάλλεται για την απόκτηση Διδακτορικού Διπλώματος
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Abstract

In this study, the extraction of polyphenols from *Olea europaea* leaves was investigated, using a biomolecule-based deep eutectic solvent (DES), composed of glycerol and the amino acid glycine. The study, which is presented here, describes for the first time the use of an eco-friendly DES as a highly effective solvent for the extraction of polyphenols from olive tree leaves.

The first part was to optimise DES concentration (C_{DES}) and the liquid-to-solid ratio ($R_{L/S}$), by employing a Box-Behnken experimental design. Following this, a kinetic assay was undertaken to assess the effect of temperature. Comparative evaluation using 60% ethanol, 60% methanol and water showed that the DES used was significantly more efficient in extracting polyphenols and flavonoids, yielding extracts with higher antiradical activity and reducing power. Liquid chromatography-photodiode array-mass spectrometry (LC-PDA-MS) examination of the DES extract showed that the major polyphenols were luteolin and apigenin glycosides, as well as the secoiridoid derivative oleuropein.

In the second part, it was evaluated the effect of methyl-β-cyclodextrin (m-β-CD) on the efficiency of polyphenol extraction from *O. europaea* leaves. The process developed was based on a Box-Behnken experimental design and response surface methodology, to assess the simultaneous effect of m-β-CD concentration ($C_{m-β-CD}$), liquid-to-solid ratio ($R_{L/S}$) and temperature (T). The extraction kinetics also showed that the extraction rate was slowed down in the presence of m-β-CD, yet the higher extraction capacity of the DES/m-β-CD medium was confirmed. Characterization of the extracts obtained with DES/m-β-CD and DES by means of LC-PDA-MS demonstrated that there was no selective extraction of any particular polyphenol, suggesting that m-β-CD acted merely as an extraction booster.

In the third part, the stability of the extracts was checked. In particular, the extracts (DES/m-β-CD, DES, 60% ethanol and water) were allowed to stand at 4 °C (cooling temperature), 22 °C (room temperature) and 50 °C (water bath) for 20 days. After this period, the reducing power (P_R) was monitored to trace changes in the antioxidant potency of the extracts. Examination of the polyphenolic profiles using LC-PDA-MS showed that after storage for 20 days at 50 °C, some major polyphenols occurring in olive leaves (OLL) suffered extended degradation.

In the fourth and final part, the use of a novel natural DES was studied that enabled the effective extraction of OLL polyphenols and their testing as radical scavengers, in the presence or absence of m-β-CD, using descriptive kinetics. Testing extended to include interactions with ascorbic acid, a natural powerful antioxidant, by implementing response surface methodology. The kinetic study showed that m-β-CD may hinder the radical scavenging effect of OLL extracts, yielding lower stoichiometry upon reaction with the radical probe DPPH.

Περίληψη

Σε αυτή τη μελέτη διερευνήθηκε η εκχύλιση αντιοξειδωτικών πολυφαινολών από τα φύλλα ελιάς (*Olea europaea*), με τη χρήση ενός καινοφανούς βαθέως εύτηκτου διαλύτη (DES), βασισμένου σε βιομόρια, που αποτελείται από γλυκερόλη και γλυκίνη. Η μελέτη που παρουσιάζεται εδώ περιγράφει για πρώτη φορά τη χρήση ενός φιλικού προς το περιβάλλον βαθέως εύτηκτο διαλύτη ως εξαιρετικά αποτελεσματικού διαλύτη για την εκχύλιση πολυφαινολών από φύλλα ελιάς.

Στο πρώτο μέρος βελτιστοποιήθηκε η συγκέντρωση του DES (C_{DES}) και η αναλογία υγρού προς στερεό ($R_{L/S}$), χρησιμοποιώντας ένα πειραματικό σχέδιο Box-Behnken. Εν συνεχεία, για να εκτιμηθεί η επίδραση της θερμοκρασίας στην απόδοση της εκχύλισης πραγματοποιήθηκε μια κινητική δοκιμασία. Συγκριτική αξιολόγηση με χρήση 60% αιθανόλης, 60% μεθανόλης και νερού έδειξε ότι το DES που χρησιμοποιήθηκε ήταν σημαντικά πιο αποτελεσματικό στην εκχύλιση πολυφαινολών και φλαβονοειδών, αποδίδοντας εκχυλίσματα με μεγαλύτερη ικανότητα απόσβεσης ελευθέρων ριζών (A_{AR}) και αναγωγική ισχύ (P_R). Τέλος, πραγματοποιήθηκε ανάλυση με υγρή χρωματογραφία-φασματοσκοπία συστοιχίας-φασματομετρία μάζας (LC-PDA-MS) του εκχυλίσματος DES, η οποία έδειξε ότι οι κύριες πολυφαινόλες ήταν οι γλυκοζίτες της λουτεολίνης και της απιγενίνης, καθώς και η ελευρωπαΐνη.

Στο δεύτερο μέρος αξιολογήθηκε η επίδραση της μεθυλ-β-κυκλοδεξτρίνης (m-β-CD) στην αποτελεσματικότητα της εκχύλισης πολυφαινολών από φύλλα *O. europaea*. Η διαδικασία που αναπτύχθηκε βασίστηκε σε μια μεθοδολογία επιφανειακού σχεδιασμού και σε ένα πειραματικό σχέδιο Box-Behnken, για να εκτιμηθεί η ταυτόχρονη επίδραση της συγκέντρωσης της m-β-CD ($C_{m-β-CD}$), του λόγου υγρού προς στερεό ($R_{L/S}$) και της θερμοκρασίας (T). Η κινητική της εκχύλισης έδειξε επίσης ότι ο ρυθμός εκχύλισης επιβραδύνθηκε παρουσία m-β-CD, αλλά επιβεβαιώθηκε η υψηλότερη ικανότητα εκχύλισης του μέσου DES/m-β-CD. Τέλος, πραγματοποιήθηκε ανάλυση με LC-PDA-MS του εκχυλίσματος με DES/m-β-CD και DES, όπου αποδείχτηκε ότι δεν υπήρχε επιλεκτική εκχύλιση οποιασδήποτε συγκεκριμένης πολυφαινόλης, υποδηλώνοντας ότι η m-β-CD ενήργησε απλώς ως ενισχυτικό εκχύλισης.

Στο τρίτο μέρος πραγματοποιήθηκε έλεγχος σταθερότητας των εκχυλισμάτων. Πιο συγκεκριμένα, τα εκχυλίσματα (με χρήση DES/m-β-CD ή DES ή 60% αιθανόλη ή νερό) μετά την παρασκευή τους αφέθηκαν σε διάφορες θερμοκρασίες, όπως ψύξης (4 °C), κανονικές συνθήκες (22 °C) και θέρμανσης (50 °C) για 20 μέρες. Μετά το πέρας του διαστήματος αυτού, η P_R παρακολουθήθηκε για την ανίχνευση αλλαγών στην αντιοξειδωτική ισχύ των εκχυλισμάτων. Η εξέταση του πολυφαινολικού προφίλ με τη χρήση LC-PDA-MS έδειξε ότι μετά από αποθήκευση επί 20 ημέρες στους 50 °C, ορισμένες πολυφαινόλες που υπάρχουν στα φύλλα της ελιάς (OLL) υπέστησαν εκτεταμένη αποικοδόμηση.

Στο τέταρτο και τελευταίο μέρος μελετήθηκε η χρήση ενός νέου φυσικού DES που επέτρεψε την αποτελεσματική εκχύλιση των πολυφαινολών από OLL και τη δοκιμή τους ως δεσμευτές ελευθέρων ριζών, παρουσία ή απουσία m-β-CD, χρησιμοποιώντας περιγραφική κινητική. Οι δοκιμές επεκτάθηκαν για να συμπεριλάβουν αλληλεπιδράσεις με το ασκορβικό οξύ, ένα φυσικό ισχυρό αντιοξειδωτικό, εφαρμόζοντας την μεθοδολογία επιφάνειας απόκρισης. Η κινητική μελέτη έδειξε ότι η m-β-CD μπορεί να παρεμποδίσει τη δέσμευση ελευθέρων ριζών των εκχυλισμάτων από OLL, αποδίδοντας χαμηλότερη στοιχειομετρία κατά την αντίδραση με την ελεύθερη ρίζα DPPH.

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Nomenclature

A_0, Initial absorbance at 515 nm

A_{AR}, Antiradical activity (µmoL DPPH g^{-1})

A_t, Absorbance at 515 nm at any time t

c, Initial DPPH concentration (moL L^{-1})

c_1, Initial antioxidant concentration (moL L^{-1})

C_{AA}, Ascorbic acid concentration (mg L^{-1})

C_{DES}, DES concentration (%, w/v)

C_{DPPH}, DPPH concentration (µmoL L^{-1}, µM)

C_{TFn}, Total flavonoid concentration (mg RtE L^{-1})

C_{TP}, Total polyphenol concentration (mg GAE L^{-1})

D_e, Diffusivity (m s^{-1})

E_a, Activation energy (kJ moL^{-1})

h, Initial extraction rate (mg g^{-1} min^{-1})

k, Pseudo zero-order decay constant (days^{-1})

k, Second-order extraction rate constant (g mg^{-1} min^{-1} or M^{-1} s^{-1})

k_1, Second-order rate constant of the first abstracted H-atom (M^{-1} s^{-1})

n_t, Total stoichiometry (dimensionless)

$P_{R(0)}$, Initial reducing power (µmoL AAE g^{-1} dw)

P_R, Reducing power (µmoL AAE g^{-1})

R_0, Initial reaction rate (M^{-1} s^{-1})

$R_{L/S}$, Liquid-to-solid ratio (mL g^{-1})

T, Temperature (ºC or K)

t, Time (min or days)

Y_{TFn}, Yield in total flavonoids (mg RtE g^{-1})

$Y_{TP(s)}$, Yield in total polyphenols at saturation (mg GAE g^{-1})

Y_{TP}, Yield in total polyphenols (mg GAE g^{-1})

ε, Molar absorptivity (M^{-1} cm^{-1})

Abbreviations

AA, Ascorbic acid

AAE, Ascorbic acid equivalents

DES, Deep eutectic solvents

DPPH, 2,2-Diphenyl-1-picrylhydrazyl radical

DW, Dry weight

GAE, Gallic acid equivalents

HBA, Hydrogen bond acceptor

HBD, Hydrogen bond donor

LC-DAD-MS, Liquid chromatography-diode array-mass spectrometry

LTTM, Low-transition temperature mixture

MAE, microwave assisted extraction

MW, Molecular weight

M-β-CD, Methyl-β-cyclodextrin

OLL, Olive tree (*Olea europaea*) leaves

PLE, Pressurized liquid extraction

RtE, Rutin equivalents

SFE, Supercritical fluid extraction

SLE, Solid-liquid extraction

TPTZ, 2,4,6-Tripyridyl-*s*-triazine

UAE, Ultrasonic assisted extraction

Chapter 1:

Literature review

1.1. Olive tree products

The olive tree, *Olea europaea* L., is the best known and most widely spread species of the Oleaceae family, its cultivation extending mainly to countries in the Mediterranean area (**Fig. 1**) (Roca *et al.,* 2007). It is a traditional symbol of abundance, glory, and peace, and its leafy branches were historically used to crown the victorious in friendly games and bloody war (El and Karakaya, 2009). Extended periods of sunlight irradiation, as well as pathogen and insect attack, require plants to synthesise high levels of polyphenols for environmental defence (Lockyer *et al.,* 2012).

Fig. 1: Distribution map of *O. europaea* L.

O. europaea L. is widely studied for its alimentary use, the fruits and the oil are important components in the daily diet of a large part of the world's population. Both the cultivation of olive trees and olive oil extraction generate every year substantial quantities of products generally known as "olive by-products" and having no practical applications (Abaza *et al.,* 2015).

The olive tree has a long history of medicinal and nutritional values. Over the centuries, extracts from olive leaf have been used for promoting health and preservation. Economically, the fruit of olive is an important commodity as it yields nutritious edible oil with potential medicinal functions (Ghanbari *et al.,* 2012).

1.1.1. Olive fruit

Table olives are a highly functional food with a balanced content of fats made up mainly of monounsaturated oleic acid. Eating olives also provides energy, fiber, vitamins, and minerals and contributes to the daily intake of nutritional antioxidants (Boskou *et al.,* 2015).

Olives contain high concentrations of phenolic compounds ranging between 1-3% of the fresh pulp weight (Silva *et al.,* 2006). The major compounds present in olive fruits are anthocyanins (cyanidin and delphinidin glucosides), flavonols (mainly quercetin-3-rutinoside), flavones (luteolin and apigenin glucosides), phenolic acids (hydroxybenzoic, hydroxycinnamic, others), phenolic alcohols (tyrosol and hydroxytyrosol), secoiridoids (oleuropein, dimethyloleuropein, ligstroside, nuzhenide), and verbascoside, a hydroxycinnamic acid derivative (Boskou *et al.,* 2015). Oleuropein, hydroxytyrosol and tyrosol are the most abundant polyphenols in olive fruits (Silva *et al.,* 2006).

The concentration of phenols and related compounds are dependent on the maturity of the fruit. Oleuropein is present in high amounts and responsible for the bitter taste of immature and unprocessed olive fruits; hydroxytyrosol is more abundant in the processed fruit and olive oil (Zoidou *et al.,* 2010; Bianchi, 2003; Soler-Rivas *et al.,* 2000). In early growth, oleuropein is most abundant, and it reaches 14% of the dry matter of young fruit. In green picked cultivars, the oleuropein level may still be high even at harvest time, albeit lower than 14%. However, in some varieties the level can fall to zero when the olives are completely black (Zoidou *et al.,* 2010; Bianchi, 2003).

1.1.2. Olive oil

The composition of olive oil is primarily triacylglycerols (~99%) and secondarily free fatty acids (oleic, linoleic, palmitic, others), mono- and diacylglycerols, and an array of lipids such as hydrocarbons (squalene), sterols, aliphatic alcohols, tocopherols (mainly α-tocopherol), pigments (chlorophylls and carotenoids), phenolic, volatile and aroma compounds. Some of these compounds contribute to the unique character of the oil (Boskou *et al.,* 2006a).

The phenolic content of olive oils is also very much influenced by the manufacturing process. The type of mill used for pressing and the centrifugation procedure seemed to have an important impact. The "extra-virgin" olive oil is obtained by the first physical, cold pressing of the olive paste and is rich in phenolic components. The phenolic concentration in these oils is

the result of a complex interaction between several factors, including cultivar, degree of maturation and climate (Soler-Rivas *et al.,* 2000).

The phenolic compounds of olive oil belong to different classes (Bendini *et al.,* 2007; Boskou *et al.,* 2006b): phenolic acids (such as gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, caffeic, syringic, *p-* and *o*-coumaric, ferulic, cinnamic, others), phenyl ethyl alcohols or simple phenols (hydroxytyrosol and tyrosol), flavonoids (apigenin, luteolin, others), lignans (pinoresinol, acetoxypinoresinol, others) and secoiridoids (oleuropein, ligstroside, others). The major phenolic compounds in olive oil are oleuropein, hydroxytyrosol and tyrosol (Tuck and Hayball, 2002).

The phenolic compounds present in olive oil are strong antioxidants and radical scavengers (Tuck and Hayball, 2002); and have anti-inflammatory and antimicrobial activity (Tripoli *et al.,* 2005). Subsequent studies (human, animal, *in vivo* and *in vitro*) have demonstrated that olive oil phenolics reduce the risk of chronic disease development, such as atherosclerosis, cardiovascular disease, and certain types of cancer (Tasioula-Margari and Tsabolatidou, 2015).

1.1.3. Olive leaves

O. europaea leaves are a significant by-product in olive-oil production (Apostolakis *et al.,* 2014) and several potential antioxidant molecules, essentially belonging to the phenolic family. The secoiridoid oleuropein is the main compound reported in olive leaf extracts together with other secoiridoids derived from tyrosol structure, such as ligstroside or oleuroside, as well as verbascoside (Quirantes-Piné *et al.,* 2013) and several flavonoids (apigenin, kaempferol, luteolin) as well as simple phenolic compounds such as caffeic acid, tyrosol, hydroxytytrosol (Talhaoui *et al.,* 2014).

However, the phenolic composition of olive leaves varies according to plant variety, harvesting season, the cultivation zone, agronomical practices, leaf age, leaf maturity and storage conditions, and the detection of these phenolics is dependent on the extraction method, in terms of both the compounds identified and their quantities (Lockyer *et al.,* 2012; Petridis *et al.,* 2012).

Olive leaves are considered as a cheap raw material (Salah *et al.,* 2012), which can be used as a useful source of high-added value products (polyphenolic compounds) with superb biological properties (El and Karakaya, 2009). Chemical, agronomical and medicinal researches have contributed together to highlight the interest in the use of olive leaves as a potential source of phenolic compounds for the production of functional food and nutraceuticals (Talhaoui *et al.,* 2015).

1.2. Olive leaf polyphenolic compounds

Polyphenolic compounds are a large class of secondary plant metabolites possessing an aromatic ring bearing one or more hydroxyl groups, including their functional derivatives (Lockyer *et al.,* 2012). An initial classification of phenolic compounds could be based on the number of phenol units in the molecule dividing them into simple phenols and polyphenols. Thus, these compounds may be classified into different groups depending upon the number of phenol rings that they bear and on the structural elements that bind these rings to one.

Olive leaf extracts have attracted the interest of researchers from different scientific disciplines mainly due to the distinctive phenolic composition allegedly related to potent biological activities (Kontogianni and Gerothanassis, 2012).

Polyphenolic compounds in olive leaves are numerous and of diverse nature (Abaza *et al.,* 2015). They are grouped with regard to major molecular characteristics as phenols, phenolic acids, flavonoids, lignans, and secoiridoids. In **Fig. 2** are presented the main classes of some polyphenolic compounds in olive leaves.
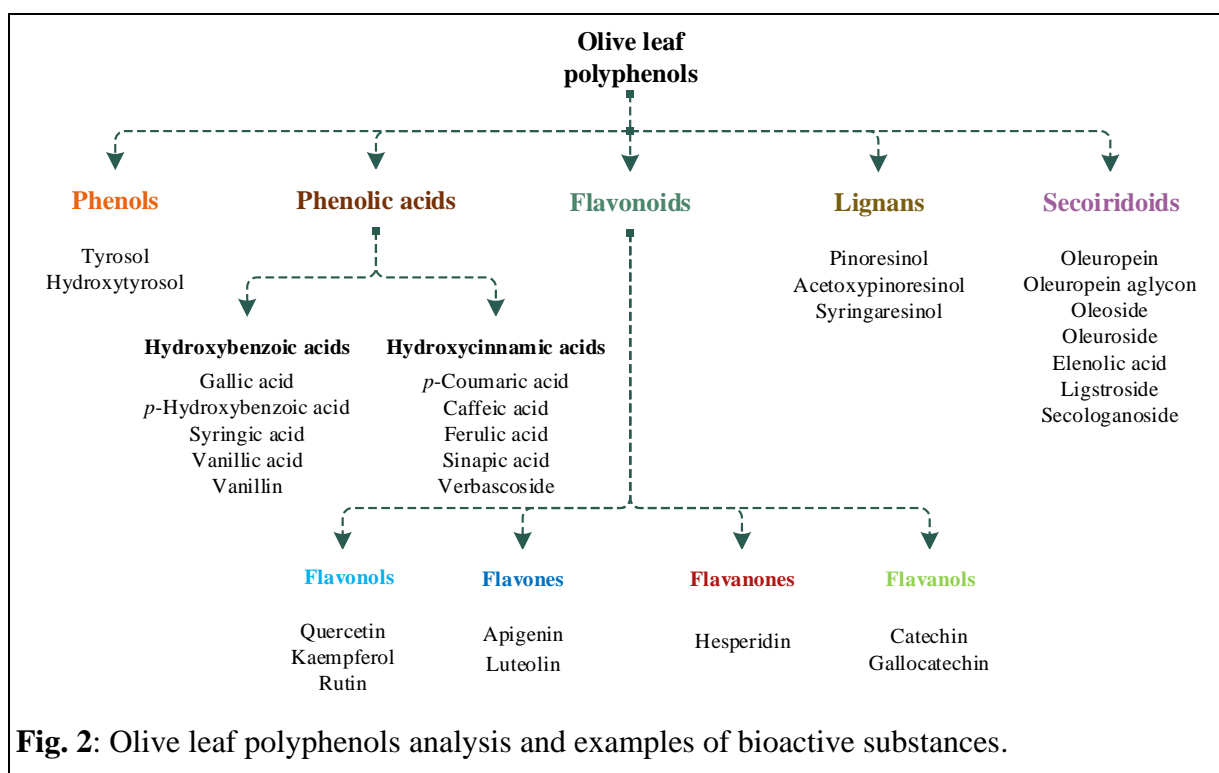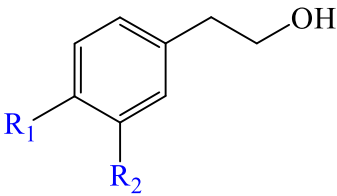
Fig. 2: Olive leaf polyphenols analysis and examples of bioactive substances.

The total polyphenol content and the total flavonoid content of olive tree leaves were determined to be 2,058 mg GAE (gallic acid equivalent) per 100 g and 858 mg CTE (catechin equivalent) per 100 g, respectively, reflecting values similar to that of red-grape peel (El and Karakaya, 2009).

1.2.1. Phenols

Hydroxytyrosol, or 3,4-dihydroxyphenylethanol, is one of the hydroxyaromatic components of secoiridoids. It is a very bioactive alcoholic *ortho*-diphenol (De Leonardis *et al.*, 2008). In addition, hydroxytyrosol in turn consists of two bounded molecular structures, such as catechol and ethanol (**Table 1**) (Ranalli *et al.*, 2006). Considerable differences in the content of tyrosol and hydroxytyrosol have also been found in the fruits during growth and ripening of the drupe the increase in their levels consistently correlates with hydrolysis of the components with higher molecular weights (Omar, 2010). Native hydroxytyrosol is rarely in the free form in nature with the exception of ripened olives where it occurs through the hydrolysis of oleuropein (De Leonardis *et al.*, 2008). Hydroxytyrosol has been widely described as one of the main components of simple phenols in olive leaves (Talhaoui *et al.*, 2015). Tyrosol is another simple phenol derived from oleuropein, but is found in low concentrations in the olive leaf (Boss *et al.*, 2016).

Table 1: Examples of phenols on differences in their chemical structure.
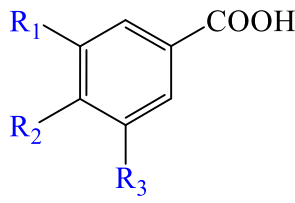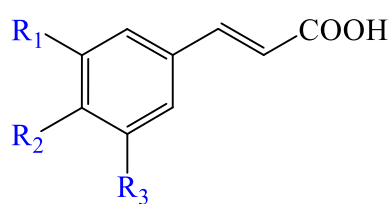
Examples of phenols	Chemical structure
Tyrosol R_1:-OH, R_2:-H Hydroxytyrosol R_1:-OH, R_2:-OH	

1.2.2. Phenolic acids

Phenolic acids are one such group of aromatic secondary plant metabolites widely spread throughout the plant kingdom (Robbins, 2003). The name "phenolic acids", in general, describes phenols that possess one carboxylic acid functionality. However, when describing plant metabolites, it refers to a distinct group of organic acids. These naturally occurring phenolic acids contain two distinguishing constitutive carbon frameworks: the hydroxybenzoic and hydroxycinnamic structures (Bendini *et al.*, 2007). Although the basic skeleton remains the same, the numbers and positions of the hydroxyl groups on the aromatic ring create the variety.

A variety of classes can be found in olive leaves (**Table 2**) such as hydroxybenzoic (e.g., gallic, syringic, vanillic, vanillin, *p*-hydroxybenzoic, others) and hydroxycinnamic (e.g., caffeic, sinapic, ferulic, *p*-coumaric, verbascoside, others) acids (Abaza *et al.*, 2015; Khaliq *et al.*, 2015; Bendini *et al.*, 2007; Robbins, 2003).

Table 2: Classification and examples of phenolic acids on differences in their chemical structure.

Phenolic acids	Examples of phenolic acids	Chemical structure
Hydroxybenzoic acids	Gallic acid R_1:-OH, R_2:-OH, R_3:-OH	
	Syringic acid R_1:-OCH$_3$, R_2:-OH, R_3:-OCH$_3$	
	Vanillic acid R_1:-H, R_2:-OH, R_3:-OCH$_3$	
	p-Hydroxybenzoic acid R_1:-H, R_2:-OH, R_3:-H	
Hydroxycinnamic acids	Caffeic acid R_1:-OH, R_2:-OH, R_3:-H	
	Sinapic acid R_1:-OCH$_3$, R_2:-OH, R_3:-OCH$_3$	
	Ferulic acid R_1:-OCH$_3$, R_2:-OH, R_3:-H	
	p-Coumaric acid R_1:-H, R_2:-OH, R_3:-H	

1.2.3. Flavonoids

Flavonoids have the general structure of a 15-carbon skeleton (**Fig. 3**), which consists of two phenyl rings (A and B) and heterocyclic ring (C). This carbon structure can be abbreviated C_6-C_3-C_6 (Kumar and Pandey, 2013; Iwashina, 2000).
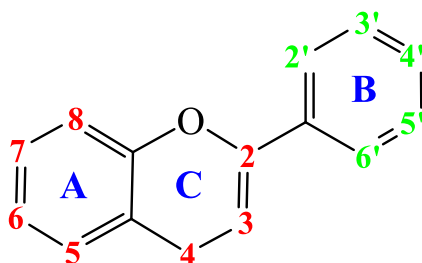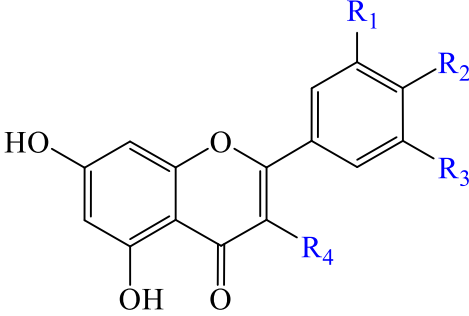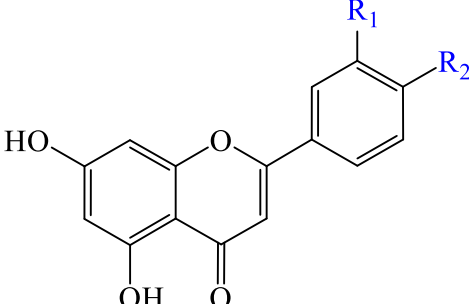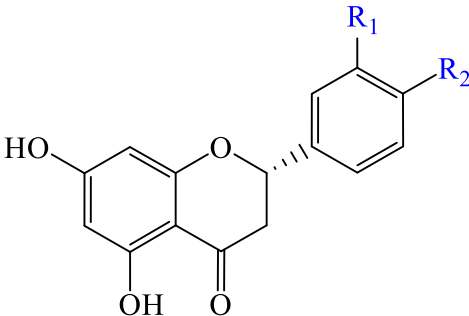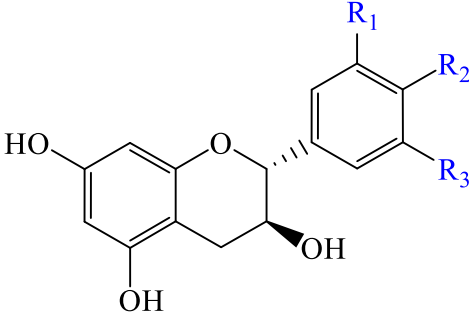
Fig. 3: Overall structure of flavonoids.

Flavonoids are widespread secondary plant metabolites, largely planar molecules and their structural variation comes in part from the pattern of modification by hydroxylation, methoxylation, prenylation, or glycosylation (Bendini *et al.,* 2007). Over four thousand (> 4,000) flavonoids, based on the C_6-C_3-C_6 skeleton, have been found in plants, and are divided

into several classes (Iwashina, 2000), i.e., anthocyanins, flavones, flavonols, flavanones, dihydroflavonols, chalcones, aurones, flavan and proanthocyanidins, isoflavonoids, biflavonoids, etc. A variety of classes can be found in olive leaves (**Table 3**) such as flavonols (e.g., quercetin, kaempferol, rutin, others), flavones (e.g., apigenin, luteolin, others), flavanones (e.g., hesperetin, others), and flavanols (e.g., catechin, gallocatechin, others) (Khaliq *et al.,* 2015; Kumar and Pandey, 2013; Bendini *et al.,* 2007).
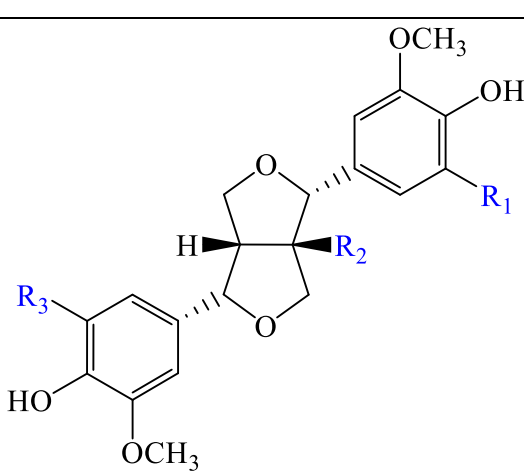
Table 3: Classification and examples of flavonoids on differences in their chemical structure.

Flavonoids	Examples of flavonoids	Chemical structure
Flavonols	Quercetin R_1:-OH, R_2:-OH, R_3:-H, R_4:-OH Kaempferol R_1:-H, R_2:-OH, R_3:-H, R_4:-OH Rutin R_1:-H, R_2:-OH, R_3:-OH, R_4:-*O*-rutinose	
Flavones	Luteolin R_1:-OH, R_2:-OH Apigenin R_1:-H, R_2:-OH	
Flavanones	Hesperetin R_1:-OH, R_2:-OCH$_3$	
Flavanols	Catechin R_1:-H, R_2:-OH, R_3:-OH Gallocatechin R_1:-OH, R_2:-OH, R_3:-OH	

1.2.4. Lignans

Lignans are naturally occurring phenylpropanoid dimers (C_6-C_3 unit; e.g., coniferyl alcohol), in which the central carbons of the side chains link the phenylpropane units (Satake *et al.,* 2015). In addition, lignans are considered important phytonutrients for robust health. Recently published research indicates that olive oil lignans may have an active role in protecting against breast cancer and contribute to the beneficial nutritional profile of this functional food (Boskou, 2015). Lignans such as pinoresinol and its derivatives (acetoxypinoresinol, hydroxypinoresinol) and the syringaresinol are among the constituents of the olive leaves phenol fraction (Kalogeropoulos and Tsimidou, 2014; Taamalli *et al.,* 2012a; Bendini *et al.,* 2007) and these substances are shown in the **Table 4**.

Table 4: Examples of lignans on differences in their chemical structure.

Examples of lignans	Chemical structure
Pinoresinol R_1:-H, R_2:-H, R_3:-H Acetoxypinoresinol R_1:-H, R_2:-OCOCH$_3$, R_3:-H Syringaresinol R_1:-OCH$_3$, R_2:-H, R_3:- OCH$_3$	

1.2.5. Secoiridoids

Secoiridoids are a group of coumarin-like compounds that are produced from the secondary metabolism of terpenes as precursors of various indole alkaloids, and are only present in plants of the *Oleaceae* family, which includes *O. europaea* (Silva *et al.,* 2006; Soler-Rivas *et al.,* 2000). Oleuropein (**Fig. 4**) is an ester of hydroxytyrosol and elenolic acid glucoside, whilst verbascoside is a conjugated glucoside of hydroxytyrosol and caffeic acid (Lockyer *et al.,* 2012).

The natural mechanism that occurs when the olive tree forms free hydroxytyrosol is enzymatic hydrolysis, and specific native β-glucosidase and esterase are implicated (De Leonardis and Macciola, 2010). As shown in the **Fig. 5**, during processing part of the oleuropein is hydrolysed and thus some compounds (hydroxytyrosol and elenolic acid) are produced which impart the excellent organoleptic properties and especially its bitter taste (Yuan *et al.,* 2015; Menendez *et al.,* 2007; Soler-Rivas *et al.,* 2000). Enzymatic hydrolysis not only involves mild

conditions and simple operation methods, but also can obtain high purity product (Yuan *et al.,* 2015).

Fig. 4: Structure of oleuropein.

Fig. 5: Enzymatic hydrolysis of oleuropein and its products.
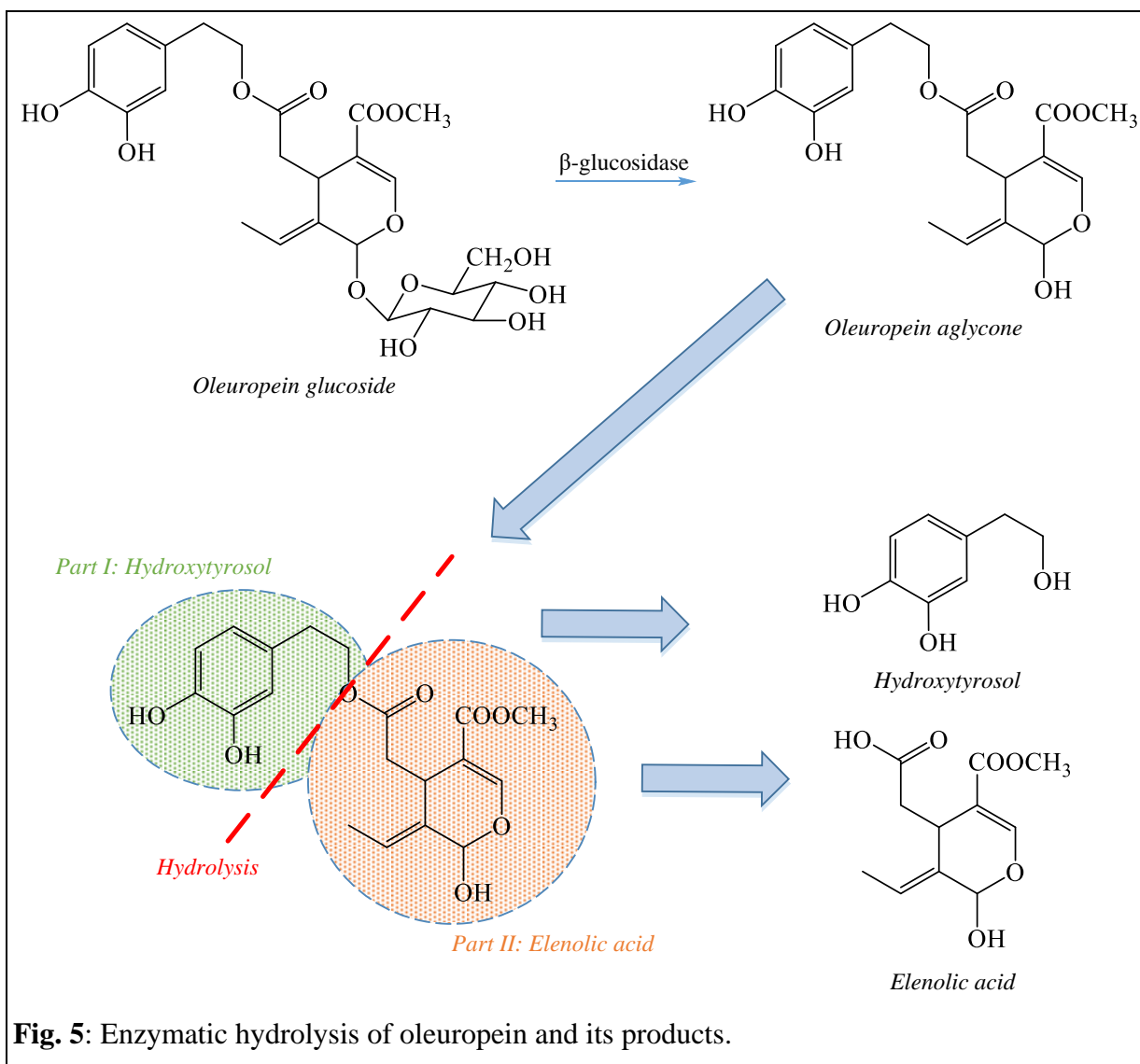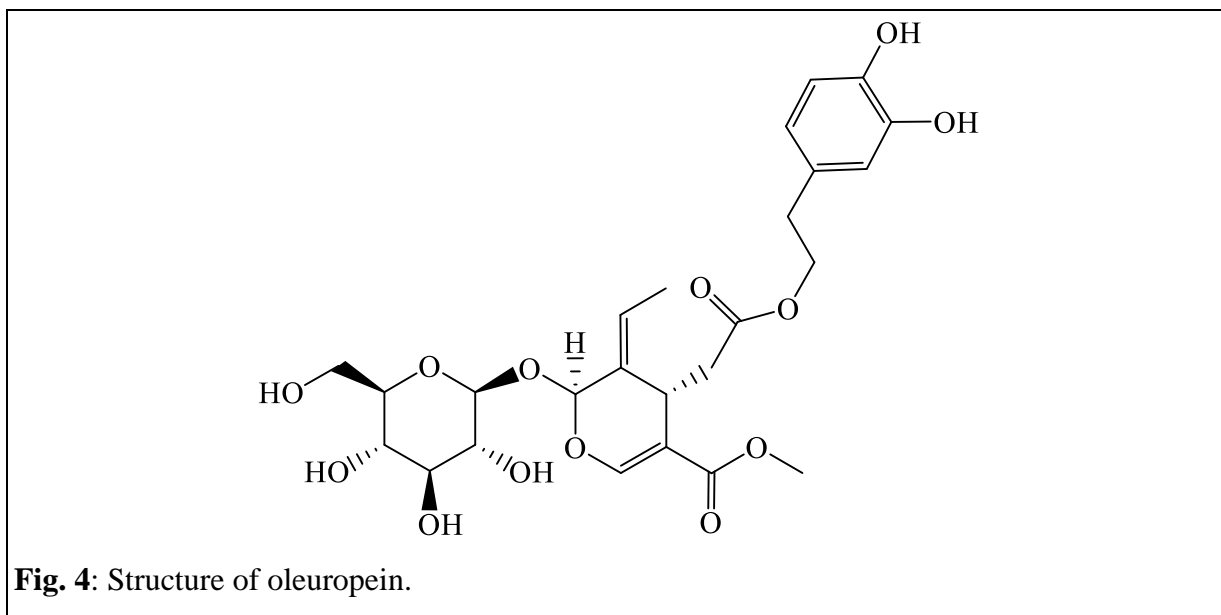
The glucosidases catalyze the hydrolysis of the glucosidic bonds and are an important group of enzymes for many biochemicals, biomedical and industrial applications. β-Glucosidases of microbial origin were investigated for substituting NaOH treatment in olive processing (Ozdemir *et al.,* 2014).

Various substances belong to the group phenol secoiridoids can be found in olive leaves (**Table 5**) and they form through opening of the five-membered ring of iridoids (Ranalli *et al.,* 2006). The main secoiridoid components determined in the olive tree leaf extracts for the cultivars Koroneiki and Kalamon were: secologanoside, dimethyloleuropein, oleuropein diglucoside, oleuropein, oleuroside, and ligstroside (Kiritsakis *et al.,* 2010).

Table 5: Examples of secoiridoids on differences in their chemical structure.

Examples of secoiridoids	Chemical structure
Oleuropein R_1:-OH, R_2:-CH$_3$ Dimethyloleuropein R_1:-OH, R_2:-H Ligstroside R_1:-H, R_2:-CH$_3$	
Elenolic acid R_1:-CH$_3$, R_2:-H Oleoside R_1:-H, R_2:-Glc	
Secologanoside R_1:-H, R_2:-Glc	

1.3. Olive leaf bioactivities and health benefits

Olive leaf polyphenols have many reported pharmacological activities (**Fig. 6**) based on preclinical (*in vitro*, *ex vivo*, and *in vivo*) studies in addition to few clinical studies. These activities suggest high potential for the prevention and treatment of diseases and the promotion of human health. Although many review articles and book chapters have been dedicated for appraising the biological activities of olive leaf polyphenols, Obied *et al.* (2012) had noticed that a systematic pharmacological review of those activities has not yet been attempted. However, one of the most attractive ways for cancer prevention and therapy today is induction of tumor cell death and/or stimulation of a host immune response by certain phytochemicals derived from medicinal herbs and dietary plants (Mijatovic *et al.,* 2011).

Olive leaves used in the human diet as an extract, an herbal tea, and a powder, and they contain many potentially bioactive compounds that may have antioxidant, antihypertensive, antiatherogenic, anti-inflammatory, hypoglycemic, and hypocholesterolemic properties (El and Karakaya, 2009). Olive leafs traditionally used for centuries to prevent and treat different diseases. They have used to enhance the immune system, in heart disease, and as an antimicrobial agent (Al-Attar and Abu Zeid, 2013).
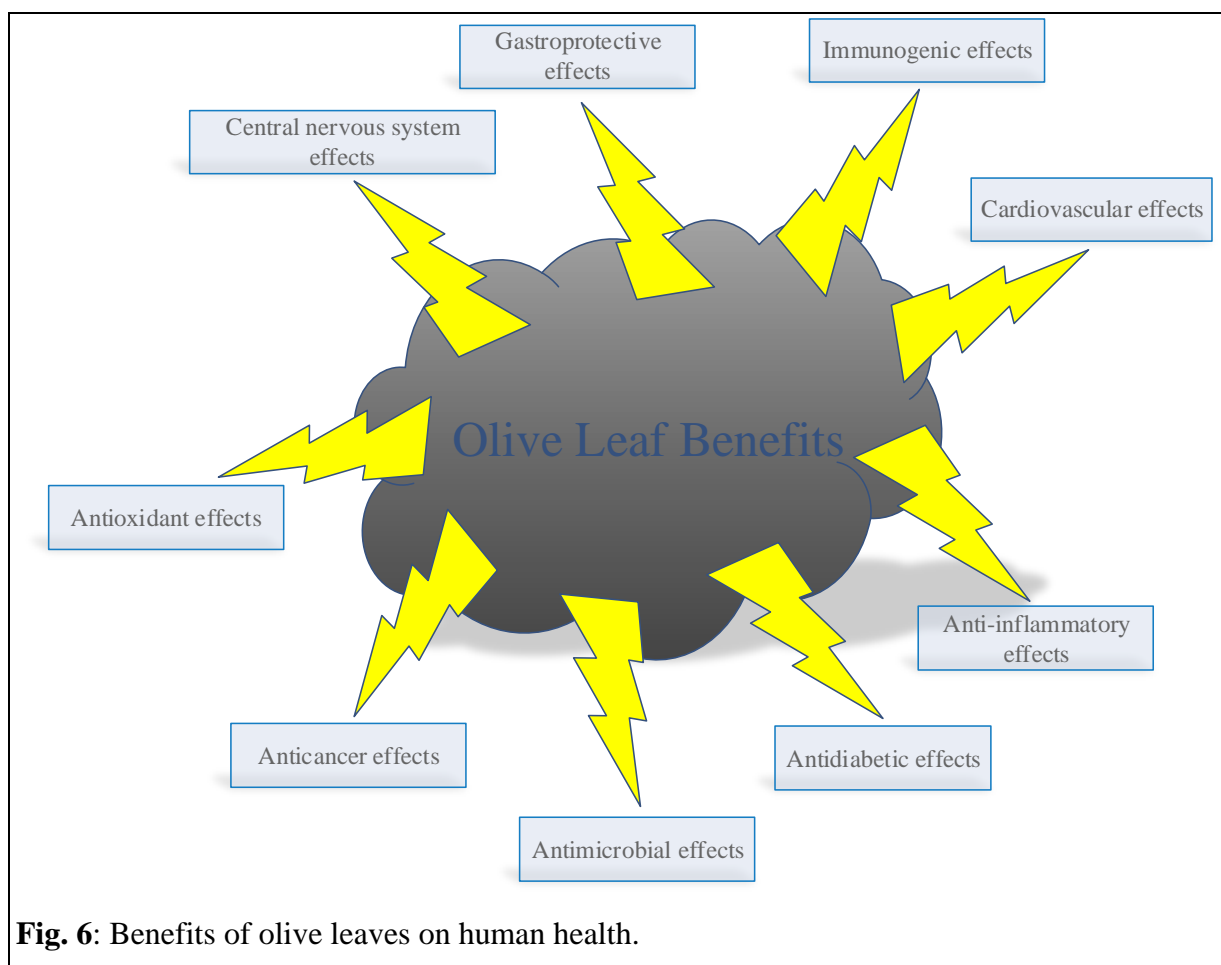
Fig. 6: Benefits of olive leaves on human health.

Several reports demonstrated that olive leaves could decrease blood pressure (antihypertensive effect) (Romero *et al.,* 2016; Wong *et al.,* 2014; Nekooeian *et al.,* 2011; Susalit *et al.,* 2011; Khayyal *et al.,* 2002), improvement of vascular function and reduced pro-oxidative and pro-inflammatory status (Romero *et al.,* 2016). Moreover, they have antiplatelet effects and may offer a degree of protection from thrombosis and other cardiovascular diseases (Singh *et al.,* 2008). Olive leaves extracts as a source of antioxidants (oleuropein and hydroxytyrosol) are able to reduce the frequency of oxidative stress-related metabolic diseases such as diabetes (Jemai *et al.,* 2009). Wainstein *et al.* (2012) suggested one treatment with olive leaf extract is associated with a beneficial hypoglycemic effect in patients with diabetes.

The leaves also possess antioxidant and antimicrobial properties against some microorganisms such as bacteria, fungi, and mycoplasma (Abaza *et al.,* 2011; Bulotta *et al.,* 2011; Lee and Lee, 2010; Jemai *et al.,* 2008; Pereira *et al.,* 2007; Škerget *et al.,* 2005; Markin *et al.,* 2003; Furneri *et al.,* 2002; Benavente-García *et al.,* 2000). In addition, they have anti-HIV properties (Lee-Huang *et al.,* 2003), anti-proliferative and apoptotic effects (Han *et al.,* 2009), protective effect against human leukemia (Abaza *et al.,* 2007), lipid-lowering activity (Jemai *et al.,* 2008), and others. Olive leaf polyphenols found active against different strains of *Helicobacter pylori* (Sudjana *et al.,* 2009). Therefore, Dekanski *et al.* (2009) indicated that the gastroprotective potential of olive leaves extracts is probably related to its ability to maintain the cell membrane integrity, by its antilipid eroxidative activity that protects the gastric mucosa against oxidative damage and by its ability to strengthen the mucosal barrier, the first line of defence against exogenous damaging agents.

The leaves claimed to play an important protective role in cancer and other inflammation-related diseases. Both inflammatory and cancer cell models have shown that olive leaf polyphenols are anti-inflammatory and protect against DNA damage initiated by free radicals. The various bioactive properties of olive leaf polyphenols are a plausible explanation for the inhibition of progression and development of cancers (Boss *et al.,* 2016). Goulas *et al.* (2009) evidenced that olive leaves extracts have health protecting biological activity as antioxidants and as potent inhibitors of cancer and endothelial cells proliferation at low micromolar concentrations. Furthermore, olive leaves extracts protect central nervous system from the destruction brought on by age-related degenerative conditions such as Alzheimer's and Parkinson's diseases (Sabry *et al.,* 2014).

1.4. Extraction of polyphenolic compounds from olive leaves

The use of bioactive compounds in different commercial sectors such as pharmaceutical, food and chemical industries signifies the need of the most appropriate and standard method to extract these active components from plant materials (Azmir *et al.,* 2013). However, Abaza *et al.* (2015) mentioned that several factors might influence the qualitative and quantitative phenolic composition of olive leaves among which we can cite date of collection, drying conditions, cultivation zone, extraction procedure and conditions (pH, solvent, temperature and time), and cultivar.

1.4.1. Sample preparation techniques

After collection, fresh olive leaves are washed with distilled water to eliminate any traces of dust (Abaza *et al.,* 2015). Olive leaves have to be dried for utilizing as a food additive or before extracting the bioactive compounds or for storage (Erbay and Icier, 2010). They are often dried before extraction to reduce their moisture content and to avoid the interference of water on the process (Bahloul *et al.,* 2009). The immediate drying of these products is the most important operation in post-harvest processing to avoid quality losses and to prevent possible degradation due to microbiological or biochemical reactions (Abaza *et al.,* 2015; Ahmad-Qasem *et al.,* 2013b).

Traditional methods of drying, such as shade or sun drying, are still practiced for drying. However, the high temperatures or long drying times in conventional air-drying, may cause serious damage to the quality attributes of the dried product such as flavour, colour, nutrients and stability of bioactive substances (Bahloul *et al.,* 2009). Therefore, the selection of proper drying conditions is of prime importance for decreasing thermal stress and maintaining the quality of the dried product. 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 (Ratti, 2001). Nevertheless, despite many advantages, freeze-drying has always been recognized as the most expensive process for manufacturing a dehydrated product and it requires a previous freezing that, in certain ways, could also affect quality (Ahmad-Qasem *et al.,* 2013b; Ratti, 2001). Consequently, 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 procedure.

1.4.2. Conventional solvent extraction

Solvent extraction is the main method adopted by most researchers to extract phenolics from olive leaves. Many factors may affect the efficiency of solvent extraction: solvent type, pH, temperature, number of extraction steps, time of extraction, liquid-to-solid ratio, and particle size of the solid matrix (Souilem *et al.,* 2016; Dent *et al.,* 2013).

The ideal solvent should have a low toxicity, a low flammability, a low risk of explosion, and a low potential for artifact formation. It should also be safe, economical and environmental-friendly. In the ideal case, the extraction method selected should be exhaustive, i.e., to yield as much of the desired metabolites or as many compounds as possible (Bogdanov, 2014; Seidel, 2012). A solvent of an appropriate polarity is used to extract metabolites following the principle of "like dissolves like" (Seidel, 2012). In replacing toxic reagents, conventional organic solvents commonly employed during the extraction procedure are gradually substituted by newly designed solvents (An et al., 2017). These non-conventional solvents include ionic liquids and their derivatives (Ratti, 2014; Suresh and Sandhu, 2011) and deep eutectic solvents (DES) (Hayyan et al., 2016; Li and Row, 2016).

The polarities of the polyphenols range from polar to non-polar, thus a wide range of conventional solvents (water, acetone, methanol, ethanol, or their mixtures with water) for their extraction has been studied (Dent et al., 2013). In the case of olive leaf extracts, because of the nature of the phenolic compounds (polar), the solvent of choice is most of the times alcoholic mixtures with water (Zeitoun et al., 2016; Talhaoui et al., 2014; Taamalli et al., 2012a; Altıok et al., 2008). Wissam et al. (2016) showed that 80% acetone, 40% methanol and 40% ethanol are more efficient in the extraction of polyphenolic compounds. Although, high yield was achieved using methanol and acetone for the extraction of phenols from olive leaves, both are not a food grade solvent due to their high toxicity (Dasgupta and Wahed, 2014; Kavet and Nauss, 1990). Therefore, ethanol was selected as the most appropriate solvent for the extraction of phenolic compounds from olive leaves for production of extracts with high phenol content and high antioxidant activity (Lafka et al., 2013).

Glycerol is a conventional solvent that is defined simply as a polyol (sugar alcohol), and it is used extensively in many industrial applications, especially in the food and pharmaceutical industries (AlOmar et al., 2016). However, the fact that glycerol is poorly combustible means that it cannot even be used as a fuel and it is regarded by many to be a waste product (Abbott et al., 2011). In addition, glycerol use is still limited in organic transformations, mainly for two reasons: (i) the intrinsic reactivity of the polyol backbone leading to the formation of side products and (ii) the very poor solubility of the vast majority of organic compounds (Hamel et al., 2014). Therefore, to overcome these disadvantages, researchers have been working to enhance the physicochemical properties of glycerol by different methods (Hamel et al., 2014; Smarrito-Menozzi et al., 2013). One of these methods is preparing of deep eutectic solvents (DES) containing glycerol as hydrogen bond donor (HBD). A recent review has assessed the use of glycerol as a green solvent (Gu and Jérôme, 2010), due to its low cost, wide availability and biodegradability (Lupacchini et al., 2017).

1.4.3. Deep eutectic solvent extraction

Deep eutectic solvents (DES) and low-transition temperature mixtures (LTTMs) are an innovative class of eco-friendly liquids comprised of bio-molecules, such as a polyol serving as the hydrogen bond donor (HBD), and an organic salt, which is the hydrogen bond acceptor (HBA) (Georgantzi *et al.,* 2017). DES are fully inclusive of LTTMs (Wagle *et al.,* 2014). DES display attractive attributes, like low vapour-pressure, high thermal stability, non-flammability, high solvability, chemical stability, low volatility, tunability, low cost, non-toxic, biodegradable and sustainable. These features make DES ideal extraction solvents, possessing unique characteristics, whereas limitations associated with similar materials, such as conventional organic solvents and ionic liquids, may be overcome (Lupacchini *et al.,* 2017; Mainberger *et al.,* 2017; Hayyan *et al.,* 2016; Wagle *et al.,* 2014).

In this regard, LTTMs are compatible with foods, pharmaceuticals and cosmetics, but also environmentally benign. The ability to tailor the physicochemical properties of LTTMs is central to customising their extraction behaviour, as a number of essential parameters (viscosity, polarity, surface tension, hydrogen bonding) that play significant roles in mass transport properties governing an extraction process, maybe effectively regulated (Dai *et al.,* 2015). A large number of different compounds have been used in the synthetic process of DES as HBD and HBA (Li and Row, 2016). The HBA is often a quaternary salt while HBD can include amines, carboxylic acids, alcohols or carbohydrates (An *et al.,* 2017). A few studies have been used glycerol, 2,3-butanediol, L-lactic acid, oxalic acid, ethylene glycol, others as HBD and choline chloride, glycine, L-alanine, L-glutamic acid, nicotinamide, sodium acetate trihydrate, ammonium acetate, sodium-potassium tartrate tetrahydrate, trisodium citrate dehydrate, others as HBA (An *et al.,* 2017; Dedousi *et al.,* 2017; Georgantzi *et al.,* 2017; Jancheva *et al.,* 2017; Karageorgou *et al.,* 2017; Kottaras et al., 2017; Patsea *et al.,* 2017; Bakirtzi *et al.,* 2016; Li and Row, 2016; Manousaki *et al.,* 2016; Mouratoglou *et al.,* 2016).

Recent investigations with LTTMs composed of glycerol or L-lactic acid (HBD) and sodium acetate (HBA), two low-cost biomolecules, have shown that polyphenol extraction yields were much higher than those obtained with water and were comparable to those obtained with aqueous ethanol (Manousaki *et al.,* 2016; Mouratoglou *et al.,* 2016). In other study (Kottaras *et al.,* 2017) was carried out to screen combinations of L-lactic acid used as HBD, with the natural amino acids glycine and L-alanine, but also choline chloride, as HBAs in an effort to identify (i) pairs that provide stable LTTMs and (ii) LTTMs that can afford high extraction yields of antioxidant phenolics from industrial solid wastes.

1.4.4. Effect of extraction conditions

Extraction conditions (e.g., time, temperature, and liquid-to-solid ratio) must be optimized to efficiently extract olive leaf polyphenols without oxidative degradation (Jemai *et al.,* 2009). Several researchers focused on reducing the extraction time (it usually ranges from 5 to 24 h) for complete recovery of phenolic compounds in order to adopt extraction process to industrial scale (Stamatopoulos *et al.,* 2014). In addition, high temperature could promote oxidative degradation of polyphenols and lead to solvent loss by volatilization, higher energy costs and more extraction impurities (Ahmad-Qasem *et al.,* 2013a). Thus, the authors propose low extraction temperatures (e.g., 40 ºC, it usually ranges from 28 to 60 ºC) for high extraction times (Stamatopoulos *et al.,* 2014; Spigno *et al.,* 2007). Time and temperature of extraction are important parameter to be optimised even in order to minimize energy cost of the process (Spigno *et al.,* 2007). In the literature, the solvent-to-solid ratio for olive leave extract preparation varied from 20 to 50 mL g^{-1} (Dedousi *et al.,* 2017; Mourtzinos *et al.,* 2016; Şahin and Şamlı, 2013; Pereira *et al.,* 2007). Increasing the solvent-to-solid ratio leads to better recovery yield of polyphenols in the obtained extract.

1.4.5. Extraction techniques

Conventional extraction techniques like solid-liquid extraction (SLE) and novel (non-conventional) extraction techniques like supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), ultrasonic assisted extraction (UAE) and microwave assisted extraction (MAE), have been used to isolate phenolic compounds from *Olea europaea* leaves (Abaza *et al.,* 2015; Taamalli *et al.,* 2012a). However, in addition to selecting the appropriate method, the extraction yield depends on several parameters such as solvent type, pH, temperature, the duration of extraction, the particle size, and the solvent-to-solid ratio. Therefore, these parameters should be taken into consideration in order to attain optimal extraction yields (Sifaoui *et al.,* 2016; Lafka *et al.,* 2013; Taamalli *et al.,* 2012a).

The most commonly extraction system used has been the SLE by maceration of the olive leaves in a solvent (Talhaoui *et al.,* 2015). The selection of an appropriate solvent is of paramount importance and profoundly defines yield and composition of the extracts produced. Most common solvents to date are of petrochemical origin but they have several inherent disadvantages, being flammable and toxic, while their production is associated with fossil resources (Georgantzi *et al.,* 2017). However, the use of non-toxic solvents is preferable for natural extracts production since it leads to the development of functional foods with health-promoting properties (Stamatopoulos *et al.,* 2014).

The major challenges of conventional extraction are longer extraction time, requirement of costly and high purity solvent, evaporation of the huge amount of solvent, low extraction selectivity and thermal decomposition of thermo labile compounds (Azmir *et al.,* 2013). Thus, other modern extraction and isolation techniques have been used as alternative (Talhaoui *et al.,* 2015). These techniques are referred as non-conventional extraction techniques and some of the most promising techniques are UAE, MAE, SFE, and PLE. These modern techniques require considerably smaller amounts of solvents with much higher extraction efficiency and lower environmental impact (Souilem *et al.,* 2016). In addition, can be regarded as a possible tool not only from a laboratory point of view but also for the natural products and food industries (Taamalli *et al.,* 2012a). Therefore, compared with conventional techniques, UAE 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 (Ahmad-Qasem *et al.,* 2013a). This is because this method is a powerful aid in accelerating various steps of the analytical process (homogenization, extraction, and others) (Talhaoui *et al.,* 2015).

The SFE is more environmentally friendly, can be used to obtain various bioactive compounds without any solvent residue and safety hazard (Lafka *et al.,* 2013). SFE with carbon dioxide (SC-CO$_2$) is the most favored method for isolation of mainly non-polar or lipophilic natural compounds from spices and agricultural by-products (Xynos *et al.,* 2012). In addition, the absence of light and air during extraction reduces the degradation of analytes that occur in traditional extraction techniques (Taamalli *et al.,* 2012a).

The PLE is a technique which uses conventional solvents and performs a fully automated extraction under constant pressure and various controllable parameters like temperature, static extraction time, extraction cycles, and others (Abaza *et al.,* 2015). The PLE technique limits the use of organic solvents, hereby making possible the use of solvent allowed for alimentary uses such as water and ethanol, while obtaining higher extraction yields and faster extraction processes (Xynos *et al.,* 2014). It is characterized by reduced operational cost and controlled extraction conditions, which provides consistent qualitative and quantitative composition of the extract (Xynos *et al.,* 2012).

Compared with other techniques, MAE has several advantages (e.g., short-extraction time, low-energy requirement, reduced solvent consumption, high extraction efficiency, and minimum degradation of target components). Moreover, MAE offers the possibility of obtaining products labelled as "green" according to environmental standards, with high quality and lower cost (Şahin *et al.,* 2017; Taamalli *et al.,* 2012a; Taamalli *et al.,* 2012b; Rafiee *et al.,* 2011).

1.5. Applications of olive leaves in agro-food industries

Olive leave polyphenols can be formulated into dietary supplements, foods, beverages, cosmetics, pharmaceutical products, and health fortificant for feed. Olive leaves are a natural material having high nutritional value and many biological activities. Incorporation of olive leaf extract in numerous foods can increase biological activities of diet and can provide efficient animal and human nutrition (Souilem *et al.,* 2016). Olive leaves have to be dried for utilizing as a food additive or before extracting the valuable compounds or for storage (Erbay and Icier, 2010).

1.5.1. Animal feeding and dietary supplementation with olive leaves

Olive leaves contain a high level of non-digestible fiber (39.9 to 62.6% dw), lignin (16.3 to 30.5% dw) and the content of crude protein varies between 9.5 and 12.9% dw (Delgado-Pertíñez *et al.,* 2000). Botsoglou *et al.* (2013a; 2013b; 2012a; 2012b) studied was to evaluate the effect of supplementation of the layer hen diet with olive leaves (10 g/kg feed) on lipid oxidation and fatty acid profile of α-linolenic acid (or very-long-chain *n*-3 fatty acids) enriched hen eggs during refrigerated storage, and to compare this effect with α-tocopheryl acetate supplementation.

In other studies was to evaluate lipid and protein oxidation during frozen storage of *n*-3 enriched pork patties as affected by diet supplementation of olive leaf extracts and α-tocopherol (or α-tocopheryl acetate) (Botsoglou *et al.,* 2014; 2012c). Furthermore, in other study was to investigate the effect of olive leaf supplementation on diet digestibility, growth performance, tocopherol content of meat and therefore on its oxidative stability and intrinsic quality condition, in pigs (Paiva-Martins *et al.,* 2009). Parsaei *et al.* (2014) showed that dietary supplementation with olive leaves had positive effects on blood lipids profile, liver enzymes and immunity of broiler chickens, but Shafey *et al.* (2013) indicated that dietary supplementation reduced abdominal fat of chickens. Addition of *O. europaea* leaves in rabbit diet showed a small reduction of oxidative processes, particularly in stored raw rabbit meat (Trebušak *et al.,* 2014).

1.5.2. Enrichment of table olives with polyphenols

Lalas *et al.* (2011) investigated the enrichment of table olives of Kalamon cultivar with two polyphenols (oleuropein and hydroxytyrosol) from olive leaves. As indicated by their results, treated table olives showed an increase of 457% in the case of oleuropein and 109% in the case of hydroxytyrosol. Sensory evaluation of treated table olives displayed an increase in bitterness. However, treated and untreated table olives showed equal overall acceptability and preference.

1.5.3. Enrichment of oils with olive leaves

The oils with higher contents of unsaturated fatty acids, especially polyunsaturated fatty acids, are more susceptible to oxidation. In order to overcome the stability problems of oils and fats, synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ter-butyl hydroquinone (TBHQ) have been used as food additives (Jaber *et al.,* 2012; Bouaziz *et al.,* 2008). Their addition to oils has been decreased for their suspected action as promoters of carcinogenesis (Jaber *et al.,* 2012; Erbay and Icier, 2010). Therefore, there is a great interest in substituting these synthetic compounds with natural antioxidants. Because of the richness of antioxidant phenols in olive leaves are natural antioxidants.

Bouaziz *et al.* (2008) indicated the treatment with olive leaves extract hydrolysate (400 ppm) increases the total sterol concentration of the oils. Hydrolysate and leaves extracts are excellent antioxidants and can serve as substitutes for synthetic antioxidants. In addition, Bouaziz *et al.* (2010) studied to enrich refined olive and pomace oils with extracts and pure compounds from olive leaves and to report on the changes that occurred with regard to phenolic compounds during storage time. Jaber *et al.* (2012) showed that olive leave chlorophyll pigment extract (400 ppm) could stabilize refined olive oil. Olive leaf extracts were proven to be good protectors for olive and sunflower oils at levels of 150 ppm (Lafka *et al.,* 2013). Sonda *et al.* (2014) added olive leaves (3%) to olive fruits before the extraction process, and the taste panel found an improvement of the olive oil sensorial quality. In other similar study (Malheiro *et al.,* 2013), after the addition of different olive leaves percentages during extraction of olive oils, the preliminary sensorial evaluation indicated that the overall sensorial quality increases, and the positive attributes, like green fruity and bitter taste, increased.

Farag *et al.* (2007) suggested that crude olive leaf juice can be used in practical operation to extend the frying life of frying oils. Adding olive leaf juice to sunflower oil at 180 °C induced excellent antioxidant activity at 800 ppm and improved stability. Zribi *et al.* (2013) recently concluded that enrichment of refined soybean oil with olive leaf extract significantly decreased the hydrolysis of triglycerides and fatty acid oxidation and *trans*-fatty acid contents, increasing the oxidative stability of pan-frying. These positive effects are due to the hydroxytyrosol that is initially present in olive leaf extracts.

Chiou *et al.* (2007) studied the enrichment of French fries using olive leaf extracts with palm oil, olive oil, and sunflower oil. Olive leaf extracts were supplemented at levels of 120 and 240 mg total polyphenols per kg of oil, and potatoes were pan fried in both enriched and non-supplemented oils under domestic frying conditions. Oil supplementation with a polyphenol-rich olive leaf extract succeeded in producing French fries containing polyphenols like oleuropein, and fried oils containing a higher polyphenol content than the original ones

before supplementation. Moreover, polyphenols seemed to have survived the pan-frying procedure better when absorbed by the fried food than better when they remained in the frying oil. Consequently, Farag *et al.* (2006) suggested adding crude olive leaf juice to increase their shelf-life of oils without any deleterious effect on human health. In general, the data for histopathological examinations were in accordance with results of biochemical measurements of serum rat liver and kidney function tests.

1.5.4. Encapsulation of olive leaf extracts

Microencapsulation is defined as a technology of packaging solids, liquids, or gaseous materials in miniature, sealed capsules that can release their contents at controlled rates under specific conditions (Desai and Park, 2005).

In the food industry, the microencapsulation process can be applied for a variety of reasons, which have been summarized as follows (Fang and Bhandari, 2010):

(i) protection of the core material from degradation by reducing its reactivity to its outside environment;

(ii) reduction of the evaporation or transfer rate of the core material to the outside environment;

(iii) modification of the physical characteristics of the original material to allow easier handling;

(iv) tailoring the release of the core material slowly over time, or at a particular time;

(v) to mask an unwanted flavor or taste of the core material;

(vi) dilution of the core material when only small amounts are required, while achieving uniform dispersion in the host material;

(vii) to help separate the components of the mixture that would otherwise react with one another.

Food ingredients of acidulants, flavoring agents, sweeteners, colorants, lipids, vitamins and minerals, enzymes and microorganisms, are encapsulated using different technologies (Desai and Park, 2005).

For stabilizing the olive leaf extract and phenols in it, encapsulation studies were performed (Erbay and Icier, 2010). Kosaraju *et al.* (2006) carried out the encapsulation of olive leaf extract in chitosan microspheres by using a spray-drying process. They found retained activity of phenolic compounds in the chitosan matrix even after spray drying. Mourtzinos *et al.* (2007) encapsulated the olive leaf extract with β-cyclodextrin by mixing of components in the aqueous media and the subsequent freeze-drying process. They concluded that olive leaf extract encapsulated with β-cyclodextrin prevents phenolic oxidation at higher temperature. Moreover,

this encapsulation increased the aqueous solubility of phenolic residue by more than 150%. Therefore, the encapsulated olive leaf extract can be used as a food additive with the advantage of higher aqueous solubility (Mourtzinos *et al.,* 2007). Concluded, encapsulation preserves the antioxidant activity and the polyphenol concentration (Souilem *et al.,* 2016).

1.6. Aims and objectives

The current investigation had as a scope:

1. The development of a novel natural deep eutectic solvent (DES), based on biomolecules, with optimised composition, which would enable a high recovery of olive tree leaves (OLL) polyphenols.

2. The optimisation of the extraction process with regard to basic parameters, including liquid-to-solid ratio ($R_{L/S}$), temperature (T) and % water content (WC), to ensure maximisation of the extraction yield.

3. The examination of the effect of methyl-β-cyclodextrin (m-β-CD) as an extraction additive on both the extraction yield and polyphenolic profile of the OLL extracts.

4. The stability of the extracts obtained, and the role exerted by m-β-CD.

5. The antioxidant behaviour of the extracts as affected by the presence of m-β-CD.

An overview of the studies carried out to achieve the above-mentioned goals may be seen below (**Fig. 7**):
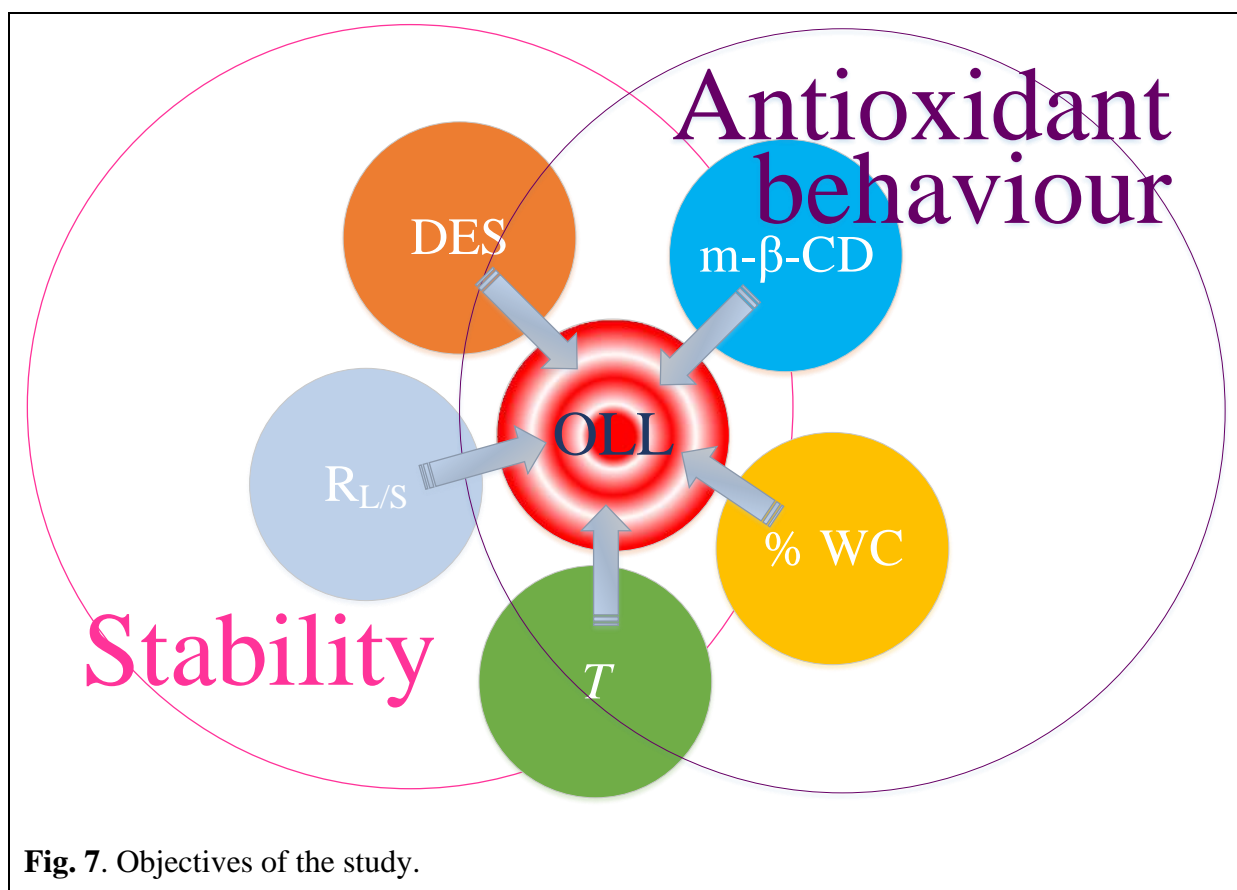
Fig. 7. Objectives of the study.

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Chapter 2:

Highly efficient extraction of antioxidant polyphenols from *Olea europaea* leaves using an eco-friendly glycerol/glycine deep eutectic solvent

Abstract

A number of biological activities have been attributed to polyphenolic substances occurring in the leaves of the olive tree (*O. europaea*), an olive oil industry waste material, and for this reason several extraction processes have been developed for the efficient recovery of these phytochemicals. The study presented herein describes for the first time the use of an eco-friendly deep eutectic solvent (DES) as a highly effective solvent for the extraction of polyphenols from olive tree leaves. Initially, a central composite design was implemented to optimise solvent composition and liquid-to-solid ratio and then a kinetic assay was performed to investigate diffusivity (D_e) and temperature effects. The maximum total polyphenol yield was 106.25 mg gallic acid equivalents per g dry weight, achieved at 70 ºC. This value was by 18% higher than that achieved with 60% aqueous methanol. Furthermore, the DES extract exhibited significantly higher antiradical activity and reducing power. Temperature increase up to 70 ºC was shown to boost D_e and the activation energy determined for the process was 29.55 kJ moL^{-1}. Characterisation of the extract by means of liquid chromatography-diode array-mass spectrometry showed oleuropein and certain flavone glycosides to be the major polyphenolic constituents.

Keywords: Antioxidants; Deep eutectic solvents; *Olea europaea*; Polyphenols

2.1. Introduction

The generation of food waste biomass encompasses a spectrum of activities, such as agricultural practices, industrial manufacturing and household consumption and contemporary environmental concepts, such as the circular economy, are regarded as the driving force for the implementation of "zero waste policies". The high volume of waste deriving from the food industry raises serious concerns, pertaining to both economic and environmental aspects, but a large amount of this residual biomass has a significant potential used as raw material for the production of novel commodities and specialty feedstock through biorefining procedures (Mirabella *et al.,* 2014).

The recovery of precious secondary metabolites from waste plant tissues is an attractive prospect in this regard, since many parts of plant material rejected during fruit and vegetable processing, such as peels, stems, seeds and leaves, bear a considerable load of such phytochemicals, including polyphenols. This particular class of compounds embraces numerous chemical structures of variable biological properties and functionalities, which are related with antioxidant and antimicrobial activity, as well as chemoprotective activity against cancer and other degenerative diseases (Li *et al.,* 2014). Olive leaves (OLL) are a waste material of the olive oil industry and they are rejected during olive fruit cleaning. OLL contain relatively high amounts of polyphenols compared with other food wastes (Mouratoglou *et al.,* 2016) and possess a peculiar polyphenolic profile, composed mainly of oleuropein, a major bioactive phenolic, but also several flavone glycosides (Rosello-Soto *et al.,* 2015; Talhaoui *et al.,* 2015). OLL polyphenols have been a subject of extensive and thorough studies, owed to their pharmacological potency (Obied *et al.,* 2012) and for this reason OLL polyphenol recovery has been a primal research field (Abaza *et al.,* 2015).

A crucial step in the recovery process is the molecule extraction, and conventional solvent extraction is the tool of preference, implemented on industrial scale to extract bioactive compounds from plant matrices (Barba *et al.,* 2016). However, food industries are facing the challenge to move towards sustainable process strategies, to maximize valorization of wastes for higher profitability and reduced environmental aggravation (Misra *et al.,* 2015). In this direction, emerging technologies are increasingly adopted by food industries, on the recognition of their potential to recover high-added value compounds effectively and in a sustainable framework. With regard to extraction on industrial scale, conventional methodologies have some major drawbacks, such as low extract recovery and long extraction duration and intensive heating, resulting in high energy consumption. Additionally, due to toxicity and the increasing prices of fossil resources, replacement of traditional solvents is a primary objective. Thus green

extraction processes are being oriented toward extraction intensification, increased mass and heat transfer, reduced equipment size, and reduction of processing steps (Rombaut *et al.,* 2014).

Deep eutectic solvents (DES) are novel liquids, composed of inexpensive, recyclable and non-toxic materials, which can be natural substances (e.g. sugars, organic acids and salts, etc.) (Paiva *et al.,* 2014). DES synthesis is eco-friendly, facile and straightforward and properties such as low vapour pressure, absence of flammability and water miscibility make DES ideal solvents for a range of sustainable and environmentally benign applications. Recently, the use of such solvents for the extraction of natural products has been attracting interest, because of their unique potency that allows for extraction yields higher than those achieved with conventional solvents (Mouratoglou *et al.,* 2016). Considering the above concepts and perspectives, the present examination was carried out to test a newly synthesized, eco-compatible DES, with regard to its potential in recovering bioactive polyphenolic phytochemicals from OLL. The approach employed included first optimization of basic extraction parameters and then a kinetic assay to assess the effect of temperature on the extraction process. The results were evaluated by comparison with other environmentally benign solvents, and characterization of the antioxidant activity and polyphenolic profile of the extracts.

2.2. Materials and methods

2.2.1. Chemicals

Solvents used for liquid chromatography-mass spectrometry were HPLC grade. Glycerol and aluminium chloride were from Fisher Scientific (New Jersey, U.S.A.). Ferric chloride hexahydrate was from Acros Organics (Geel, Belgium). Gallic acid, glycine, Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, rutin (quercetin 3-*O*-rutinoside) and 2,4,6-tripyridyl-*s*-triazine (TPTZ) were from Sigma-Aldrich (Steinheim, Germany). Sodium acetate trihydrate was from Penta (Prague, Czeck Republic).

2.2.2. Preparation of the DES

DES preparation was carried out according to a previously reported methodology (Mouratoglou *et al.,* 2016). Briefly, glycerol (HBD) was mixed with glycine (HBA) and water at a molar ratio 7:1:3. The mixture was heated in a stoppered glass vial at 80-90 ºC for approximately 90 min, under stirring, until a perfectly transparent liquid was formed. The DES was stored in screw-cap glass vial, in the dark, at room temperature. The DES was periodically inspected for the appearance of crystals, within a period of several weeks.

2.2.3. Plant material

Olive leaves (OLL) were from the *O. europaea* variety Agrielia Kalamon, collected from an olive tree plantation located at Avlida (Evia, Central Greece). Sampling and OLL handling was performed as described in an earlier study (Mylonaki *et al.,* 2008). More specifically, in order to obtain a uniform amount of leaves, the leaves were collected from several vicinal trees and from different parts of each tree, so as to minimise the effect of sun exposure and differences related to different maturation stages. After collection, the leaves were immediately transferred to the laboratory, randomly pooled and air-dried in-house.

2.2.4. Experimental design

A central composite design with one central point was deployed (Tzima *et al.,* 2015) to test the effect of concentration of the DES (C_{DES}) and the liquid-to-solid ratio ($R_{L/S}$) on the total polyphenol yield (Y_{TP}). For this scope, appropriate amounts of dry OLL powder were mixed with 25 mL of aqueous DES and stirred at 600 rpm for 120 min, at 50 °C. C_{DES} and $R_{L/S}$ used were predetermined by the experimental design (**Table 6**). The response (Y_{TP}) at each design point was measured and recorded (**Table 7**).

Table 6: Coded and actual levels of the independent variables used for the central composite design.

Independent variables	Code units	Coded variable level		
		-1	**0**	**1**
C_{DES} (w/v, %)	X_1	50	65	80
$R_{L/S}$	X_2	20	35	50

Table 7: Measured and predicted responses for all design points considered for the experimental design.

Design point	Independent variables		Response (Y_{TP}, mg GAE g^{-1} dw)	
	C_{DES} (X_1)	R_{moL} (X_2)	**Measured**	**Predicted**
1	-1	-1	82.33	81.23
2	-1	1	80.86	80.13
3	1	-1	91.29	91.67
4	1	1	89.45	90.21
5	-1	0	80.78	82.61
6	1	0	94.01	92.87
7	0	-1	78.02	78.74
8	0	1	77.49	77.46
9	0	0	79.36	80.02

2.2.5. Kinetics and temperature assay

Amount of 1.56 g of dry OLL powder was mixed with 50 mL of solvent, [C_{DES} = 80% (w/v), $R_{L/S}$ = 32 mL g^{-1}] and extractions were performed as described above, within a temperature range of 40-70 ºC. Sampling was accomplished at regular intervals (5-280 min) to measure Y_{TP}. The second-order model, as previously described (Cavdarova and Makris, 2014), was obtained by plotting t/Y_{TP} as a function of t (**Fig. 10**). Then kinetic parameters including second-order extraction rate constant (k), initial extraction rate (h) and extraction yield at saturation ($Y_{TP(s)}$) were determined. Likewise, the effect of temperature was estimated by calculating the activation energy (E_a), according to a previous study (Makris, 2016). Diffusivity was estimated as described in details elsewhere (Blidi *et al.,* 2015).

2.2.6. Determinations

2.2.6.1. Total polyphenol yield (Y$_{TP}$)

A well-established protocol was used (Blidi *et al.,* 2015). Briefly, 0.78 mL of distilled water, 0.02 mL of sample extract and 0.05 mL of Folin-Ciocalteu reagent (2 M) were added in a 1.5-mL Eppendorf tube. After exactly 1 min, 0.15 mL of aqueous sodium carbonate 20% (w/v) was added, and the mixture was allowed to stand at room temperature in the dark, for 60 min. The absorbance was read at 750 nm (A_{750}) in a Shimadzu UV-1700 UV-Vis Spectrophotometer (Tokyo, Japan), and the total polyphenol concentration (C_{TP}), expressed in mg L^{-1}, was calculated from a calibration curve, using gallic acid as a standard. Yield in total polyphenols (Y_{TP}) was determined as mg gallic acid equivalents (GAE) per g of dry weight (dw), using the following equation:

$$Y_{TP} \text{ (mg GAE g}^{-1} \text{ dw)} = \frac{C_{TP} \times V}{m} \tag{1}$$

where, V is the volume of the extraction medium (in L) and m the dry weight of OLL (in g).

2.2.6.2. Total flavonoid yield (Y$_{TFn}$)

A previously published protocol was used (Makris, 2016). An aliquot of 0.25 mL sample extract was mixed with 0.75 mL AlCl$_3$ reagent (0.16% (w/v) AlCl$_3$ in 5% (v/v) acetic acid in methanol) and allowed to stand for 30 min, at room temperature. The absorbance was obtained at 415 nm (A_{415}) and the total flavonoid concentration (C_{TFn}), expressed in mg L^{-1}, was calculated from a calibration curve, constructed with rutin (quercetin 3-*O*-rutinoside) as the calibration standard. Yield in total flavonoids (Y_{TFn}), expressed as mg rutin equivalents (RtE) per g of dw OLL, was determined using the following equation:

$$Y_{TFn} \text{ (mg RtE g}^{-1} \text{ dw)} = \frac{C_{TFn} \times V}{m} \qquad (2)$$

where, V is the volume of the extraction medium (in L) and m the dry weight of OLL (in g).

2.2.6.3. Reducing power (P_R)

A previously reported methodology was employed (Blidi *et al.*, 2015). Sample extract (0.05 mL) was mixed thoroughly with 0.05 mL FeCl$_3$ solution (4 mM in 0.05 M HCl), and incubated for 30 min in a water bath at 37 ºC. Following this, 0.90 mL TPTZ solution (1 mM in 0.05 M HCl) were added, and the absorbance was recorded at 620 nm after exactly 5 min. P_R was determined as µmoL ascorbic acid equivalents (µmoL AAE) per g of dw OLL, was determined using the following equation:

$$Y_{P_R} \text{ (µmoL AAE g}^{-1} \text{ dw)} = \frac{C_{P_R} \times V}{m} \qquad (3)$$

where, V is the volume of the extraction medium (in L) and m the dry weight of OLL (in g).

2.2.6.4. Antiradical activity (A_{AR})

The assay was performed using a well-established methodology (Shehata *et al.*, 2015). A volume of 0.025 mL sample extract was mixed with 0.975 mL DPPH solution (80 µM in methanol) and the absorbance at 515 nm was read immediately after mixing ($A_{515(i)}$) and after exactly 30 min ($A_{515(f)}$). The A_{AR} was determined as µmoL DPPH per g of dw OLL, using the following equation:

$$A_{AR} \text{ (µmoL DPPH g}^{-1} \text{ dw)} = \frac{C_{DPPH}}{C_{TP}} \times \left(1 - \frac{A_{515(f)}}{A_{515(i)}} \right) \times Y_{TP} \qquad (4)$$

where, C_{DPPH} is the initial molar concentration of DPPH (µmoL L^{-1}); C_{TP} is the total polyphenol concentration of the extract, expressed as mg GAE L^{-1} and Y_{TP} is the total polyphenol yield of the extract, expressed as mg GAE g^{-1} dw.

2.2.7. Qualitative liquid chromatography-diode array-mass spectrometry (LC-DAD-MS)

A previously described methodology (Blidi *et al.*, 2015) was employed to tentatively characterise the principal polyphenolic metabolites, with some modifications. Briefly, the equipment used was a Finnigan MAT Spectra System P4000 pump, coupled with a UV6000LP diode array detector and a Finnigan AQA mass spectrometer. A Fortis RP-18 column, 150 × 2.1 mm, 3 µm, was used, at 40 ºC. Analyses were performed with electrospray ionization (ESI) in positive ion mode, with acquisition set at 5 and 50 eV, capillary voltage 4 kV, source voltage 25 V, detector voltage 650 V and probe temperature 350 ºC. Eluent (A) and eluent (B) were 1%

acetic acid and methanol, respectively. The flow rate was 0.2 mL min^{-1}, and the elution programme used was as follows: 0-2 min, 10% B; 2-40 min, 100% B; 45 min, 100% B.

2.2.8. Statistics

Extractions were repeated at least twice and all determinations at least in triplicate. Values reported are averages and standard deviations. Linear and non-linear regressions were performed with SigmaPlot® 12.0. Central composite experimental design and all associated statistics were performed with JMP® 10.

2.3. Results and discussion

2.3.1. DES synthesis and optimisation of solvent composition

In an earlier investigation (Bakirtzi *et al.,* 2016), DES composed of L-lactic acid and glycine at a molar ratio 3:1 was shown to be unstable, yielding a plastic solid. This mixture was successfully tailored by adding 3 moles of water, to give a stable DES, which was found to be a very efficient solvent for the extraction of polyphenolic antioxidants from various medicinal herbs, compared with a few other L-lactic acid-based DES that contained organic salts as HBAs. This finding evidenced that glycine might be a HBA with a high potency in forming DES with enhanced extraction efficiency. In this context, attempts were undertaken to combine glycerol (HBD) with glycine, in the view of synthesising an efficient and more cost-effective solvent, which could have an important prospect for polyphenol recovery. To this purpose, glycerol was combined with glycine starting from a molar ratio 1:1, but up to ratio 6:1 all mixtures were unstable, giving crystallisation. A ratio of 7:1 was of improved stability as the crystallisation observed was limited, but stability was assured through tailoring with 3 moles of water. Thus the final composition of the DES was glycerol:glycine:water (7:1:3). This DES was used for further examination.

The next step was the optimisation of the extraction process with respect to DES concentration (C_{DES}) and the liquid-to-solid ratio ($R_{L/S}$), two critical parameters that largely define extraction yield (Blidi *et al.,* 2015). For this scope, a central composite design with one central point was used and the extraction efficiency was estimated by determining the total polyphenol yield (Y_{TP}). The effect of the simultaneous variation of the independent variables (C_{DES} and $R_{L/S}$) was illustrated in the form of a 3D plot (**Fig. 8**). Variations in $R_{L/S}$ provoked rather weak changes in Y_{TP}, but switching of C_{DES} had a pronounced impact in this regard. This is in line with the outcome of recent studies, which demonstrated that water content between 10 and 50% may greatly enhance the extraction performance of various DES (Dai *et al.,* 2013a). This is because mixing the DES with water regulates properties such as polarity and viscosity,

which are directly related with the extraction capacity of a solvent (Dai *et al.*, 2013b). Depending on the polyphenols to be extracted, the ideal water content may vary up to approximately 64% (v/v) (Jancheva *et al.*, 2017).
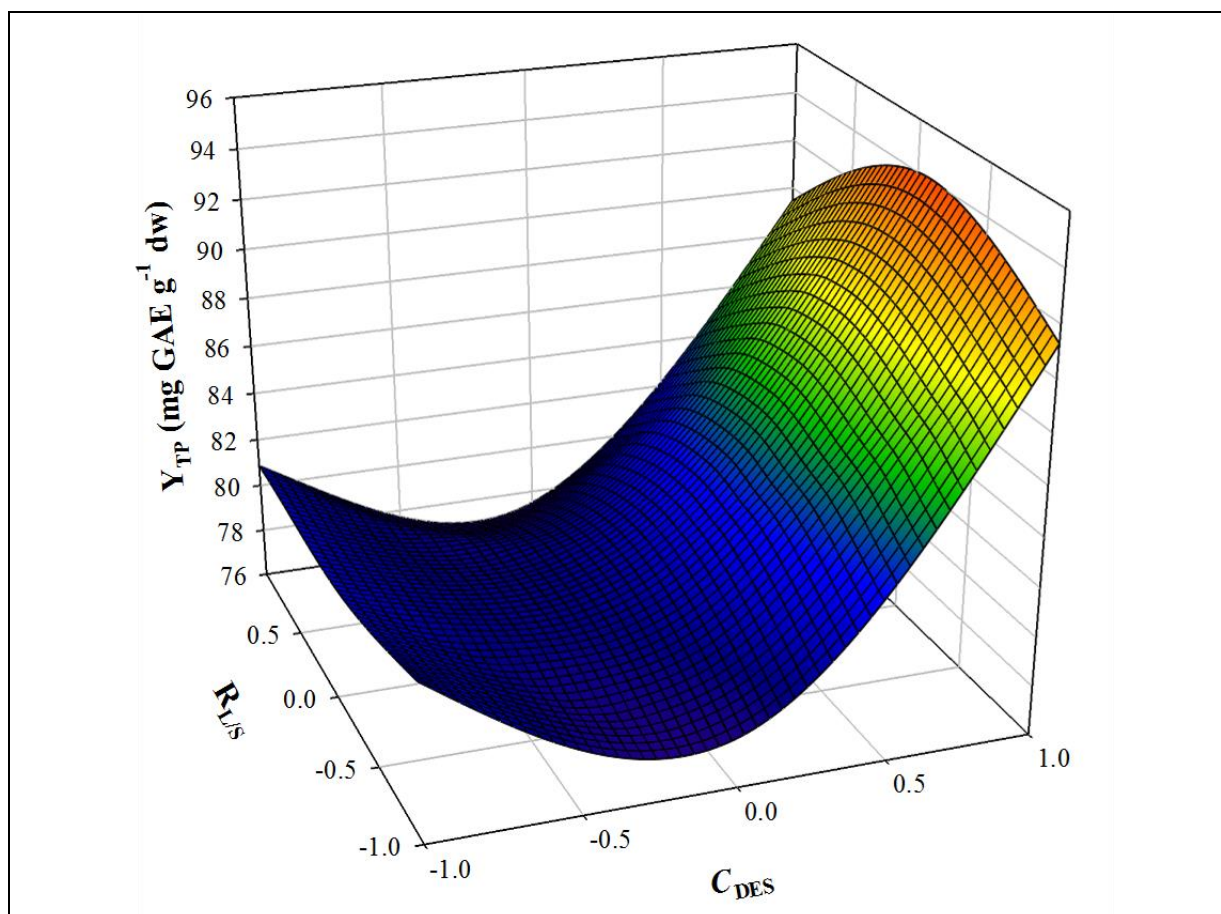
Fig. 8: 3D plot displaying the effect of simultaneous variation of C_{DES} and $R_{L/S}$ on Y_{TP}. OLL Extractions were carried out at 50 ºC, under continuous stirring at 600 rpm, for 120 min.

To assess quantitatively the combined effect of both independent variables, the desirability function was used (**Fig. 9**). It was found that only C_{DES} and its quadratic term were significant ($p < 0.005$) and therefore removal of the non-significant terms gave the following model (mathematical equation):

$$Y_{TP} \text{ (mg GAE g}^{-1} \text{ dw)} = 80.02 + 5.26X_1 + 7.71X_1^2$$

(5)

$$(R^2 = 0.97, p = 0.0043)$$
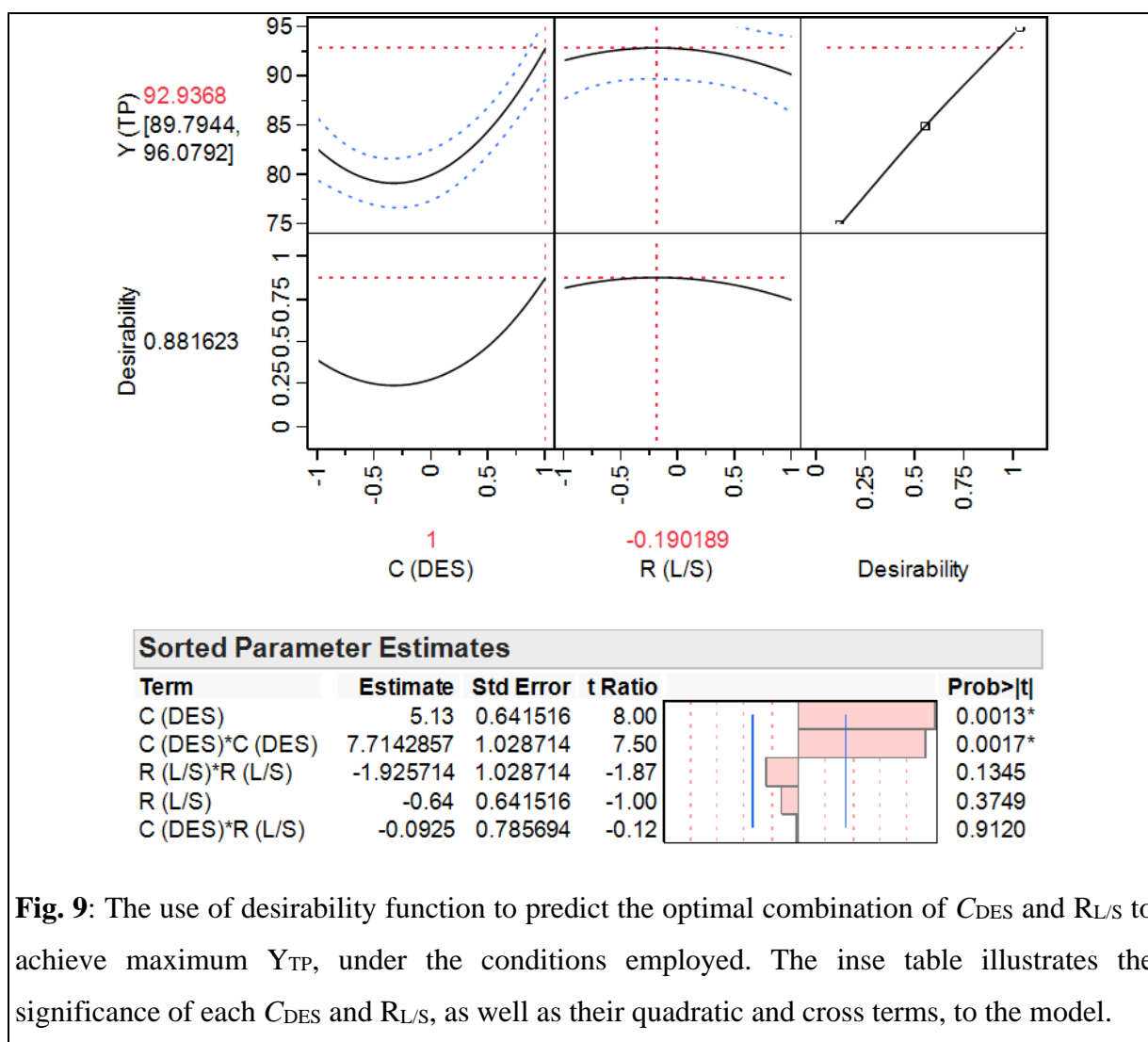
Fig. 9: The use of desirability function to predict the optimal combination of C_{DES} and $R_{L/S}$ to achieve maximum Y_{TP}, under the conditions employed. The inse table illustrates the significance of each C_{DES} and $R_{L/S}$, as well as their quadratic and cross terms, to the model.

Using the fitted model, the optimal condition was found to be C_{DES} = 80% (w/v). Although $R_{L/S}$ exerted a non-significant effect on the model, the optimal value determined (32 mL g^{-1}) was adopted, because it was within the range reported for polyphenol extraction with DES (Jancheva *et al.,* 2017). Under these conditions, the estimated maximum Y_{TP} was 92.94±3.15 mg GAE g^{-1} dw.

2.3.2. Extraction kinetics - Effect of temperature

In order to further evaluate the extraction capacity of the solvent, the optimal conditions (C_{DES} = 80% (w/v), $R_{L/S}$ = 32 mL g^{-1}) were used to trace extraction kinetics, with the aim to estimating some basic kinetic parameters and the effect of temperature. The model best fitted to the data generated was second-order kinetics (**Fig. 10**), as previously shown for OLL extraction with aqueous glycerol and aqueous ethanol (Apostolakis *et al.,* 2014), but also with various glycerol-based DES for polyphenol extraction from *Satureja thymbra* (Jancheva *et al.,* 2017). It can be seen in **Table 8** that an increase in *T* from 40 to 70 ºC revealed kinetics obeying the Arrhenius model, giving E_a = 29.55 kJ mol^{-1}. This value was higher than that determined for polyphenol

extraction from red grape pomace with aqueous glycerol (13.94 kJ moL^{-1}) (Trasanidou *et al.,* 2016), but lower than those reported for polyphenol extraction with aqueous glycerol from onion solid wastes (31.52 kJ moL^{-1}) (Katsampa *et al., 2015*) and *Artemisia* (37.64 kJ moL^{-1}) (Shehata *et al., 2015*). However, similar data for polyphenol extraction with DES are not available in the literature.
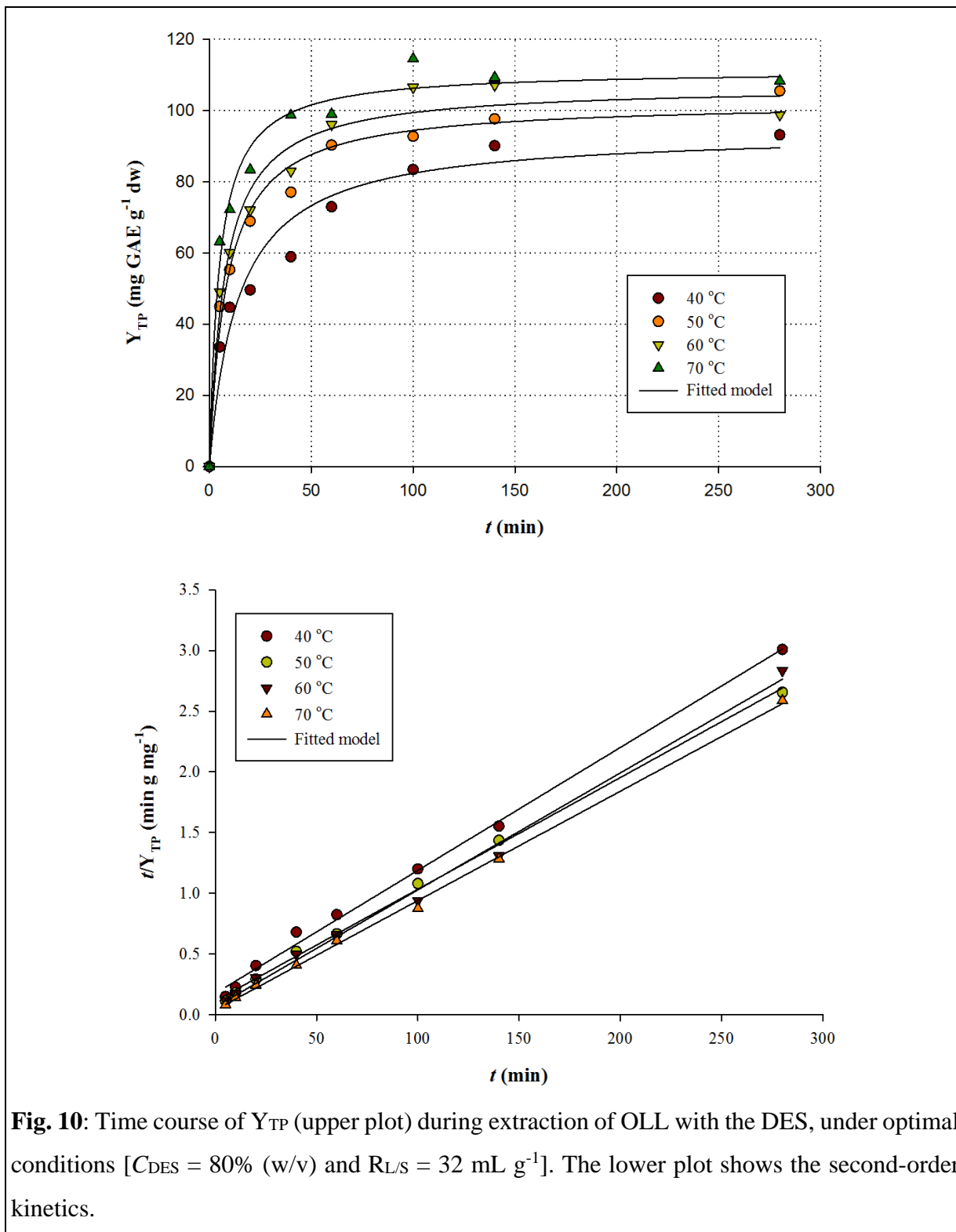
Fig. 10: Time course of Y_{TP} (upper plot) during extraction of OLL with the DES, under optimal conditions [$C_{DES} = 80\%$ (w/v) and $R_{L/S} = 32$ mL g^{-1}]. The lower plot shows the second-order kinetics.

Table 8: Parameters of second-order kinetics, determined for the extraction of TP from OLL, using 80% (w/v) aqueous DES. Extractions were performed under continuous stirring at 600 rpm and $R_{L/S}$ = 32 mL g^{-1}.

T (ºC)	Kinetic parameters				
	k ($\times 10^{-3}$)	h	$Y_{TP(s)}$	D_e	E_a
	(g mg^{-1} min^{-1})	(mg g^{-1} min^{-1})	(mg GAE g^{-1})	(m s^{-1}) $\times 10^{-12}$	(kJ moL^{-1})
40	0.750	6.63	94.04	0.127	
50	1.157	12.12	102.36	0.138	29.55
60	1.240	14.16	106.87	0.205	
70	1.864	23.10	111.33	1.041	

The effect of temperature on diffusivity (D_e) followed a similar trend, but a significant increase, approximately by 5-fold, was recorded when the temperature was raised from 60 to 70 ºC. Such a steep change might be associated with a sharp decrease in viscosity, since according to Stokes-Einstein equation, diffusion (D) is reciprocally related with viscosity (Karakashov *et al.,* 2015). It has been proposed that aqueous DES solutions could be disintegrated through hydrogen bond rupture, by the energy provided through temperature increase, hence abolishing their unique properties (Jancheva *et al.,* 2017). Such a theory could explain this unusual behaviour, yet it is to be proven. It should be emphasised that such a phenomenon has not been described in studies on polyphenol extraction with conventional solvents, where diffusivity increases in response to raising the temperature were much smoother (Trasanidou *et al.,* 2016; Katsampa *et al.,* 2015; Shehata *et al.,* 2015). On the other hand, if DES disintegration occurred, then a drop in its extraction capacity would have been observed, leading to lower Y_{TP} and Y_{TFn}, but this was not the case. Therefore, the steep increase in D_e recorded at 70 ºC might be attributed to a weakening of hydrogen bonds between the HBD and HBA, and not complete rupture.

2.3.3. Comparison with conventional eco-friendly solvents

To bring out the extraction capacity of the DES used, extraction of OLL was carried out under optimised conditions, at 70 ºC, and the results were compared with those obtained using distilled water and 60% ethanol (AE), but also 60% methanol (AM), which was used as a positive control. The results showed that extraction with the DES afforded almost 18% higher Y_{TP} than AE, over than 24% higher than AM and approximately 29% higher than water (**Fig. 11**, upper plot). To further illustrate the higher capacity of the DES, Y_{TFn} was also determined. In the same fashion, extraction with the DES gave 9.6, 15.3 and 43.8% higher Y_{TFn}, compared with AE, AM and water, respectively (**Fig. 11**, lower plot). Regarding A_{AR}, extracts with AM and AE had comparable levels of 656.0 and 643.8 µmoL DPPH g^{-1} dw, but the DES extract

exhibited A_{AR} = 1097.8 μmoL DPPH g^{-1} dw (**Fig. 12**, upper plot). Likewise, AM and AE extracts had P_R values of 381.6 and 394.9 μmoL AAE g^{-1} dw, but P_R for the DES extract was 445.1 μmoL AAE g^{-1} dw (**Fig. 12**, lower plot). These findings clearly demonstrated that the DES used was far more efficient than AE and water, yielding extracts with higher total polyphenol and total flavonoid content, but also with superior antioxidant properties.
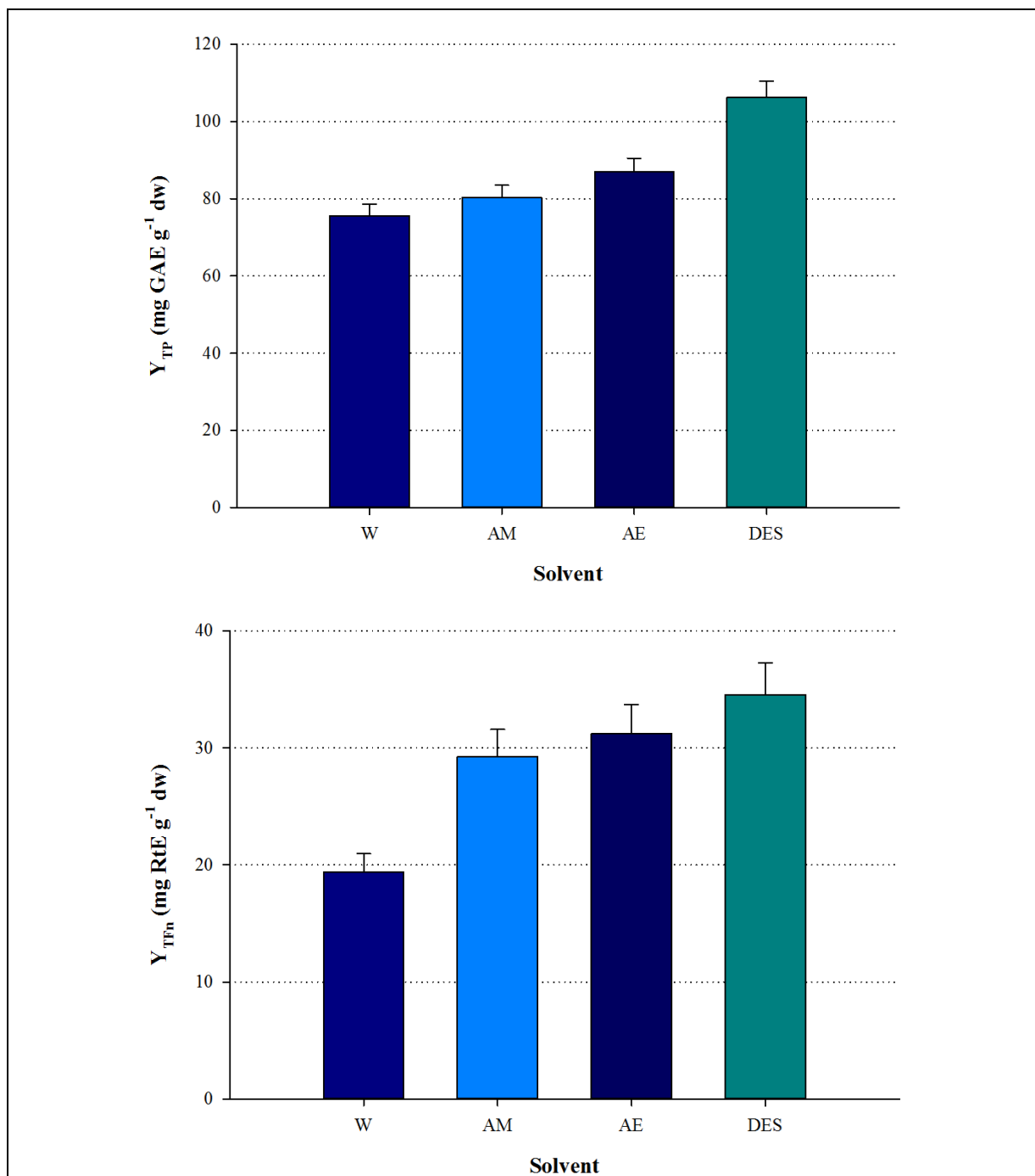
Fig. 11: Comparative diagram for Y_{TP} (upper plot) and Y_{TFn} (lower plot), obtained using 80% (w/v) DES, 60% aqueous methanol (AM), 60% aqueous ethanol (AE) and distilled water (W). Extractions were carried out at 70 ºC, at $R_{L/S}$ = 32 mL g^{-1}, under continuous stirring at 600 rpm, for 280 min.
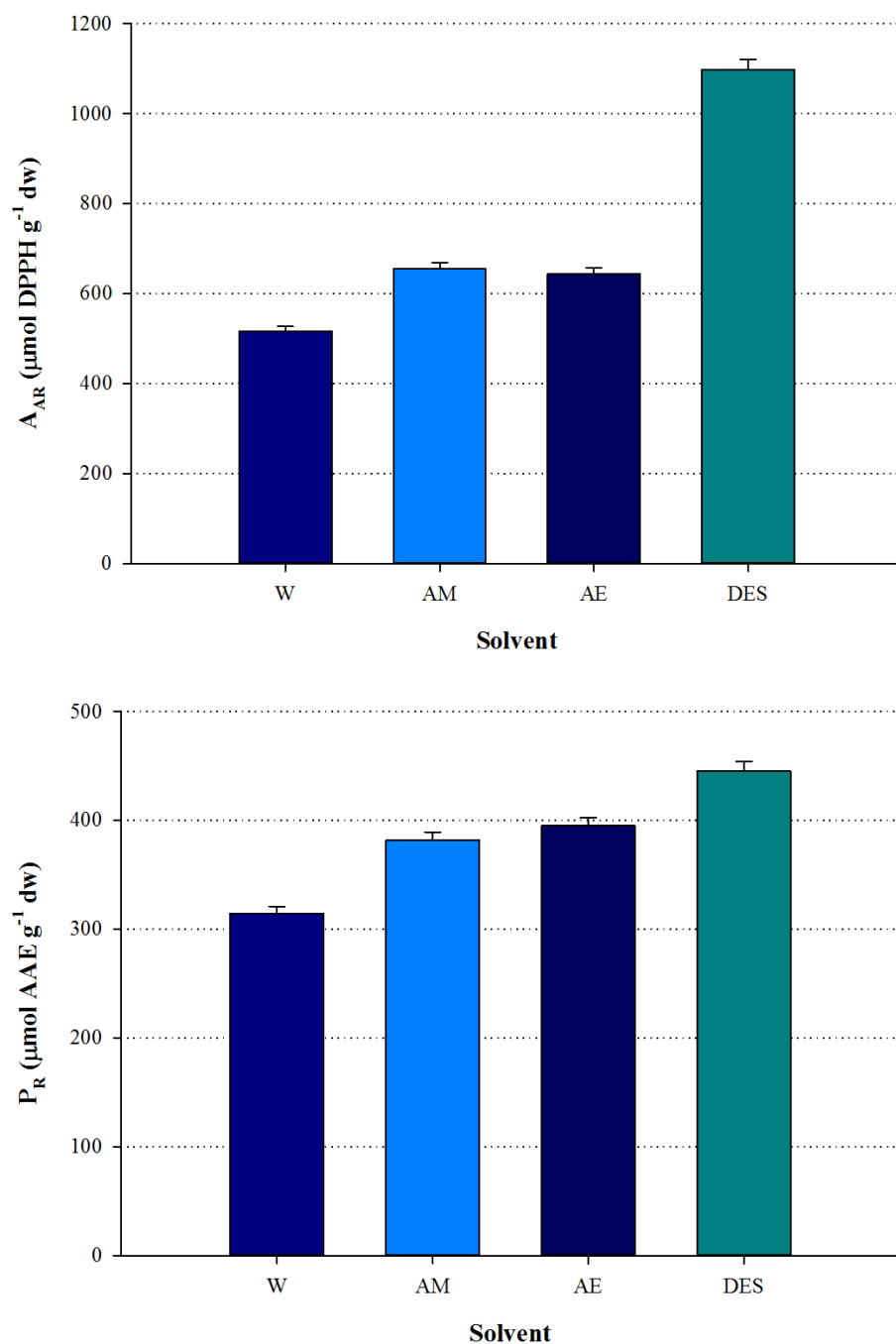
Fig. 12: Comparative diagram for A_{AR} (upper plot) and P_R (lower plot), determined for OLL extracts obtained using 80% (w/v) DES, 60% aqueous methanol (AM), 60% aqueous ethanol (AE) and distilled water (W). Extractions were carried out at 70 ºC, at $R_{L/S}$ = 32 mL g^{-1}, under continuous stirring at 600 rpm, for 280 min.

At this point, it should be emphasised that the antioxidant activity seen in the OLL extracts has been proposed to be the integration of interactions amongst the polyphenolic constituents, rather than that of the individual substances (Lee and Lee, 2010). Such a claim has been demonstrated by studies that assessed the antiradical behaviour of mixtures of pure antioxidants and extracts (Karvela *et al.,* 2012). However, compounds such as hydroxytyrosol and luteolin

7-*O*-glucoside were shown to be major radical scavengers in OLL extracts (Goulas *et al.,* 2010) and it is likely that their levels in OLL extracts may affect significantly their antioxidant potency. However, since the polyphenolic composition and hence the antioxidant activity of OLL is subject to seasonal variations (Goulas *et al.,* 2010) and age (Laguerre *et al.,* 2009), then methodologies that maximise extraction yield, as the one proposed herein, could assure extracts with high antioxidant activity.

2.3.4. Characterisation of principal polyphenols

The DES extract that displayed the highest Y_{TP} was analysed by LC-DAD-MS to tentatively characterise its polyphenolic profile. At this point it should be stressed that although olive leaf extracts from various Greek native varieties have been examined (Mitsopoulos *et al.,* 2016), data on the particular variety Agrielia Kalamon used in this study, to the best of our knowledge, are inexistent. In total, ten compounds could be identified including secoiridoid, flavone and flavonol derivatives (**Table 9**). Critical comparison with previous studies on olive leaf extract composition (Mourtzinos *et al.,* 2016; Apostolakis *et al.,* 2014; Mylonaki *et al.,* 2008) showed the presence of two oleoside derivatives (compounds 1 and 2) and a quercetin derivative with $m/z = 603$ (compound 5), which was heretofore unreported. Other common representatives, including oleuropein (compound 10), a luteolin di-glycoside (compound 4), which most probably corresponds to luteolin 4′,7-*O*-di-glucoside, two luteolin rutinosides (compounds 6 and 7) and an apigenin rutinoside (compound 9) were also present. On the other hand, no rutin (quercetin 3-*O*-rutinoside) or verbascoside, which are commonly encountered constituents (Jerman Klen *et al.,* 2015), were detected.

Table 9: UV-Vis and mass spectrometric data of the major polyphenols detected in the OLL extracts, obtained with 80% (w/v) DES, at 70 ºC.

No	Rt (min)	UV-Vis	$[M + H]^+$ (*m/z*)	Other ions (*m/z*)	Tentative identity
1	14.24	288, 322(s)	391	413 $[M + Na]^+$, 261	Oleoside
2	18.80	288, 332	391	413 $[M + Na]^+$, 229	Oleoside
3	22.86	248, 348	765	579, 503, 233	Luteolin derivative
4	23.36	266, 340	611	449, 287	Luteolin di-glycoside
5	24.96	264, 318, 368	603	475, 303	Quercetin derivative
6	25.90	248, 280(s), 356	595	617 $[M + Na]^+$, 449, 287	Luteolin rutinoside
7	26.39	248, 354	595	617 $[M + Na]^+$, 449, 287	Luteolin rutinoside
8	26.69	252, 280	541	563, 361, 137	Oleuropein isomer
9	27.30	244, 340	579	271	Apigenin rutinoside
10	28.38	252, 280	541	563, 385, 345	Oleuropein

2.4. Conclusions

In this investigation, a newly synthesised DES composed of two biomolecules, glycerol and glycine, was tested for its effectiveness in extracting polyphenolic antioxidants from OLL. Following a first stage of parameter optimisation, this particular DES was demonstrated to be highly efficient, giving significantly increased polyphenol yield compared with conventional bio-solvents, such as aqueous ethanol and water. The DES extract also displayed stronger antioxidant effects. Thus this solvent is proposed as an eco-friendly means of boosting polyphenol recovery through solid-liquid extraction and this may also pave the way for more effective valorisation of other phytochemicals, from agri-food waste biomass. Since the solvent is composed of non-toxic substances naturally occurring in foods, the extracts may be directly used as bioactive constituents in food/cosmetic/pharmaceutical formulations, where this would be convenient, thus negating the need of downstream processing for solvent removal. This would eventually lead in lower cost and energy consumption, in line with a sustainable process. Currently, work is in progress to test a broader range of glycine-based DES, in an effort to further improve polyphenol extraction.

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Chapter 3:

Methyl-β-cyclodextrin as a booster for the extraction for *Olea europaea* leaf polyphenols with a bio-based deep eutectic solvent

Abstract

A novel deep eutectic solvent (DES) with optimized composition was used to evaluate the effect of methyl-β-cyclodextrin (m-β-CD) on the efficiency of polyphenol extraction from *O. europaea* leaves (OLL). The process developed was based on a Box-Behnken experimental design and response surface methodology, to assess the simultaneous effect of m-β-CD concentration ($C_{m-β-CD}$), liquid-to-solid ratio ($R_{L/S}$) and temperature (T). Under optimized conditions ($C_{m-β-CD} = 9\%$, $R_{L/S} = 40$ mL g^{-1}, $T = 51$ °C), the yield in total polyphenols (Y_{TP}) was 116.65±3.60 mg gallic acid equivalents per g dry weight. This value was significantly higher than that determined for the extraction performed with 60% aqueous ethanol. The extraction kinetics also showed that the extraction rate was slowed down in the presence of m-β-CD, yet the higher extraction capacity of the DES/m-β-CD medium was confirmed. Characterization of the extracts obtained with DES/m-β-CD and DES by means of liquid chromatography-mass spectrometry demonstrated that there was no selective extraction of any particular polyphenol, suggesting that m-β-CD acted merely as an extraction booster.

Keywords: Antioxidants; Deep eutectic solvents; Methyl-β-cyclodextrin; *Olea europaea*; Polyphenols

3.1. Introduction

Nowadays it is regarded imminent to emerging circular economy that the waste biomass deriving from the agricultural and food sector should not be simply treated as waste, but rather as a bioresource with high potential in value-added substances, chemicals and fuels. Agro-industrial activity accounts for the production of a large volume of wastes, including processing residues, such as leaves, branches, peels, roots, stems and seeds. This biomass is usually undervalorized; in spite its richness in an array of high value-added substances. A highly regarded class of phytochemicals of broad occurrence in agri-food wastes is polyphenols, embracing a bewildering diversity of structures (Tsao, 2010). A plethora of these compounds exhibits biological properties of particular interest, including anti-inflammatory and chemopreventive activities (Brglez Mojzer *et al.,* 2016) and therefore they are considered to have an undisputed perspective as bioactive agents in food, pharmaceutical and cosmetics industry.

Mediterranean countries are facing pressure to deal with problems associated with agri-food wastes, typical to their native principal crops, such olives. Olive industry wastes not properly treated are a primal environmental issue for olive-producing countries, and therefore strategies aiming at valorising olive wastes, especially those allowing a sustainable recovery of valuable natural components, are gaining acceptance. Olive leaves, a regular olive-processing residue, may bear a high load of polyphenols with potential pharmacological applications (Obied *et al.,* 2012), hence various extraction methodologies have been developed for their effective recovery. However, green procedures for effective olive leaf polyphenol retrieval are rather limited but the use of eco-friendly extraction techniques may represent unprecedented opportunities to face such challenge in a sustainable manner.

A selective solvent with a high solubility of the target compounds is a salient feature of processes destined to achieve a high yield of target compounds in a short process time. Most of the extraction procedures are based on regulating solvent properties to increase solubility of the solute molecules, by modifying solvent polarity. Apart from its physical-chemical capacity in dissolving the target compound(s), the toxicity of a solvent and the environmental impact by its use should also be considered. In this view, deep eutectic solvents (DES), which are composed of inexpensive, recyclable and non-toxic natural substances (e.g. sugars, organic acids and salts etc.) appear as the most promising prospect. This is because characteristics including low vapour pressure, absence of flammability and water miscibility make DES ideal solvents for a range of sustainable and eco-friendly applications (Alonso *et al.,* 2016).

Cyclodextrins are natural cyclic oligosaccharides originating from enzymic cleavage of starch, and they comprise of 6, 7 or 8 glucose units linked by α(1→4) glycosidic bond.

Cyclodextrin structure has a truncated cone shape, with a hydrophobic cavity inside and a hydrophilic external surface; therefore, cyclodextrins can form inclusion complexes with sparingly water-soluble molecules (such as polyphenols), increasing their solubility (Pinho *et al.,* 2014). Furthermore, cyclodextrin encapsulation of polyphenols contributes in higher stability and controlled release (Munin and Edwards-Lévy, 2011).

Recent studies showed that combining 2-hydroxypropyl-β-cyclodextrin with aqueous glycerol may effectively increase yield of polyphenol extraction (Kyriakidou *et al.,* 2016; Mourtzinos *et al.,* 2016). Similar results were reported for water extraction of apple flavonols, using various cyclodextrins (Parmar *et al.,* 2015). In the light of such evidence, the study carried out aimed at testing the efficiency of polyphenol extraction from OLL, using a combination of methyl-β-cyclodextrin (m-β-CD) with a glycerol-based DES (Athanasiadis *et al.,* 2017). The process was first optimized by implementing a central composite design and performance assessment was based on kinetics and extract characterization, using representative antioxidant tests and liquid chromatography-mass spectrometry.

3.2. Materials and methods

3.2.1. Chemicals and reagents

HPLC grade solvents were used for liquid chromatography. Folin-Ciocalteu reagent was from Fluka (Steinheim, Germany). Ferric chloride hexahydrate and ascorbic acid was from Acros Organics (Geel, Belgium). Glycerol and aluminium chloride were from Fisher Scientific (New Jersey, U.S.A.). Sodium acetate was from Penta (Prague, Czeck Republic). Methyl-β-cyclodextrin, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), rutin (quercetin 3-*O*-rutinoside), glycine and 2,4,6-tripyridyl-*s*-triazine (TPTZ) were from Sigma-Aldrich (Steinheim, Germany).

3.2.2. Plant material and DES synthesis

Details regarding collection and handling of *O. europaea* leaves (OLL) have been analytically described elsewhere (Mylonaki *et al.,* 2008). The material used for extraction was dried OLL powder, with an average mean particle diameter of approximately 0.5 mm. For DES synthesis, the optimised conditions were used (Athanasiadis *et al.,* 2017). Briefly, the DES was prepared using glycerol as the hydrogen bond donor (HBD) and glycine as the hydrogen bond acceptor (HBA), at a molar ratio HBD:HBA:water of 7:1:3. Aqueous solution 80% (w/v) of this DES was employed for all extractions performed.

3.2.3. Batch extraction process

A suitable amount of OLL powder was mixed with the DES and m-β-CD in a 50-mL glass vial and extractions were carried out under continuous stirring on a magnetic stirrer, at 600 rpm, for 180 min. The amount of OLL powder, the concentration of m-β-CD and the temperature of the extraction were defined by the experimental design (**Table 10**). After the completion of each extraction, samples were centrifuged at 20,000 × g and the clear supernatant was used for all analysis, after dilution 1:20 with water.

Table 10: Actual values and coded levels of the independent variables used for the Box-Behnken experimental design.

Independent variables	Code units	Coded variable level		
		-1	**0**	**1**
$C_{\text{m-β-CD}}$ (%, w/v)	X_1	1	5	9
$R_{\text{L/S}}$ (mL g^{-1})	X_2	10	25	40
T (°C)	X_3	40	50	60

3.2.4. Experimental design

A Box-Behnken experimental design was used, as described previously (Karvela *et al.,* 2009), with the yield in total polyphenols (Y_{TP}) as the response. The three independent variables considered were the concentration of m-β-CD ($C_{\text{m-β-CD}}$) (X_1, varying between 1 and 9 %, w/v), liquid-to-solid ratio ($R_{\text{L/S}}$) (X_2, varying between 10 and 40 mL g^{-1}) and temperature (T) (X_3, varying between 40 and 60 °C). The range used for each variable was chosen on the basis of previous findings (Kyriakidou *et al.,* 2016) and preliminary experimentation. Each variable was coded at three levels, -1, 0 and 1 (**Table 10**), according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \, , \, i = 1, 2, 3 \tag{6}$$

The terms x_i and X_i represent the dimensionless and the actual value of the independent variable i, X_0 the actual value of the independent variable i at the central point, and ΔX_i the step change of X_i corresponding to a unit variation of the dimensionless value. For each design point, the response was determined and recorded (**Table 11**). Data from the experimental design were subjected to regression analysis using least square regression methodology to obtain the parameters of the mathematical models. Analysis of variance (ANOVA) was used to assess the significance of the model. 3D plots were obtained using the fitted model.

Table 11: Measured and predicted value of Y_{TP}, determined for individual design points.

Design point	Independent variables			Response (Y_{TP}, mg GAE g^{-1} dw)	
	X_1	X_2	X_3	Measured	Predicted
1	-1	-1	-1	74.67	75.17
2	-1	-1	1	87.20	86.88
3	-1	1	-1	104.94	105.56
4	-1	1	1	112.30	112.99
5	1	-1	-1	86.46	86.20
6	1	-1	1	91.99	91.81
7	1	1	-1	112.57	113.33
8	1	1	1	114.72	114.66
9	-1	0	0	103.27	101.78
10	1	0	0	108.38	108.13
11	0	-1	0	86.10	86.36
12	0	1	0	114.98	112.98
13	0	0	-1	99.42	97.80
14	0	0	1	104.44	104.31
15	0	0	0	102.68	103.68
16	0	0	0	101.18	103.68

3.2.5. Kinetic and diffusivity (D_e) determination

Extractions were carried out as described above, under optimised conditions and sampling was accomplished at predetermined intervals (5 - 180 min), to calculate Y_{TP}. Then Y_{TP} was plotted against time (t) and model fitting was performed with non-linear regression. The second-order model was obtained after plotting t/Y_{TP} as a function of t, according to the following equation (Apostolakis *et al.,* 2014):

$$\frac{t}{Y_{TP(t)}} = \frac{1}{kY_{TP(s)}^2} + \frac{t}{Y_{TP(s)}} \tag{7}$$

The initial extraction rate (h) and the yield in total polyphenols at saturation ($Y_{TP(s)}$) were determined graphically, from the slope ($t/Y_{TP(s)}$) and the intercept ($1/h$), respectively. The second-order extraction rate constant (k) was then calculated:

$$h = kY_{TP(s)}^2 \tag{8}$$

Diffusivity (D_e) was determined using the linearized expression of Fick's second law for non-steady state, as described previously (Shehata *et al.,* 2015):

$$1 - \frac{Y_{TP(t)}}{Y_{TP(s)}} = \frac{6}{\pi^2} e^{-\frac{D_e \pi^2 t}{r^2}} \tag{9}$$

D_e calculation was based on the line with the shallow slop (slope $= \frac{D_e \pi^2}{r^2}$), obtained by plotting

$ln\left(\frac{Y_{TP(s)}}{Y_{TP(s)} - Y_{TP(t)}}\right)$ against t. Where r is the radius of the solid particles.

3.2.6. Determinations

Total polyphenol yield (Y_{TP}) was determined using the Folin-Ciocalteu reagent and expressed as mg gallic acid equivalents (GAE) per g of dried material (Paleologou *et al.*, 2016). Total flavonoid yield was determined using the $AlCl_3$ reagent as described previously (Manousaki *et al.*, 2016) and expressed as mg rutin equivalents (RtE) per g of dried material. Ferric reducing power (P_R) was estimated using the TPTZ methodology (Paleologou *et al.*, 2016). The antiradical activity (A_{AR}) was measured with the DPPH probe. P_R and A_{AR} were expressed as µmol ascorbic acid equivalents (AAE) and µmol DPPH per g of of dried material, respectively.

3.2.7. Qualitative liquid chromatography-diode array-mass spectrometry (LC-DAD-MS)

The equipment used was a Finnigan MAT Spectra System P4000 pump, coupled with a UV6000LP diode array detector and a Finnigan AQA mass spectrometer. A Fortis RP-18 column, 150 × 2.1 mm, 3 µm, was used, at 40 °C. Analyses were carried out as described elsewhere (Paleologou *et al.*, 2016).

3.2.8. Statistical analyses

All extractions were carried out twice and all determinations in triplicate. Values reported are averages. Response surface design and associated statistics were performed with JMP™ 10. Kinetics was estimated by non-linear regression between Y_{TP} and t, using SigmaPlot™ 12.0, at least at a 95% significance level.

3.3. Results and discussion

3.3.1. Extraction optimisation

The objective of the optimisation process was to assess simultaneously the effect of $C_{m\text{-}\beta\text{-}CD}$, $R_{L/S}$ and T on Y_{TP}. To do so, a Box-Behnken experimental design with two central points (points # 15 and 16, **Table 11**) was deployed and the trend in Y_{TP} variation as a function of parallel changes in all three process (independent) variables was recorded in the form of 3D plots (**Fig. 13**). The ANOVA test indicated that all process variables considered ($C_{m\text{-}\beta\text{-}CD}$, $R_{L/S}$ and T) exerted a statistically significant effect on Y_{TP}, but the terms T^2, $C_{m-\beta-CD}^2$,

$R_{L/S} \cdot T$ and $R_{L/S} \cdot C_{m\text{-}\beta\text{-}CD}$ were non-significant. Thus, the model obtained after omitting the non-significant terms was as follows:

$$Y_{TP} \text{ (mg GAE g}^{-1} \text{ dw)} = 103.68 + 3.17C_{m\text{-}\beta\text{-}CD} + 13.31R_{L/S} + 3.26T - 1.53C_{m\text{-}\beta\text{-}CD} \cdot T - 4.01R_{L/S}^2$$

(10)

$$(R^2 = 0.99, \, p < 0.0001)$$

Fig. 13: 3D plots showing the effect of simultaneous variation of $C_{m\text{-}\beta\text{-}CD}$, $R_{L/S}$ & T on the Y_{TP}.
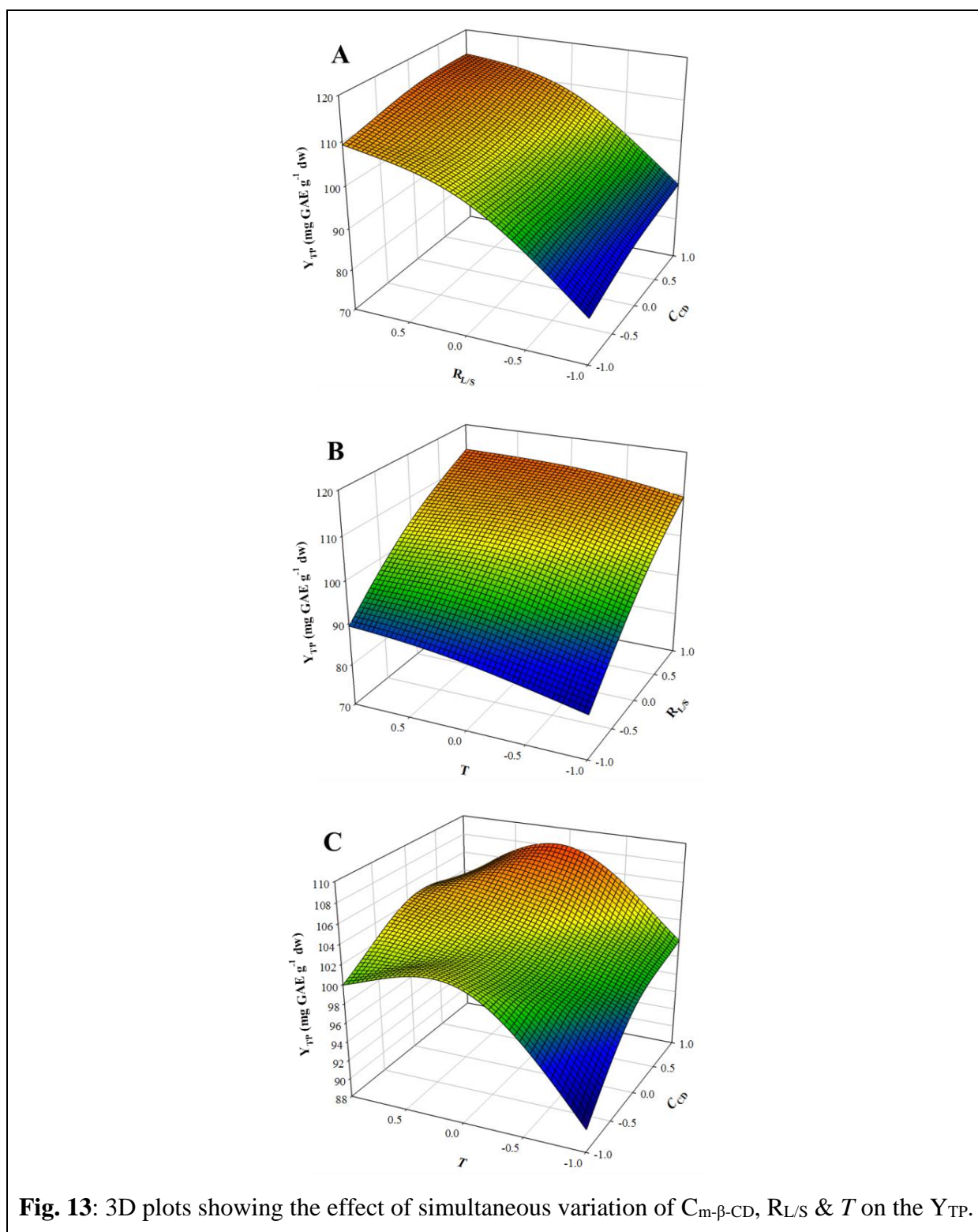
With the use of the desirability function (**Fig. 14**), the recommended settings to achieve the highest Y_{TP} were estimated to be $C_{m-\beta-CD} = 9\%$ (w/v), $R_{L/S} = 40$ mL g^{-1} and $T = 51$ °C. Under these conditions, the predicted Y_{TP} was 116.65 ± 3.60 mg GAE g^{-1} dw.

Sorted Parameter Estimates

Term	Estimate	Std Error	t Ratio		Prob>|t|
R (L/S)	13.309	0.549742	24.21		<.0001*
T	3.259	0.549742	5.93		0.0010*
C (CD)	3.174	0.549742	5.77		0.0012*
R (L/S)*R (L/S)	-4.008793	1.070671	-3.74		0.0096*
C (CD)*T	-1.52625	0.61463	-2.48		0.0476*
T*T	-2.618793	1.070671	-2.45		0.0501
R (L/S)*T	-1.06875	0.61463	-1.74		0.1327
C (CD)*R (L/S)	-0.81625	0.61463	-1.33		0.2324
C (CD)*C (CD)	1.2762069	1.070671	1.19		0.2783
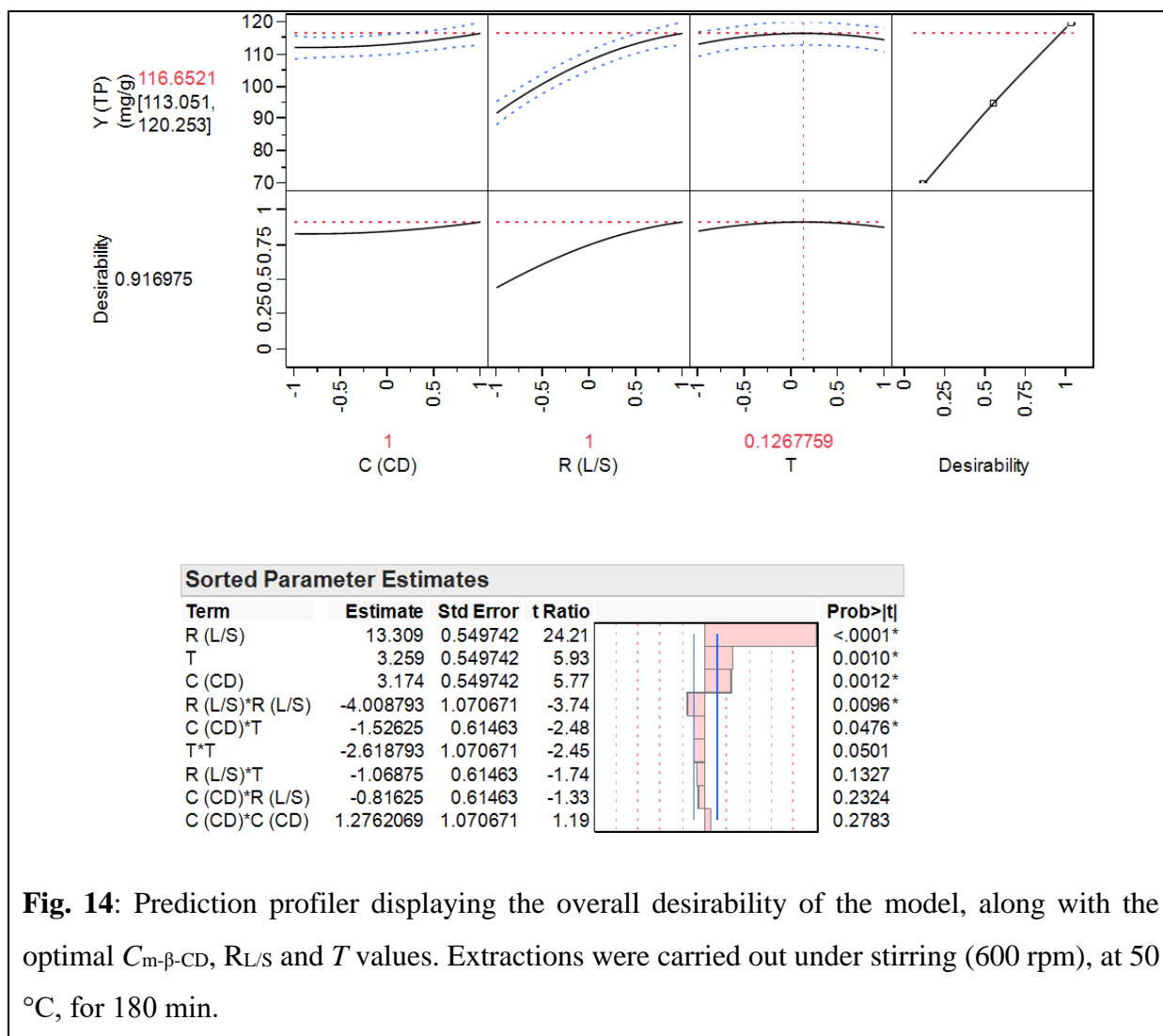
Fig. 14: Prediction profiler displaying the overall desirability of the model, along with the optimal $C_{m-\beta-CD}$, $R_{L/S}$ and T values. Extractions were carried out under stirring (600 rpm), at 50 °C, for 180 min.

The optimal $R_{L/S}$ found was close to 36.2 mL g^{-1} reported for polyphenol extraction from *Satureja thymbra*, using a DES composed of glycerol and sodium acetate (3:1) (Jancheva *et al.,* 2017), and to 33.3 mL g^{-1}, determined as optimal for the extraction of polyphenols from *Carthamus tinctorius* L. using several choline chloride-based DES (Dai *et al.,* 2014). This is a corroboration that, unlike polyphenol extraction with conventional solvents that may display optimal $R_{L/S}$ of 90 - 120 mL g^{-1} (Trasanidou *et al.,* 2016; Vetal *et al.,* 2013), polyphenol extraction with DES may be effectively performed using lower solvent volume. Such a feature is highly desirable, entailing the use of smaller equipment and lower cost. This prospect, so far disregarded, is particularly essential, highlighting another aspect of the use of DES as efficient solvents.

Likewise, the optimal T was 51 °C, suggesting that higher T did not favour polyphenol extraction. This outcome is in absolute accordance with a previous study, which demonstrated

that yield of polyphenol extraction from *Moringa oleifera* leaves with a glycerol-based DES dropped, when T was raised from 50 to 80 °C (Karageorgou *et al.,* 2017). This is also in line with recent data, which showed that yield of polyphenol extraction from red grape pomace with certain DES declined upon switching T from 60 to 80 °C (Patsea *et al.,* 2017). Such a trend was also seen in polyphenol extraction from *Equisetum palustre* L. using choline chloride-based DES (Qi *et al.,* 2015). This particular behaviour could be attributed to the nature of the DES, whose structure depends on the number and the strength of hydrogen bonds between the HBD and the HBA. The increase in extraction temperature may initially favour polyphenol extraction due to higher diffusivity, which stems from a decrease in viscosity (Karakashov *et al.,* 2015). However, further temperature increase may endow the system with energy sufficient to provoke hydrogen bond rupture and thus the extraction efficiency of the DES could be abrogated (Jancheva *et al.,* 2017).

With reference to the effect of $C_{m-\beta-CD}$, the optimal value determined by the model was 9% (w/v), which is fairly close to 7% (w/v) required for polyphenol extraction from olive leaves with aqueous glycerol (Mourtzinos *et al.,* 2016). However, 13% (w/v) 2-hydroxypropyl-β-cyclodextrin was determined as optimal for polyphenol extraction from oak acorn with aqueous glycerol (Kyriakidou *et al.,* 2016). Moreover, recovery of polyphenols from grape pomace was shown to be optimal using 1% (w/v) β-cyclodextrin in either 80% aqueous ethanol or water, whereas recoveries declined at higher β-cyclodextrin concentration (Ratnasooriya and Rupasinghe, 2012). Ligand inclusion in the m-β-CD cavity is a stoichiometric phenomenon and usually only one molecule may be incorporated into the cavity so as to become entrapped. Thus, it would normally be expected increased extraction yield as a response to raising $C_{m-\beta-CD}$, as previously demonstrated for β-cyclodextrin-aided polyphenol extraction (Rajha *et al.,* 2015).

Such a phenomenon may be attributed to the specific polyphenol/m-β-CD interactions. In an aqueous medium the driving force that enables polyphenol-m-β-CD complex formation is the displacement of water molecules outside the m-β-CD cavity and retention of the polyphenol within through apolar-apolar association (Del Valle, 2004). M-β-CD would increase solubility of the less polar polyphenols through entrapment and the eventual result seen could represent the integration of such an effect. On the other hand, polyphenols may behave as HBDs and interact with glycine of the DES, and a sort of antagonism may develop, most probably because m-β-CD might weaken polyphenol-glycine interactions. This could happen because polyphenol/m-β-CD complex formation involves strong hydrogen bond formation (Çelik *et al.,* 2015). Yet, since combinations of DES with m-β-CDs have never been previously reported, such an assumption is to be elucidated by profounder examinations.

3.3.2. Extraction kinetics

To further clarify the effect of the combination of DES/m-β-CD on the polyphenol extraction from OLL, a kinetic investigation was undertaken. The kinetic model implemented (**Fig. 15**) allowed for the determination of certain parameters that permitted a comparative evaluation. It can be seen in **Table 12** that k and h for the extractions carried out with DES and DES/m-β-CD were significantly lower ($p < 0.05$) compared with the corresponding determined for the aqueous ethanol extraction, indicating a much slower extraction rate. By contrast, differences in D_e were not so pronounced, yet extraction with aqueous ethanol displayed the highest value. This outcome was most probably attributed to the higher viscosity of the DES system, which might have resulted in low diffusivity, as observed in studies where viscous solvents, such as aqueous glycerol, were compared with water (Karakashov *et al.,* 2015) or aqueous ethanol (Paleologou *et al.,* 2016; Philippi *et al.,* 2016).
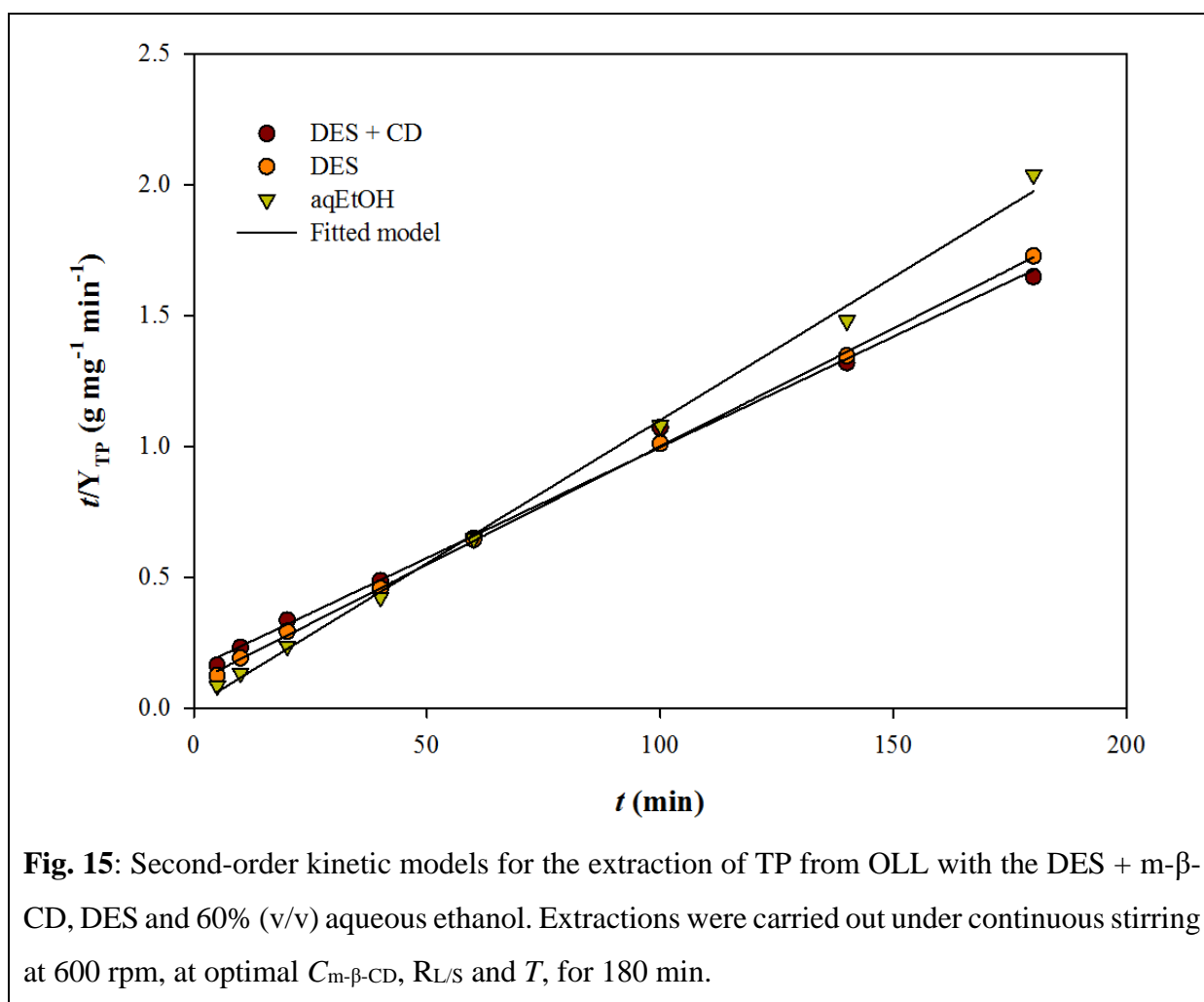
Fig. 15: Second-order kinetic models for the extraction of TP from OLL with the DES + m-β-CD, DES and 60% (v/v) aqueous ethanol. Extractions were carried out under continuous stirring at 600 rpm, at optimal $C_{m-β-CD}$, $R_{L/S}$ and T, for 180 min.

Table 12: Parameters of second-order kinetics, determined for the extraction of TP from OLL, under optimised conditions.

Solvent	Kinetic parameters			
	k ($\times 10^{-3}$)	h	D_e ($\times 10^{-12}$)	$Y_{TP(s)}$
	(g mg^{-1} min^{-1})	(mg g^{-1} min^{-1})	(m s^{-1})	(mg GAE g^{-1})
DES + m-β-CD	0.502	6.82	6.06	116.58
DES	0.867	10.46	5.42	109.93
60% EtOH	3.576	32.82	7.44	95.81

Extraction with the DES/m-β-CD afforded $Y_{TP(s)}$ = 116.58 mg GAE g^{-1} dw, which matched exactly the value predicted by the response surface model. This was a sound confirmation for the model validity. The $Y_{TP(s)}$ attained with DES/m-β-CD was by 5.7% higher than that achieved with the DES and 17.8% higher than that with aqueous ethanol. The difference in $Y_{TP(s)}$ between DES/m-β-CD and DES was not significant ($p > 0.05$), but m-β-CD enhanced significantly $Y_{TP(s)}$ of the DES/m-β-CD compared with the aqueous ethanol ($p < 0.05$). This finding pointed clearly to a boosting of the extraction yield triggered by the addition of the m-β-CD in the DES, evidencing its improved effectiveness. It is to be noted that the use of various cyclodextrins, such as β-cyclodextrin (Rajha *et al.,* 2015; Mantegna *et al.,* 2012) or 2-hydroxypropyl-β-cyclodextrin (Kyriakidou *et al.,* 2016; Mourtzinos *et al.,* 2016) in combination with aqueous solvents resulted in increased extraction yields, suggesting that cyclodextrins can effectively enhance extraction efficiency. However, such an effect is for the first time reported for combination of DES and m-β-CD.

3.3.3. Model evaluation and efficiency testing

To check the applicability of the model established through the response surface methodology, extraction was repeated under optimal conditions and afforded 113.21±3.02 mg GAE g^{-1} dw. This value was virtually equal to the maximum value predicted by the model (116.65±3.60 mg GAE g^{-1} dw). Moreover, to bring out clearly the efficiency of the DES/m-β-CD to extract OLL polyphenols, extractions were carried out with DES and 60% (v/v) aqueous ethanol, under the same conditions, and the extracts were analysed, in addition to Y_{TP}, for Y_{TFn}, A_{AR} and P_R. The results confirmed the outcome of the kinetic study, since the Y_{TP} attained with the DES/m-β-CD was by 5.3 and 19.3% higher than that reached with the DES and 60% (v/v) aqueous ethanol, respectively (**Fig. 16**, upper plot). Additionally, the extract obtained with the DES/m-β-CD displayed 16.0% and 4.5% higher Y_{TFn}, compared with the DES and 60% (v/v) aqueous ethanol, respectively (**Fig. 16**, lower plot).
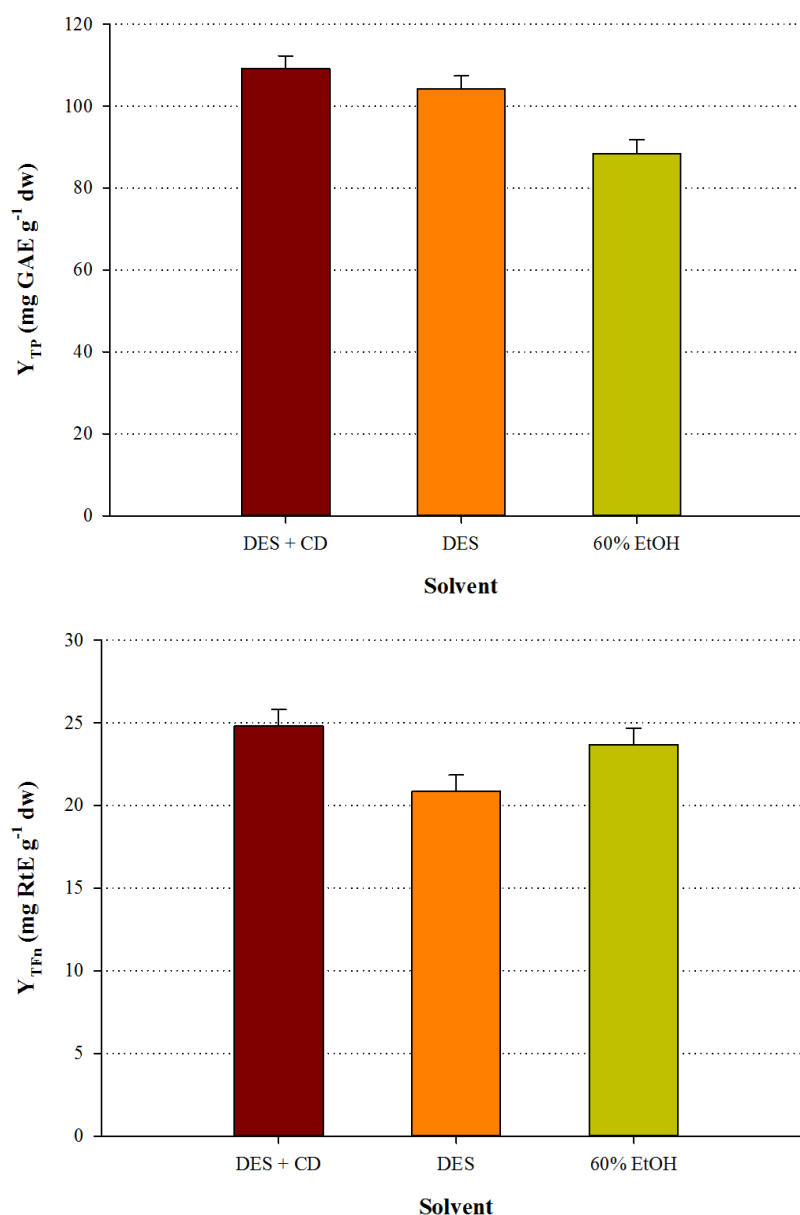
Fig. 16: Comparative diagram for Y_{TP} (upper plot) and Y_{TFn} (lower plot), obtained using DES + m-β-CD, DES and 60% aqueous ethanol (AE). Extractions were carried out under continuous stirring at 600 rpm, at optimal $C_{m-β-CD}$, $R_{L/S}$ and T, for 180 min.

However, the determination of the antioxidant activity demonstrated that the DES/m-β-CD extract was not the most potent in scavenging radicals, having an A_{AR} value that was by 4.3% lower than that seen with the DES extract (**Fig. 17**, upper plot). Although the difference in A_{AR} was not statistically different ($p < 0.05$), both DES/m-β-CD and DES extract exhibited by 40 - 43% higher A_{AR} that the aqueous ethanol extract. Similarly, the DES extract had by 5.4 and 28.5% higher P_R compared with the DES/m-β-CD and aqueous ethanol extract, respectively. These data suggested that m-β-CD did not impact the antioxidant behaviour of the DES extract, yet both DES/m-β-CD and DES extracts possessed considerably stronger antioxidant capacity compared with the aqueous ethanol.
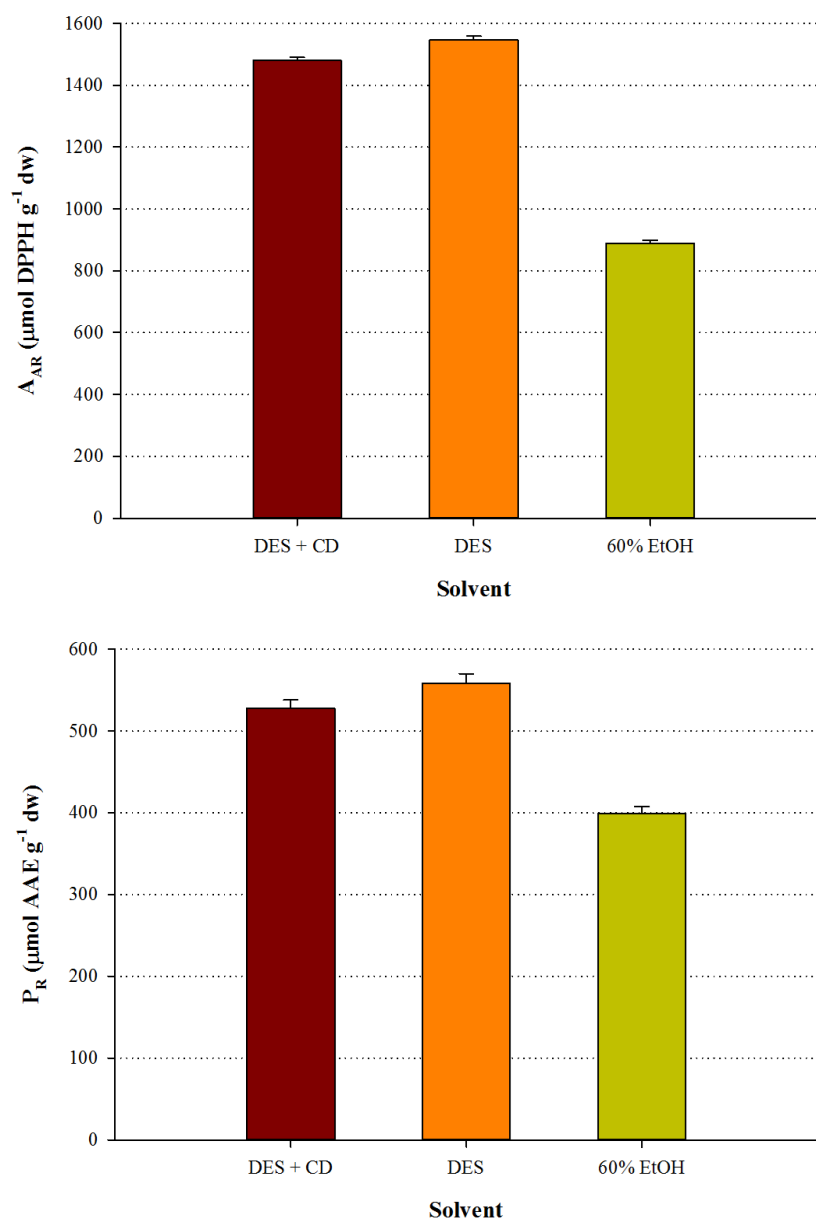
Fig. 17: Comparative diagram for A_{AR} (upper plot) and P_R (lower plot), obtained using DES + m-β-CD, DES and 60% aqueous ethanol (AE). Extractions were carried out under continuous stirring at 600 rpm, at optimal $C_{m-β-CD}$, $R_{L/S}$ and T, for 180 min.

It has been shown that inclusion complexes of 2-hydroxypropyl-β-cyclodextrin with polyphenols such as chlorogenic acid (Shao *et al.,* 2014) and quercetin (Çelik *et al.,* 2015), but also β-cyclodextrin complexes with rosmarinic acid (Medronho *et al.,* 2014), quercetin and rutin (Alvarez-Parrilla *et al.,* 2005) displayed more powerful antioxidant activity compared with the non-encapsulated polyphenols. This phenomenon has also been demonstrated for chlorogenate-rich coffee extracts (Budryn *et al.,* 2014). Nevertheless, the inclusion complex cyclodextrin/polyphenol is better stabilized with molecules having higher hydrophobicity (Hădărugă *et al.,* 2012). Therefore, not all polyphenols present in the DES extract would form equally stable complexes and this might compromise the antioxidant capacity.

Another factor that could contribute towards such an effect is probably the orientation of the encapsulated molecule in the m-β-CD cavity. For OLL, it has been demonstrated that complex of β-cyclodextrin with oleuropein, the major OLL polyphenol, involves deep insertion of the dihydroxyphenethyl moiety inside the cavity from its secondary side (Mourtzinos *et al.,* 2007). Such a fact would pose constrains with regard to oxidant/antioxidant interactions arising from steric effects, thus resulting in reduced antioxidant activity. In fact, in a more recent study, an increase from 7 to 13% (w/v) in 2-hydroxypropyl-β-cyclodextrin concentration for OLL polyphenol extraction with aqueous glycerol did not result in significantly higher A_{AR}, but rather a slight decline was observed (Mourtzinos *et al.,* 2016). This was probably the reason why the DES/m-β-CD extract did not express more powerful antioxidant properties. Therefore, the antioxidant effects observed in DES/m-β-CD extracts may not simply reflect the higher polyphenol concentration, but interactions associated with polyphenol/m-β-CD complexes as well. However, such a hypothesis remains to be elucidated.

3.3.4. Extract characterisation

Characterisation was performed by analysing the DES/m-β-CD and DES extracts with LC-DAD-MS, to investigate whether m-β-CD acted selectively towards particular OLL polyphenols. To this purpose, major constituents previously detected in OLL extracts (Athanasiadis *et al.,* 2017) were tentatively identified and chosen as indicators (**Table 13**). These substances were two luteolin glucosides (peaks #1 and 4), an apigenin rutinoside (peak #3) and the secoiridoid derivative oleuropein (peak #2). As can be seen in **Fig. 18**, the chromatographic profiles of DES/m-β-CD and DES extracts were virtually identical, which clearly showed that the DES containing m-β-CD did not selectively extract any specific phytochemical. This finding indicated that m-β-CD acted merely as an extraction booster and did not affect the composition of the extract.

Table 13: UV-vis and mass spectral data for the major polyphenolic phytochemicals tentatively identified in OLL extracts obtained under optimal conditions, using DES/m-β-CD and DES.

Peak	Rt (min)	[M+H]$^+$ (*m/z*)	Other ions (*m/z*)	Tentative identity
1	25.85	449	287	Luteolin glucoside
2	26.46	541	563[M + Na]$^+$, 361, 137	Oleuropein
3	27.14	579	601[M + Na]$^+$, 271	Apigenin rutinoside
4	27.96	449	471[M + Na]$^+$, 418, 287	Luteolin glucoside

Fig. 18: Total ion chromatograms showing the principal polyphenols detected in the extracts obtained with DES + m-β-CD (upper figure) and DES (lower figure).

3.4. Conclusions

In this study, it was demonstrated for the first time that m-β-CD can be very effectively combined with a DES, boosting polyphenol extraction from OLL. The investigations performed showed that Y_{TP} and Y_{TFn} achieved with DES/m-β-CD were significantly higher compared with those attained with aqueous ethanol. In addition, the DES/m-β-CD extract possessed improved antioxidant properties, and this might not be simply attributed to the higher polyphenol

concentration, as interactions with m-β-CD could have an important contribution. LC-DAD-MS analysis revealed that DES/m-β-CD and DES extracts had identical polyphenolic profile, suggesting that m-β-CD posed no issue of selectivity, but it acted solely towards boosting the extraction yield. Contrary to that, the aqueous ethanolic extract had poorer composition, revealing the effectiveness of the DES/m-β-CD as an extraction medium. Currently, work is in progress to ascertain the validity of the model proposed in scale-up procedures, which is anticipated to further illustrate its applicability for industrial use.

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Chapter 4:

Stability effects of methyl-β-cyclodextrin on *Olea europaea* leaf extracts in a natural deep eutectic solvent

Abstract

Polyphenol-containing extracts from olive (*Olea europaea*) leaves (OLL) were obtained using a glycerol-based deep eutectic solvent (DES) and a combination of DES with methyl-β-cyclodextrin (m-β-CD). The extracts were stored at various temperatures for a period of 20 days and the reducing power (P_R) was monitored to trace changes in the antioxidant potency of the extracts. Over the examination period and at every temperature tested, P_R displayed a constant decline, which followed pseudo zero-order kinetics. The determination of the decay constants indicated that the presence of m-β-CD acted protectively, slowing down the progression of the P_R decline. Examination of the polyphenolic profiles using liquid chromatography-diode array-mass spectrometry showed that after storage for 20 days at 50 °C, some major polyphenols occurring in OLL suffered extended degradation. The formation of a yellow pigment in the extracts stored in DES but not in aqueous ethanol suggested that polyphenol oxidation did occur during storage. It was concluded that the oxidation of some OLL components was rather responsible for the P_R decline observed.

Keywords: Antioxidants; Deep eutectic solvents; Methyl-β-cyclodextrin; *Olea europaea*; Polyphenols

4.1. Introduction

Olive leaves (OLL) are olive oil production residues, generated during olive tree pruning and in the early steps of olive cleaning. They are considered an important food industry by-product, because they bear a high load of polyphenolic substances, which have been shown to possess a versatile pharmacological potency (Obied *et al.,* 2012). This is the major reason for the development of a broad variety of methodologies of solid-liquid extraction, aiming at enhanced recovery of polyphenolic phytochemicals from OLL. These methodologies embrace primarily environmentally benign solvents, such as subcritical water and water/ethanol mixtures, but also eco-friendly effective technologies, such as microwave heating and ultrasonication (Roselló-Soto *et al.,* 2015).

The development and increasing use of the new generation solvents, called deep eutectic solvents (DES) or low-transition temperature mixtures (LTTMs), has shifted research on the use of that kind of media for purposes of natural product extraction. The emerging scientific data provide solid evidence for the superiority of DES in processes pertaining to polyphenol recovery, compared with water and hydroethanolic solutions (Bakirtzi *et al.,* 2016), opening new routes for the implementation of even more effective extraction procedures. In this frame, a recently designed novel DES, composed of glycerol and glycine, was shown to have significantly higher performance in the extraction of OLL polyphenols (Athanasiadis *et al.,* 2017a), as compared with aqueous ethanol. Following investigations revealed that incorporation of methyl-β-cyclodextrin (m-β-CD) into the DES may act as an extraction booster, giving even higher extraction yield (Athanasiadis *et al.,* 2017b).

However, apart from the increasing demand for natural antioxidants in the food, cosmetics and pharmaceutical industries, which has led to the search for natural extracts, strategies with which to increase long-term storage stability of the extracts are also required. Stability in the extraction medium over time is an important issue associated with polyphenol extraction, considering that many polyphenols are inherently unstable molecules, owed to their susceptibility to oxidation. Therefore, the examination of their stability under specific conditions is imminent and requires detailed examination. On such a ground, this examination was undertaken to investigate the stability of OLL polyphenols in extracts generated using the above mentioned extraction medium, composed of the DES and m-β-CD. Stability in the presence and absence of m-β-CD was assessed by monitoring the reducing power (P_R) of the extracts over 20 days at various temperatures, and polyphenol transformations that might be linked with changes in P_R were identified using liquid chromatography-diode array-mass spectroscopy.

4.2. Materials and methods

4.2.1. Chemicals

Solvents used for liquid chromatography were HPLC grade. Methyl-β-cyclodextrin, glycerol (99%) and ethanol (99.8%) were from Acros Organics (Geel, Belgium). Anhydrous sodium carbonate was from Carlo Erba Reactifs (Val de Reuil, France). Glycine was from NeoLab Migge Laborbedarf-Vertiebs (Heildelberg, Germany). Ascorbic acid and ferric chloride hexahydrate were from Fluka (Steinheim, Germany). 2,4,6-Tripyridyl-*s*-triazine (TPTZ) was from Aldrich (Steinheim, Germany).

4.2.2. Preparation of the DES

The DES used was synthesised according to the optimised conditions described previously (Athanasiadis *et al.,* 2017a). Briefly, glycerol (HBD) was mixed with an appropriate amount of glycine (HBA) and water to give a molar ratio HBD:HBA:water of 7:1:3, and the mixture was mildly heated under stirring until the formation of a transparent liquid. Aqueous solution 80% (w/v) of this DES was used for the extractions and stability tests.

4.2.3. Plant material

Dried and pulverised *Olea europaea* leaves (OLL) from Agrielia Kalamon variety, with average particle diameter of 0.5 mm, were used for all examinations performed. Details concerning collection and handling of the plant material have been analytically given elsewhere (Athanasiadis *et al.,* 2017a).

4.2.4. Batch extraction procedure and sample handling

Polyphenol extraction from OLL was carried out using the optimized methodology previously developed (Athanasiadis *et al.,* 2017b). Amount of 2.5 g of dried plant material was mixed with 100 mL of 80% (w/v) aqueous DES containing 9% (w/v) methyl-β-cyclodextrin (m-β-CD), to give a liquid-to-solid ratio of 32 mL g^{-1}. Extractions were performed at 70 °C, under continuous stirring at 600 rpm, for 280 min. Extractions in the absence of m-β-CD were also repeated, under identical conditions. After the completion of each extraction, samples were centrifuged in a table centrifugator (Hermle, Wehingen, Germany) at 10,000 × *g* for 10 min, and the clear extract was used for stability tests and analyses.

4.2.5. Stability test and determinations

The clear extracts were divided into aliquots of 30 mL, placed in screw-cap glass vials and stored in a refrigerator (4 °C), in a temperature-controlled dark chamber (22 °C) and in a thermostated water bath (50 °C). Sampling was randomly carried out to eliminate variance, at

regular intervals, within a period of 20 days. Reducing power (P_R) was estimated with the TPTZ assay and expressed as μmoL AAE per g dry weight (Blidi *et al.,* 2015).

4.2.6. Qualitative liquid chromatography-diode array-mass spectrometry (LC-DAD-MS)

A Finnigan MAT Spectra System P4000 pump was used, coupled with a UV6000LP diode array detector and a Finnigan AQA mass spectrometer. A Fortis RP-18 column, 150 × 2.1 mm, 3 μm, was used, at 40 °C. The analytical methodology implemented was reported elsewhere (Blidi *et al.,* 2015).

4.2.7. Statistics

Extractions and stability tests were repeated at least twice and all analyses were performed in triplicate. Values reported are means. Linear regressions were established at least at a 95% significance level. For all statistics, Microsoft Excel™ 2010 and SigmaPlot™ 10 were used.

4.3. Results and discussion

4.3.1. Kinetics of P_R evolution and the effect of m-β-CD

In order to assess extract stability at various temperatures, as well as to clarify the role of m-β-CD, P_R of the OLL extracts was monitored over a period of 20 days. P_R was chosen as a safe criterion to track changes because it is tightly associated with polyphenol structure (Pulido *et al.,* 2000) and it has been demonstrated to reflect changes related with redox phenomena (Sioumis *et al.,* 2005). In particular, P_R has been shown to be inversely correlated with polyphenol oxidation in white wines, strong evidence that P_R decline may stem from polyphenol loss or structure alteration as a result of oxidation. During the examination period, it was ascertained that P_R exhibited a declining trend, which could be very effectively described as pseudo zero-order kinetics:

$$P_R = P_R^0 - kt \qquad (11)$$

Where P_R is the reducing power, P_R^0 the initial reducing power, k the pseudo zero-order decay rate constant (μmoL AAE g^{-1} days^{-1}), and t time (days). The decay constants (k) were calculated graphically from the slope of the regression lines, after plotting P_R against t.

As can be seen in **Fig. 19**, kinetics at 4 °C showed that the presence of m-β-CD apparently accelerated P_R decline. From the data given in **Table 14**, it was illustrated that the pseudo-zero order decay constant for the P_R in the OLL extract obtained with DES/m-β-CD, at 4 °C, was higher than those determined for the extracts stored either in DES or in 60% aqueous ethanol. By contrast, P_R exhibited higher stability in DES than in 60% aqueous ethanol. However, at

both 22 and 50 °C, the extracts containing m-β-CD displayed lower k. For the extract containing m-β-CD, k was increased by almost 12% when storage temperature was increased from 4 to 50 °C, whereas the increase in the extracts lacking m-β-CD was approximately 46% and in aqueous ethanolic extracts 43%. This outcome highlighted the role of m-β-CD in P_R stability.
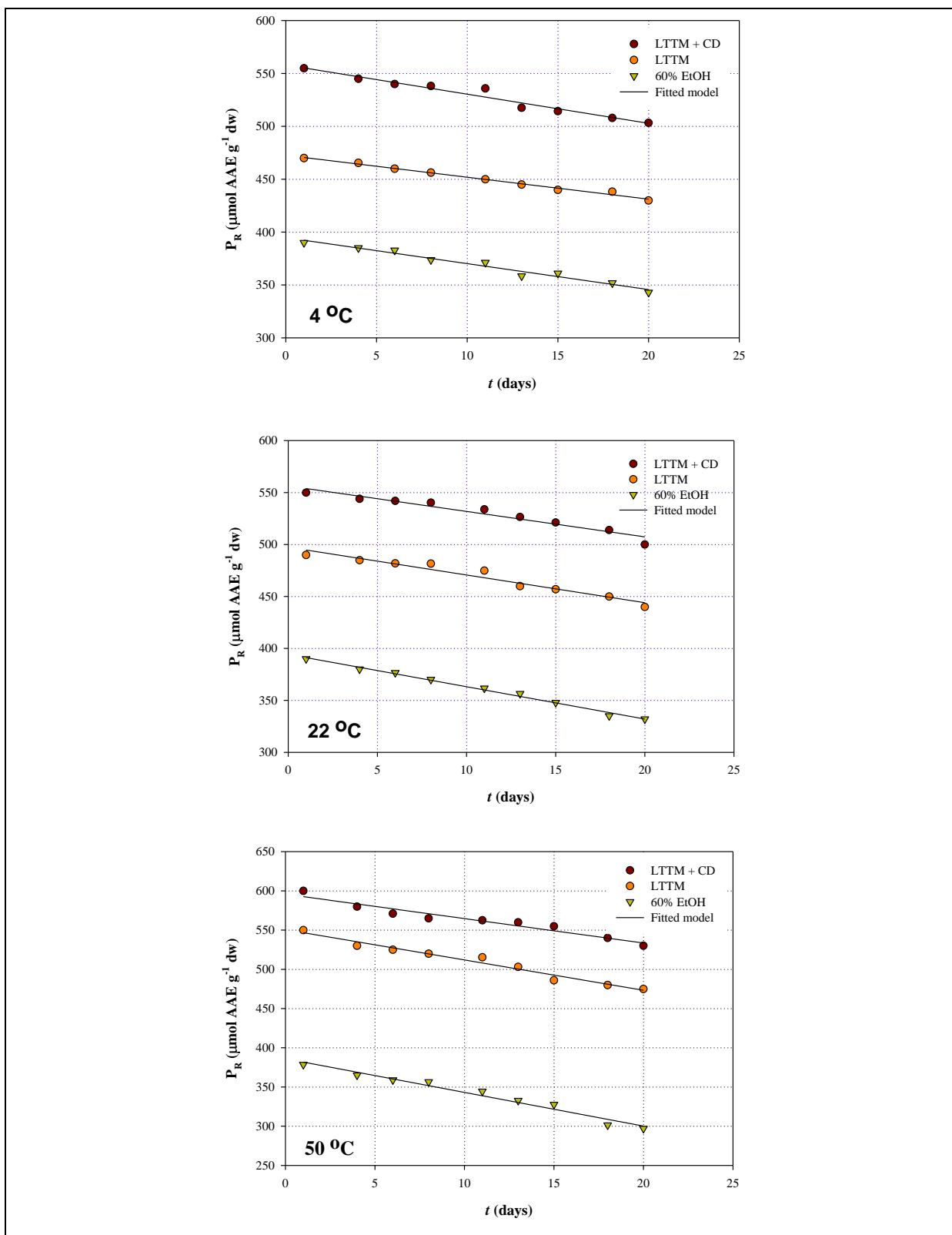
Fig. 19: Zero-order kinetics of P_R decline in OLL extracts at 4, 22 and 50 °C. Extracts were obtained using either DES/m-β-CD, only DES or 60% aqueous ethanol.

Table 14: Zero-order constant determined for P_R decay during storage of OLL extracts in the solvents tested.

Solvent	k (µmoL AAE g^{-1} days^{-1})		
	4 °C	22 °C	50 °C
DES + m-β-CD	2.75	2.45	3.11
DES	2.07	2.67	3.86
60% EtOH	2.45	3.11	4.29

Polyphenol stability in DES is an issue currently unexamined and the data available are too limited to draw safe conclusions. In a recent investigation, it was demonstrated that green tea polyphenols displayed higher stability in a DES than in conventional solvents, such as aqueous methanol and aqueous ethanol (Jeong *et al.,* 2017). The exact mechanism for this effect could not be known, yet it could be assumed that extended hydrogen bonding between polyphenols and the HBA could confer some sort of stability. Polyphenols may develop this kind of interactions, since they may behave as HBDs (Bi *et al.,* 2013). Hydrogen bonding may also be developed between a polyphenol and cyclodextrins, and in this regard the presence of m-β-CD might be critical. Polyphenols such as flavonoid glycosides but also flavonoid aglycones have been shown to form complexes with various types of cyclodextrins, including β-cyclodextrin (β-CD) and 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) (Jullian *et al.,* 2007; Alvarez-Parrilla *et al.,* 2005). The formation of flavonoid/HP-β-CD complexes contributed in higher stability of the encapsulated molecule, as opposed to the free (non-encapsulated) one (Nguyen *et al.,* 2013), a fact attributed to the protective effect of HP-β-CD cavity (Liu *et al.,* 2006). Similar conclusion regarding stability was reached by studies on polyphenol-containing extracts (Kalogeropoulos *et al.,* 2010; Mourtzinos *et al.,* 2008).

A hypothesis relating polyphenol stability with antioxidant activity has been proposed by examinations on rosmaric acid inclusion complexes by various cyclodextrins (Çelik *et al.,* 2011). A polyphenol radical, deriving from a reaction with another radical species, once engulfed in m-β-CD hydrophobic cavity may be better stabilised through resonance, by intramolecular hydrogen bonding. Thus the redox potential between the aroxyl radical and the reduced polyphenol may be lowered, rendering the polyphenol higher antioxidant potency. In fact, several studies have demonstrated that polyphenols such as quercetin and rutin (Alvarez-Parrilla *et al.,* 2005), rosmarinic acid (Medronho *et al.,* 2014; Çelik *et al.,* 2011), chlorogenic acid (Shao *et al.,* 2014), and quercetin and glycosides thereof (Çelik *et al.,* 2015), encapsulated in cyclodextrins may perform as more powerful antioxidants, compared with the non-encapsulated molecules. Therefore, the slower P_R decay recorded for OLL extracts at 22 and

50 °C in the presence of m-β-CD might be ascribed to higher polyphenol stability, as a result of effective inclusion. On the other hand, the higher decay rate found for the extract stored in DES/m-β-CD at 4 °C would appear rather paradox and further studies are required to identify the phenomena implicated to yield this outcome.

4.3.2. Modifications in the polyphenolic profile

To clarify whether alterations in the polyphenolic profile accounted for the changes in P_R observed, LC-DAD-MS investigation was undertaken for the samples stored at 50 °C, which displayed the most pronounced decline. In the trace recorded at 280 nm for the initial extract obtained with the DES/m-β-CD (**Fig. 20**, upper chromatogram), eight polyphenolic substances could be tentatively identified (**Table 15**). The extract obtained only with the DES showed identical profile (data not shown), confirming previous results that stressed the importance of m-β-CD merely as an extraction booster (Athanasiadis *et al.,* 2017b). On the basis of published data (Apostolakis *et al.,* 2014; Taamalli *et al.,* 2012), peak #1 was identified as hydroxytyrosol, peak #1b as tyrosol, peaks #5 and #8 as oleuropein and an isomer thereof, and peaks #2, 3, 6 and 7 as flavone glycosides. Furthermore, a flavonol glycoside was also detected (peak #4). When the extracts were analysed after 20 days of storage at 50 °C, some alterations in the polyphenolic profile were evident, the most prominent being an increase in hydroxytyrosol (peak #1), which pointed to oleuropein hydrolysis, and the drastic decrease in apigenin rutinoside (peak #6). Nevertheless, this decrease was not accompanied by the appearance of the apigenin aglycone; this fact raised suspicions for apigenin oxidative degradation.

Table 15: Mass spectral data for the major polyphenolic phytochemicals tentatively identified in OLL extracts obtained under optimal conditions, using DES/m-β-CD and DES.

Peak	Rt (min)	$[M+H]^+$ (*m/z*)	Other ions (*m/z*)	Tentative identity
1	6.33	153	-	Hydroxytyrosol
1b	8.72	137	-	Tyrosol
2	16.57	611	449, 287	Luteolin di-glucoside
3	18.90	449	287	Luteolin glucoside
4	19.28	611	633, 465, 303	Rutin (quercetin 3-*O*-rutinoside)
5	20.00	541	563, 379, 361	Oleuropein
6	21.01	579	433, 271	Apigenin rutinoside
7	21.88	449	471, 287	Luteolin glucoside
8	21.97	541	563, 379	Oleuropein isomer
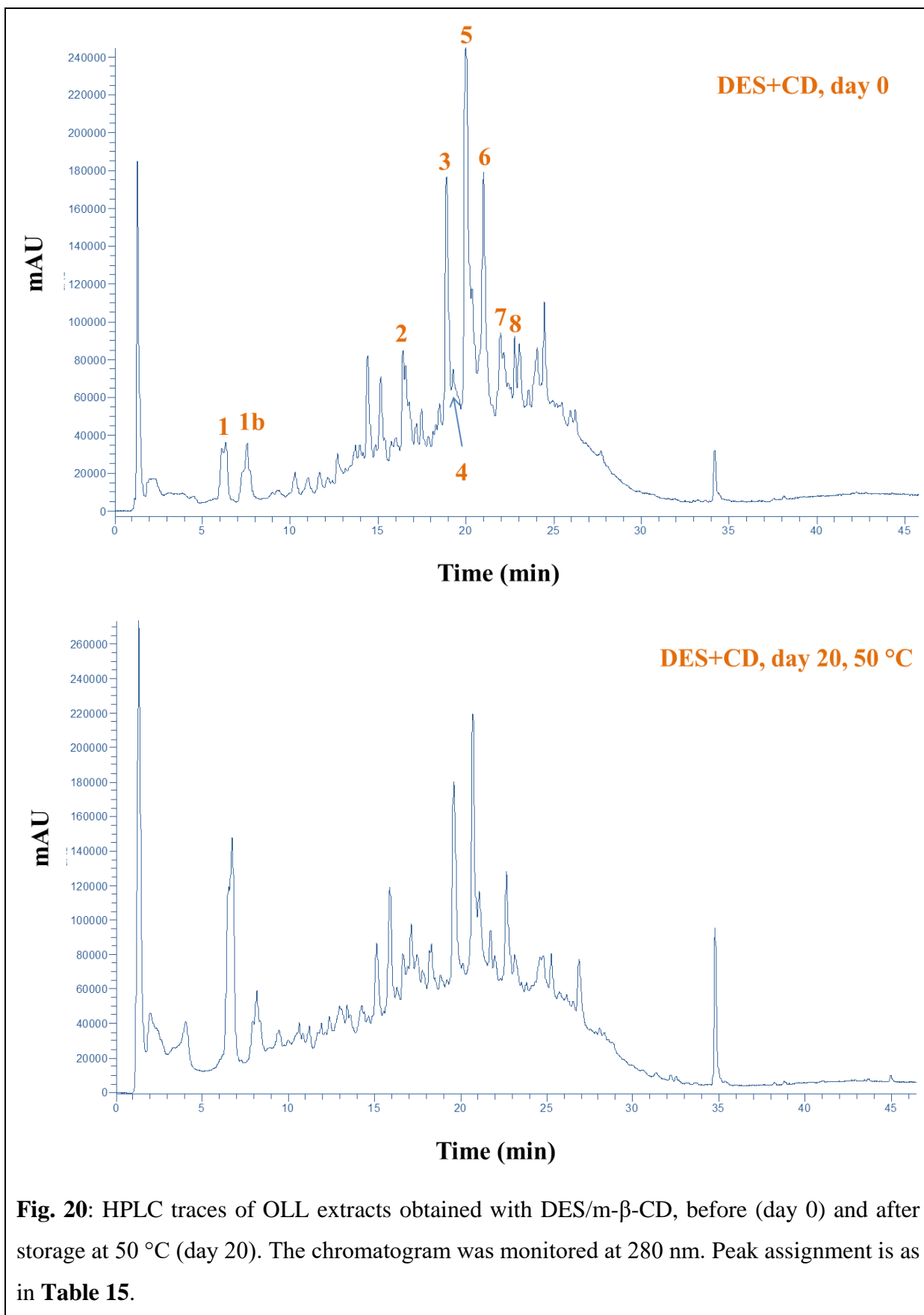
Fig. 20: HPLC traces of OLL extracts obtained with DES/m-β-CD, before (day 0) and after storage at 50 °C (day 20). The chromatogram was monitored at 280 nm. Peak assignment is as in **Table 15**.

An important finding was the detection of a product identified only in the extracts stored in DES/m-β-CD or DES, but not in those stored in 60% ethanol (**Fig. 21**, lower chromatogram).
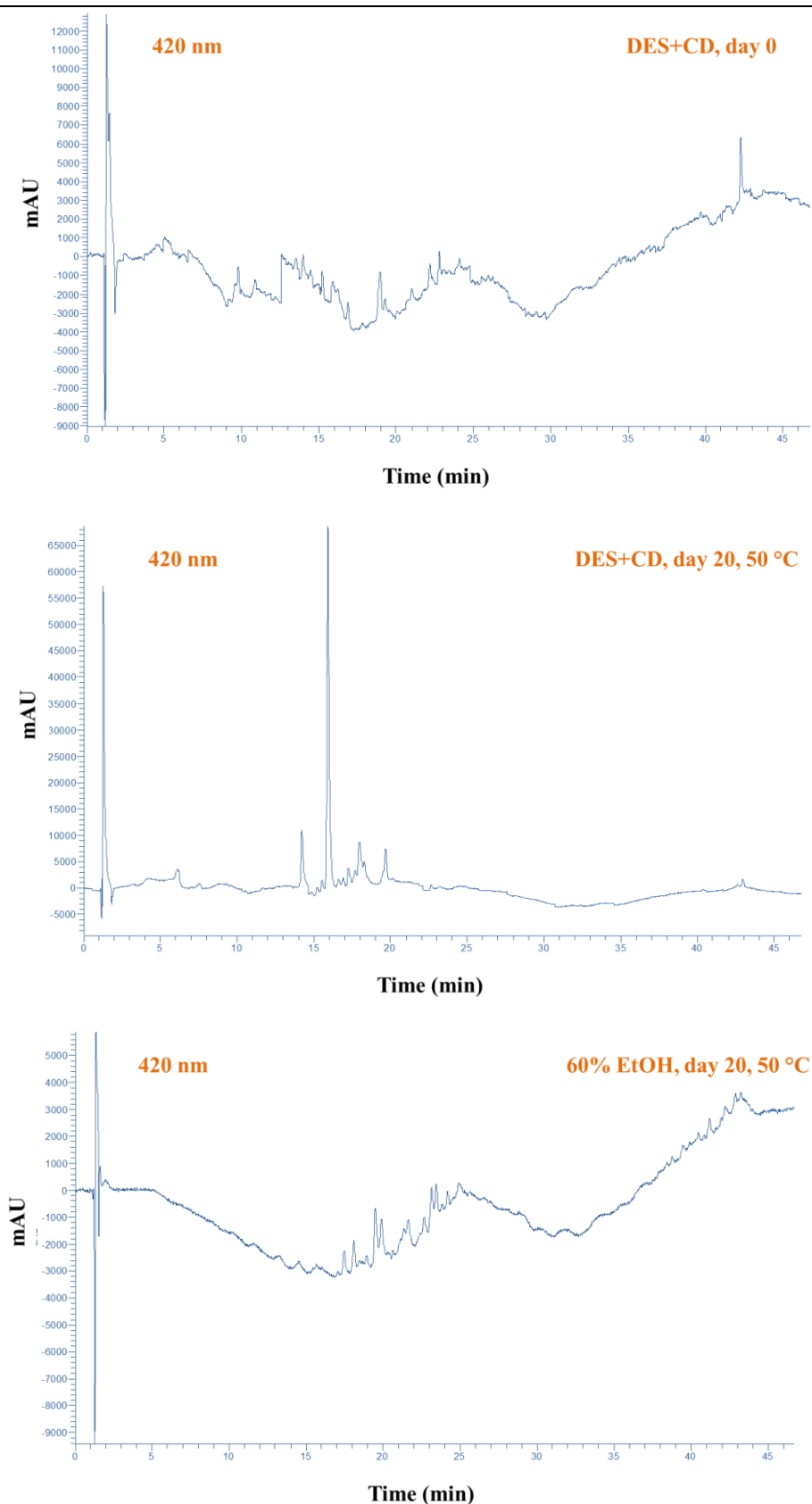
Fig. 21: HPLC traces at 420 nm, of OLL extracts obtained with DES/m-β-CD, before (day 0) and after storage at 50 °C (day 20) (upper and middle chromatograms, respectively). Lower chromatogram displays the trace of OLL extracts stored for 20 days at 50 °C, in 60% aqueous ethanol).

This fact strongly emphasised the role of DES in its formation. The substance displayed λmax at 270 and 424 nm, which indicated that it was a yellow pigment (**Fig. 22**). Liquid chromatography-mass spectrometry examination showed a pseudo-molecular ion at *m/z* 503 amu and diagnostic fragments at *m/z* 439 amu $[M - 64]^+$, *m/z* 309 amu $[M - 194]^+$ and *m/z* 249 amu $[M - 254]^+$ (**Fig. 23**).

Fig. 22: UV-vis spectrum of the compound detected in OLL extracts stored in DES/m-β-CD for 20 days, at 50 °C.

Fig. 23: Mass spectrum and putative fragmentation pattern of the yellow pigment detected in OLL extracts stored in DES/m-β-CD for 20 days, at 50 °C.
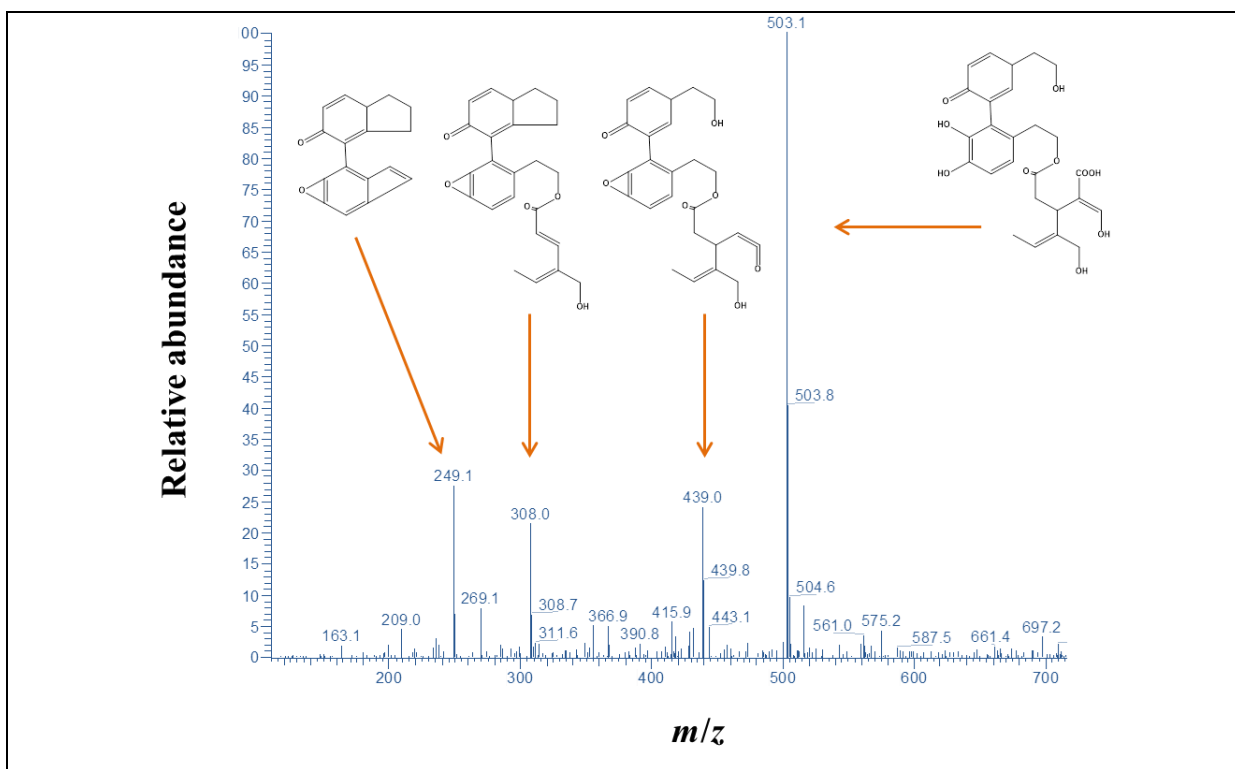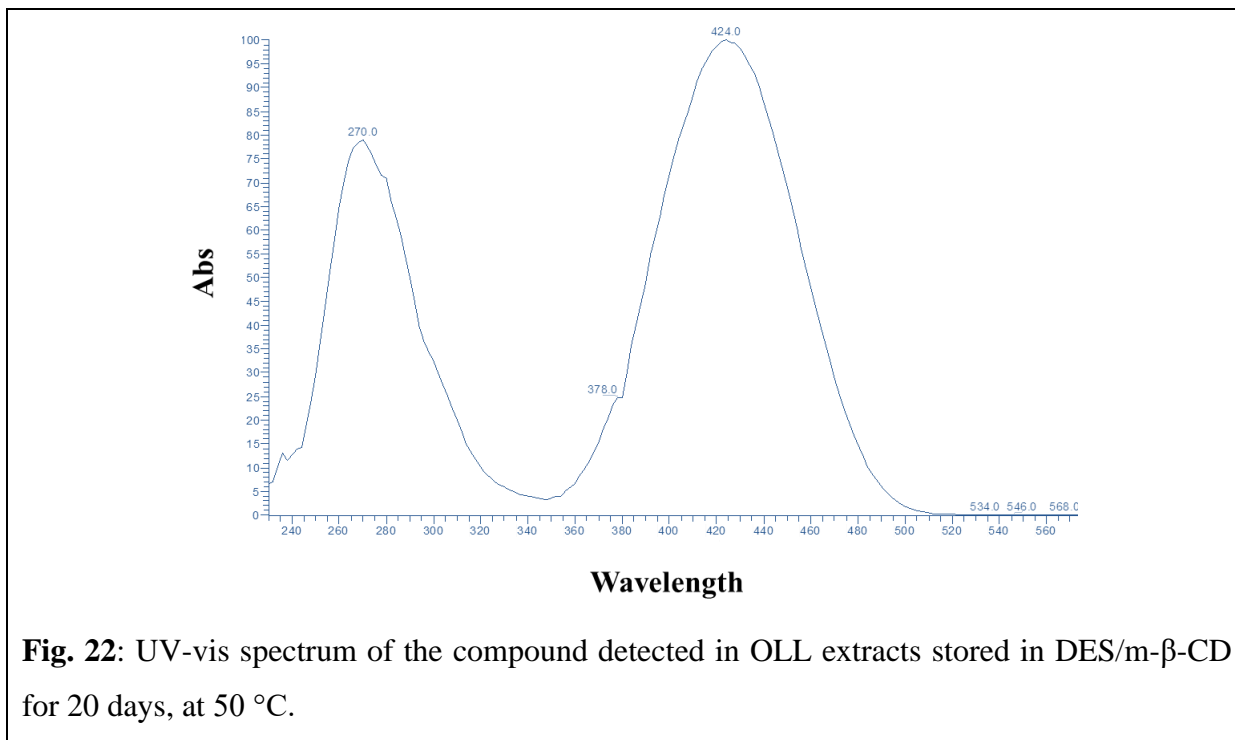
The fragment with *m/z* 439 could derive from dehydration and decarboxylation of the parent molecule, while further dehydration and fragmentation would yield the ion with *m/z* 308 amu, which in turn would provide the ion with *m/z* 249 amu. On the basis of these data, the product was identified as adduct of oleuropein aglycone with tyrosol quinone. A plausible pathway for the formation of such a product would embrace first oleuropein hydrolysis leading to the formation of the aglycone. One-electron oxidation of the hydroxytyrosyl moiety would give the semi-quinone derivative, which after nucleophilic attack by tyrosol, rearrangement and further oxidation, would yield the putatively proposed product (**Fig. 24**). This substance could possess yellow pigment-like UV features, as observed for other oxidised simple phenolics, such as chlorogenic acid (Murata *et al., 2002*). Oxidation of oleuropein itself has also been shown to yield a product absorbing at 439 nm (Tzika *et al., 2008*). On the other hand, Fenton-type oxidation would be rather precluded, because oleuropein oxidation under similar conditions was shown to give non-pigment products of higher molecular weight (Antolovich *et al., 2004*).

Fig. 24: Putative formation pathway of the yellow pigment detected in OLL extracts stored in DES/m-β-CD for 20 days, at 50 °C.
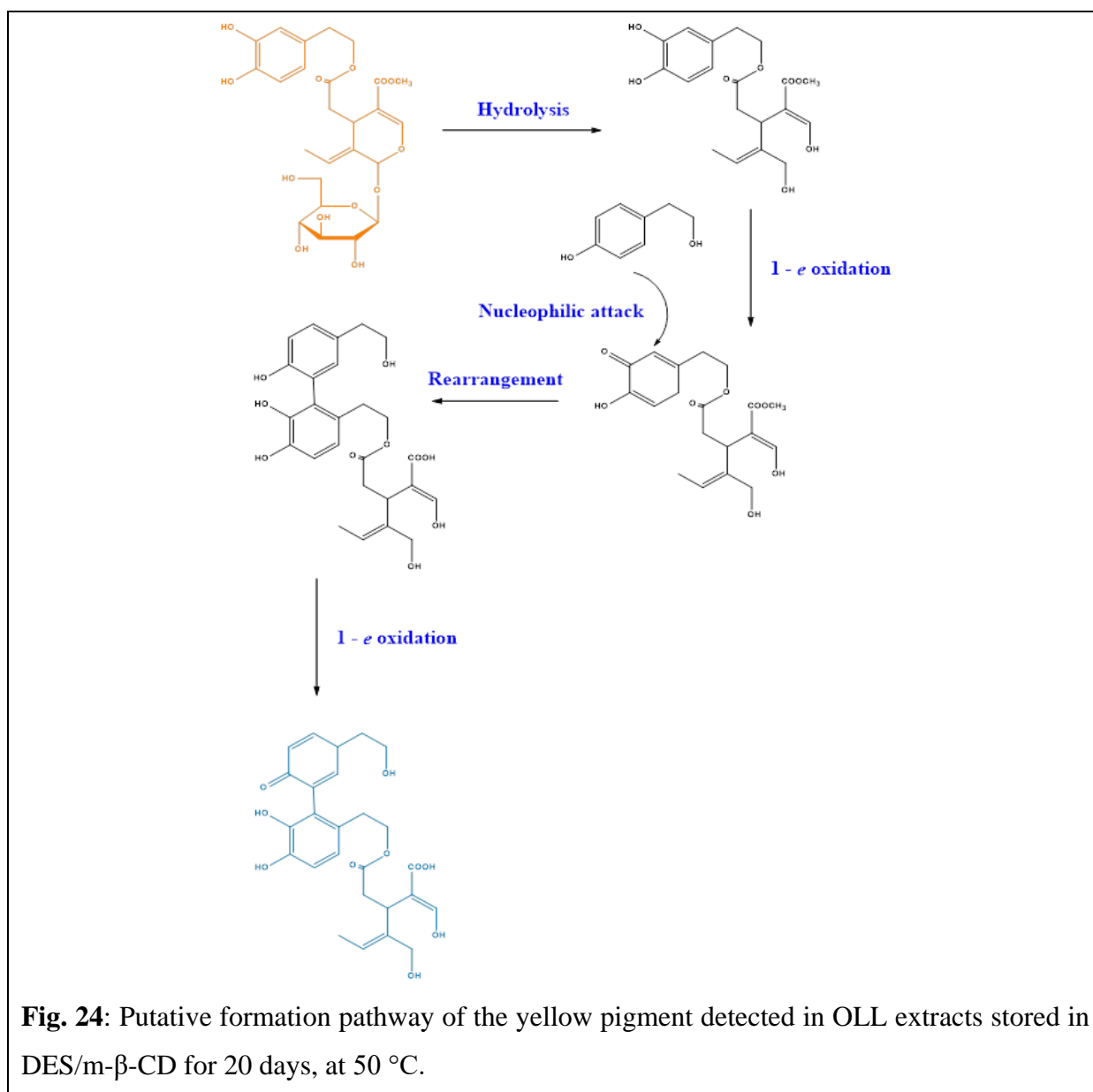
The evidence emerged from the putative structure of this compound suggested that some OLL constituents indeed underwent oxidation and this was the most probable cause of P_R decline recorded during storage of the OLL extracts. Previous investigations on white wines demonstrated that browning, that is, the formation of yellow pigments, had a statistically significant correlation with a decrease in P_R (Sioumis *et al.,* 2005). A following detailed kinetic investigation confirmed that browning development was indeed associated with a concomitant decline in P_R (Sioumis *et al.,* 2006), which was correlated with epicatechin, the major antioxidant constituent. Thus the disappearance of certain OLL polyphenols due to oxidation would be very likely to bring about a drop in the reducing potency of the extracts.

4.4. Conclusions

DES are novel solvents of variable composition and for this reason, they may present a broad spectrum of properties, whose characterisation is a matter of case experimentation. An issue of high importance in the extraction of antioxidant polyphenols using DES is their stability, since many polyphenols may readily undergo reactions, such as hydrolysis and oxidation. OLL extracts, obtained with either DES/m-β-CD or only DES were shown to suffer alterations regarding their polyphenolic profile, when stored for a period of 20 days at 50 °C. These alterations were mainly characterised by degradation of some glycosides and the formation of a yellow pigments as a result of polyphenol oxidation. It was therefore postulated that the decline in P_R seen in the stored OLL extracts could be ascribed to the oxidation of some OLL constituents. However, the kinetic study demonstrated that the incorporation of m-β-CD in the extraction solvent may result in notable retardation of the P_R decay. This finding highlighted the importance of such additives in stabilising polyphenol-containing extracts in DES. Further studies are required to clarify the exact mechanism of protection, which could lead in more efficient utilisation of m-β-CD in formulations related with food, pharmaceutical and cosmetic products.

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Chapter 5:

Effect of methyl-β-cyclodextrin on radical scavenging kinetics of olive leaf extracts and interactions with ascorbic acid

Abstract

Olive leaf (OLL) extracts contain a high load of antioxidant polyphenols, with significant pharmacological potency. In this study, the use of a novel natural deep eutectic solvent enabled the effective extraction of OLL polyphenols and their testing as radical scavengers, in the presence or absence of methyl-β-cyclodextrin (m-β-CD), using descriptive kinetics. Testing extended to include interactions with ascorbic acid, a natural powerful antioxidant, by implementing response surface methodology. The kinetic study showed that m-β-CD may hinder the radical scavenging effect of OLL extracts, yielding lower stoichiometry upon reaction with the radical probe DPPH. The extension of the reaction time to determine the total stoichiometry confirmed this effect. As a further concurrence, the interactions of OLL extracts with ascorbic acid showed lower radical scavenging performance in the presence of m-β-CD. These results were discussed on the ground of the role that m-β-CD may play in similar systems.

Keywords: Antioxidants; Deep eutectic solvents; Methyl-β-cyclodextrin; Olive leaf extracts; Polyphenols

5.1. Introduction

In recent years, there is a growing demand for natural antioxidants as replacers of synthetic ones, but also as functional additives that could provide biological systems with protection against harmful free radicals. Plant-derived antioxidant polyphenols are becoming increasingly important in this respect, as numerous of these substances have been shown to possess a very high capacity to quench free radicals (Moon and Shibamoto, 2009). This has stimulated a broad spectrum of studies regarding the use of plant extracts as rich sources of natural antioxidants. In such a frame, olive leaves (OLL), which represent a major waste generated during the production of olive oil, have gained a great deal of interest because they may bear an important load of polyphenolic phytochemicals, which may display significant pharmacological potential (Obied *et al.,* 2012).

Although the antioxidant activity may me effectively estimated in plant extracts with several tests developed for such a purpose (López-Alarcón and Denicola, 2013), a few studies have investigated in details the rate of antiradical reactions, which might represent the rate at which the antioxidants react with free radicals. Reaction kinetics information complements that of antiradical activity and may be of value for characterizing a potential antioxidant source. 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH) is widely used for quickly assessing the ability of antioxidants to transfer labile H atoms to radicals, based on the theory that a hydrogen donor is an antioxidant. This reaction is stoichiometric with respect to the number of hydrogen atoms absorbed. Therefore, the antioxidant effect can be easily evaluated by following the decrease of UV absorption at 515 nm (Goupy *et al.,* 2003).

On this theoretical background, this examination was carried out to assess the antiradical behaviour of OLL extracts, obtained using a novel methodology that involved extraction with a combination of a deep eutectic solvent (DES) with methyl-β-cyclodextrin (m-β-CD) as an extraction booster (Athanasiadis *et al.,* 2017b). OLL extracts were also generated without m-β-CD, to evaluate the effect of m-β-CD on the antiradical potency of the extracts. The investigations included a kinetic assay and also interactions with ascorbic acid, after implementing a response surface methodology.

5.2. Materials and methods

5.2.1. Chemicals

Anhydrous sodium carbonate was from Carlo Erba Reactifs (Val de Reuil, France). Methyl-β-cyclodextrin was from Acros Organics (Geel, Belgium). Folin-Ciocalteu reagent was from Fluka (Steinheim, Germany). Glycerol (99%), 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic

acid and ascorbic acid were from Sigma-Aldrich (Steinheim, Germany). Glycine (99.5%) was from NeoLab Migge Laborbedarf-Vertiebs (Heildelberg, Germany).

5.2.2. Preparation of the DES

The DES used was synthesised according to the optimised conditions described previously (Athanasiadis *et al.,* 2017a). Briefly, glycerol (HBD) was mixed with an appropriate amount of glycine (HBA) and water to give a molar ratio HBD:HBA:water of 7:1:3, and the mixture was mildly heated under stirring until the formation of a transparent liquid. Aqueous solution 80% (w/v) of this DES was used for the extractions.

5.2.3. Plant material

The material used for all extractions was dried *Olea europaea* leave (OLL) powder, with average particle diameter of 0.5 mm. Details concerning variety, methodology of leaf collection and handling of the plant material have been analytically given elsewhere (Athanasiadis *et al.,* 2017a).

5.2.4. Batch extraction procedure and sample handling

Polyphenol-containing extracts were prepared from OLL by implementing the optimized methodology previously developed (Athanasiadis *et al.,* 2017b). Briefly, amount of 2.5 g of dried OLL was mixed with 100 mL of 80% (w/v) aqueous DES containing 9% (w/v) methyl-β-cyclodextrin (m-β-CD) and extractions were carried out at 70 °C, under continuous stirring at 600 rpm, for 280 min. Extractions without m-β-CD were also performed, under identical conditions. Samples were centrifuged in a table centrifugator (Hermle, Wehingen, Germany) at $10,000 \times g$ for 10 min, and the clear extract was used for all assays.

5.2.5. Total polyphenol determination

Total polyphenols were determined with the Folin-Ciocalteu reagent, following a previously published protocol (Makris *et al.,* 2016). Results were expressed as mg gallic acid equivalents per L of extract, using a gallic acid calibration curve (30 - 600 mg L^{-1}).

5.2.6. Kinetic assay

The ability of the extracts to transfer H-atoms to DPPH was assessed by measuring changes in the absorbance at 515 nm (A_{515}). Typically, 0.975 mL of freshly prepared DPPH solution in methanol (100 μM), was mixed in a spectrometer cell with 0.025 mL of OLL extract. The decay in A_{515} was monitored over a period of 2 min to determine rate constant (k_1). The assay was extended up to 21 min for the determination of total stoichiometries (n_t).

5.2.7. Interaction with ascorbic acid

The methodology implemented aimed at investigating the interactions of OLL extracts with ascorbic acid and clarify the effect of m-β-CD. Thus, the two independent variables chosen were the total polyphenol concentration of the extracts (C_{TP}, mg GAE L^{-1}), termed as X_1, and the ascorbic acid concentration (C_{AA}, mg L^{-1}), termed as X_2. A central composite experimental design was used with two central points and both independent variables were coded between -1 (lower limit) and +1 (upper limit), using the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i}, \, i = 1, 2 \tag{12}$$

Terms x_i and X_i represent the dimensionless and the actual value of the independent variable i, respectively. X_0 is the actual value of the independent variable i at the central point of the design, and ΔX_i the step change of X_i, which corresponds to a unit change of the dimensionless value (**Table 16**). The choice for the range of values for both variables was based on preliminary runs, but also published information (Karvela *et al.,* 2012). The response considered was the antiradical activity (A_{AR}). ANOVA was performed to estimate model significance, the significance for each polynomial coefficient, and determine the overall coefficient R^2 for the mathematical model. Statistically non-significant dependent terms ($p > 0.05$) were removed from the equations, which were visualised in the form of 3D plots. The models were validated by performing experiments under the predicted optimal conditions, and comparing for each model the predicted values with the actual (measured) ones. For each design point, measured and predicted response values were recorded (**Table 17**). For each design point, C_{TP} and C_{AA} were fixed as dictated by the experimental design. A_{AR} (μmoL DPPH g^{-1} dry OLL weight) was determined as described elsewhere (Shehata *et al.,* 2015).

Table 16: Actual values and coded levels of the independent variables used for the experimental design.

Independent variables	Code units	Coded variable level		
		-1	**0**	**1**
C_{TP} (mg L^{-1})	X_1	10	40	70
C_{AA} (mg L^{-1})	X_2	10	40	70

Table 17: Measured and predicted A_{AR} values of OLL extract and AA mixtures, determined for individual design points.

Design point	Independent variables		Response (A_{AR}, µmoL DPPH g^{-1} dw)			
			No m-β-CD		m-β-CD	
	X_1	X_2	Measured	Predicted	Measured	Predicted
1	-1	-1	91.04	90.58	82.13	82.06
2	-1	1	22.85	19.14	18.87	15.04
3	1	-1	166.02	168.98	140.08	143.39
4	1	1	96.88	96.59	82.68	82.23
5	-1	0	35.50	39.68	31.81	35.71
6	1	0	120.27	117.60	102.84	99.98
7	0	-1	141.99	139.50	124.61	121.37
8	0	1	63.58	67.58	53.00	57.28
9	0	0	89.11	88.36	78.18	76.49
10	0	0	89.11	88.36	75.83	76.49

5.2.8. Statistics

Curve-fittings of the absorbance *vs* time were carried out by non-linear regression. Kinetic model was obtained by performing linear regreassion. All analyses were carried out at least at a 95% significance level, using SigmaPlot™ 12. The experimental design for the response surface methodology and all associated statistics was accomplished with JMP™ 10.

5.3. Results and discussion

5.3.1. Reaction stoichiometries and the effect of m-β-CD

The H-transfer reactions from polyphenols to DPPH can be very effectively assessed by monitoring the decay of A_{515}, using as molar absorptivity $\varepsilon = 11,240$ M^{-1} cm^{-1} and considering the purity of the reagent. The decay in A_{515} is initiated following addition of the antioxidant(s) to the DPPH solution (Brand-Williams *et al.,* 1995) and potent antioxidants may provoke a rapid decay over 1-2 min, as a result of the transfer of H-atoms of the antioxidant that possess low C-H bond dissociation enthalpies (fast step). This step is followed by a much slower decline in A_{515}, which corresponds to the donation by the antioxidant(s) the residual H-atoms (slow step) (Alluis and Dangles, 2001; Dangles *et al.,* 1999).

A simple hypothesis considers that an antioxidant AH bears n independent antioxidant subunits, which may all transfer a single H atom to DPPH with the same second-order rate constant k (Goupy *et al.,* 2003). Such a background can be described as follows:

$$A = \varepsilon \, [DPPH] \tag{13}$$

$$R = -\frac{d}{dt} \, [AH] = -\frac{d}{dt} \, [DPPH] = k \, [AH][DPPH] \tag{14}$$

As mentioned above, the initial (fast) step of the reaction actually represents donation of the most readily abstracted H-atoms from the antioxidant. Hence, the initial reaction rate R_0 could be given as:

$$R_0 = k_1 c c_0 \tag{15}$$

Where c is the initial antioxidant concentration, c_0 the initial DPPH concentration and k_1 the reaction rate constant of the first abstracted H-atom. Therefore, k would be $\frac{k_1}{n}$. Based on Beer - Lambert's law, the [DPPH] that reacts with the first H-atom may be represented as $A_0 - A_f$, where A_0 and A_f correspond to the initial and final A_{515}. Thus by replacing [DPPH] with $A_0 - A_f$, the eq. (14) can be transformed after integration, as follows:

$$ln\left(\frac{1 - \frac{A_f}{A}}{1 - \frac{A_f}{A_0}}\right) = -\frac{k_1 \, c}{\frac{A_0}{A_f} - 1} \tag{16}$$

The slope of the straight line obtained after plotting $ln\left(\frac{1 - \frac{A_f}{A}}{1 - \frac{A_f}{A_0}}\right)$ as a function of time t, equals k_1.

On such a theoretical basis, OLL extracts obtained with or without m-β-CD were assayed with the aim to clarifying the role of m-β-CD on the antiradical effects exerted by OLL polyphenols. To this purpose, the extracts generated were adjusted at a final C_{TP} of 0.1 g L^{-1} and reaction with DPPH was monitored up to 2 min (**Fig. 25**, upper plot). Determination of k_1 was performed by tracing the second order kinetics (**Fig. 25**, lower plot) and gave values of 1.925 and 2.221 $M^{-1} s^{-1}$, for the extract obtained with DES/m-β-CD and DES, respectively. The slower reaction rate of the extract obtained with DES/m-β-CD compared with that obtained only with DES could not be interpreted as weaker antiradical activity, but only as a measure of the radical scavenging rate. This is because several polyphenolic antioxidants were shown to respond differently in kinetic and stoichiometric assays based on reaction with DPPH (Villano *et al.,* 2007).
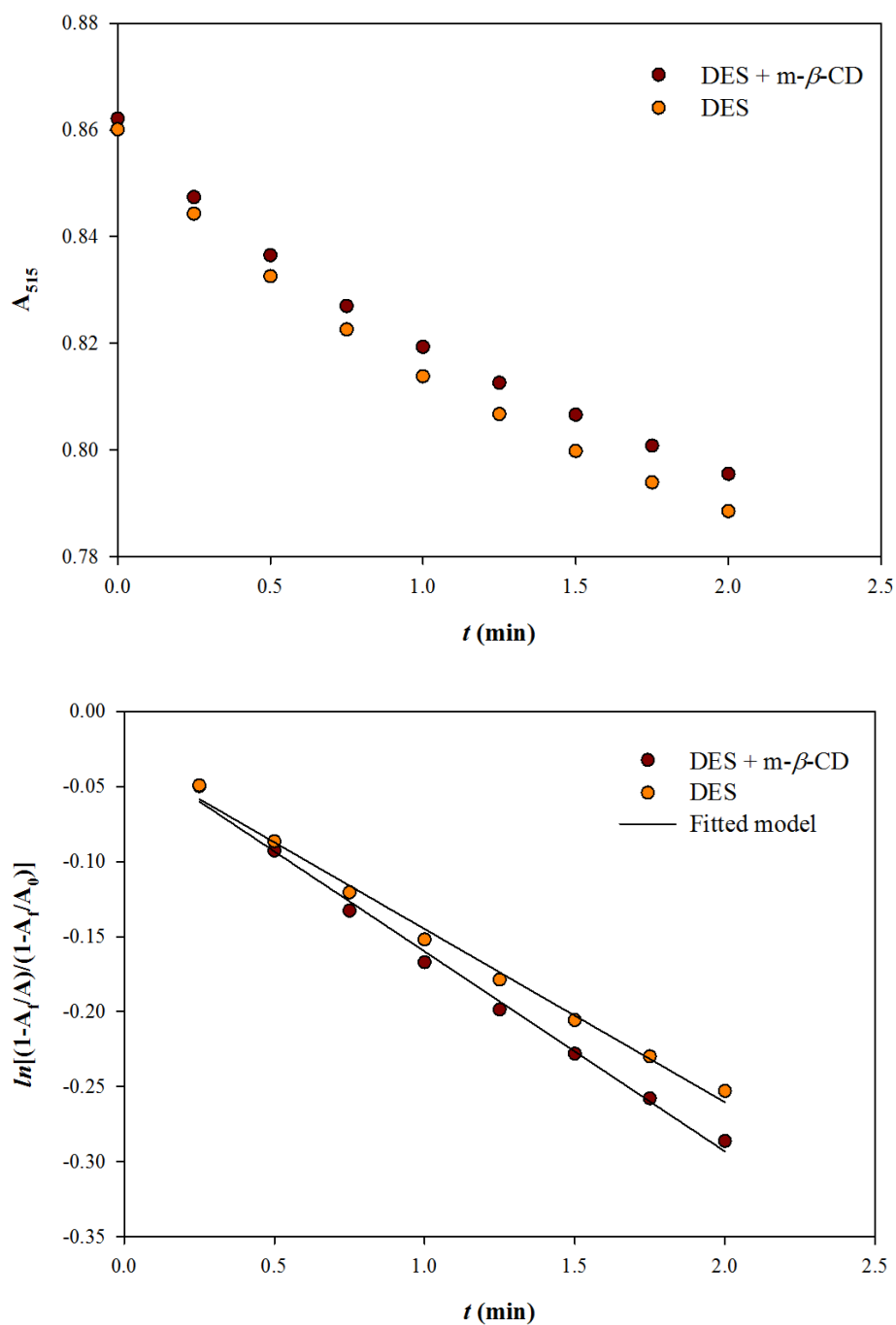
Fig. 25: Fast reaction kinetics recorded upon mixing OLL extract with DPPH. C_{TP} was adjusted to 0.1 g L^{-1}. c$_0$ was 100 µmoL L^{-1}.

Thus in order to have a more integrated picture, the total stoichiometries (n_t) were also determined by extending the reaction of each extract with DPPH, up to 21 min (**Fig. 26**), using the following equation:

$$n_t = \frac{A_0 - A_f}{\varepsilon \, C_{TP}}$$

(17)

Where C_{TP} is the total polyphenol concentration of the extracts, which as mentioned above was adjusted to 0.1 g GAE L^{-1}. Determination of n_t for the DES/m-β-CD and DES extracts gave corresponding values of 1.05×10^{-4} and 1.92×10^{-4} moL g^{-1}, indicating higher stoichiometry for the extract in the absence of m-β-CD.
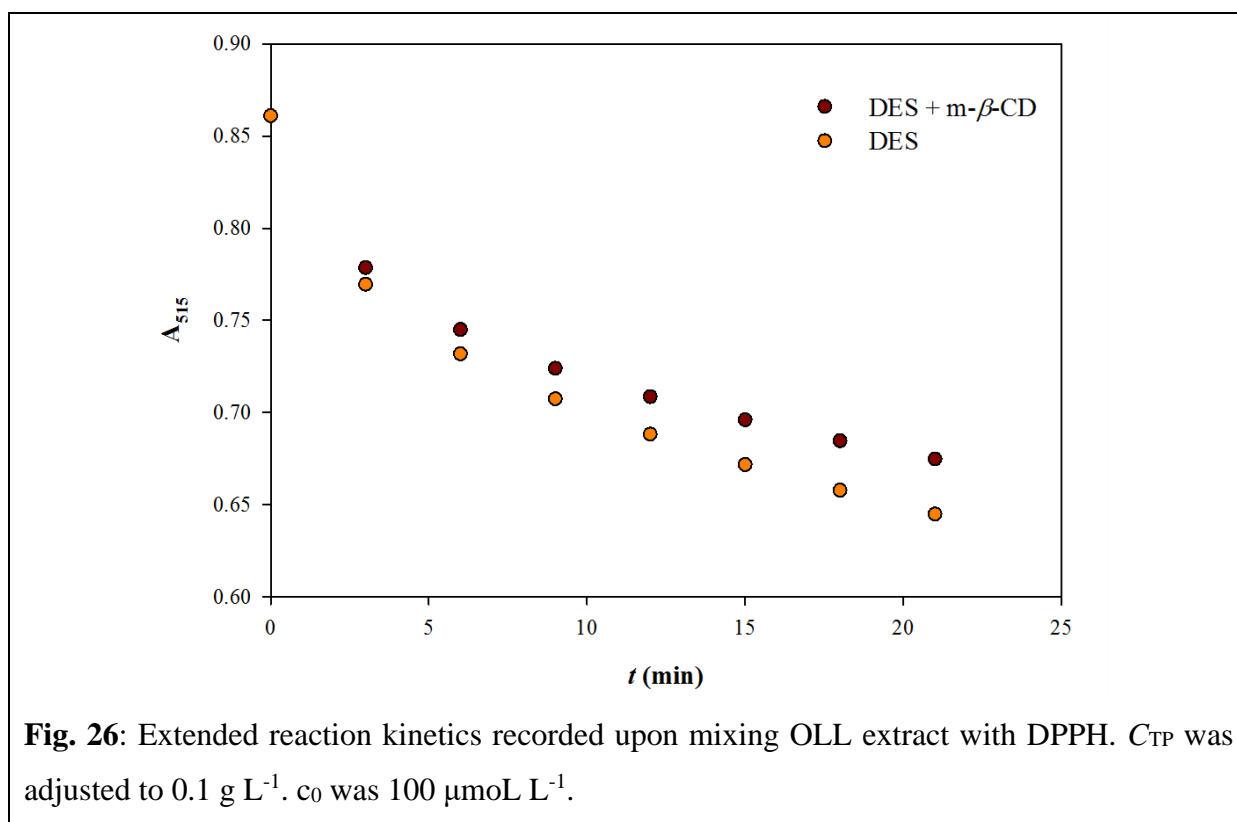
Fig. 26: Extended reaction kinetics recorded upon mixing OLL extract with DPPH. C_{TP} was adjusted to 0.1 g L^{-1}. c$_0$ was 100 µmoL L^{-1}.

Considering both k_1 and n_t, it could be argued that the extract obtained only with DES displayed superior radical scavenging potency. This finding contrasted previous ones, which demonstrated that polyphenol-containing extracts obtained with the aid of various cyclodextrins, such as *Melissa officinalis* leaf extract (Mourtzinos *et al.,* 2011) and pomegranate fruit extract (Diamanti *et al.,* 2017) exhibited increased antiradical activity. Likewise, simple phenolics such as rosmarinic acid (Medronho *et al.,* 2014), chlorogenic acid (Shao *et al.,* 2014) and *trans*-resveratrol (Lu *et al.,* 2009), and quercetin and glycosides thereof (Çelik *et al.,* 2015), showed improved antioxidant properties when they were encapsulated in cyclodextrins. However, a detailed study on inclusion complexes of tea catechins, suggested that the nature of the polyphenol, as well as the orientation of the encapsulated molecule inside the cyclodextrin cavity may affect antioxidant potency either negatively or positively (Folch-Cano *et al.,* 2010).

Hydrophobicity would be an issue in this regard, because cyclodextrin/polyphenol inclusion complexes may be better stabilized with molecules having higher hydrophobicity (Mourtzinos *et al.,* 2007). On the other hand, hydrogen bonding could also greatly affect the antioxidant behaviour of the complexed polyphenols, because if there is extended intermolecular hydrogen bond development between the encapsulated and the host molecule,

then radical scavenging is abrogated (Snelgrove *et al.,* 2001). Such a claim was made for the apparent null effect of hydroxypropyl-β-CD on caffeic acid antioxidant potency (García-Padial *et al.,* 2013), where intramolecular hydrogen bond between the hydroxyl groups of the *o*-diphenol moiety would not allow for intermolecular interactions. On the basis of the above concepts, it could be supported that there might be a slower reaction for the OLL extract with DPPH in the presence of m-β-CD. This phenomenon might be ascribed to the inclusion of OLL polyphenols inside the m-β-CD cavity, which could slow down H-atom transfer to DPPH due to steric effects. Such a hypothesis would be concurred by the fact that complexation of oleuropein, the most abundant polyphenolic antioxidant in OLL, most probably involves deep insertion of the dihydroxyphenethyl moiety inside the cavity from its secondary side, as demonstrated for OLL interactions with β-cyclodextrin (β-CD) (Mourtzinos *et al.,* 2007). The formation of similar inclusion complexes with of β-CD has also been shown for chlorogenic acid (Álvarez-Parrilla *et al.,* 2010).

5.3.2. Interactions with ascorbic acid

In an earlier study, interactions of polyphenol-containing extract with ascorbic acid (AA) were very effectively examined using response surface methodology (Karvela *et al.,* 2012). It was proposed that by combining fixed amounts of AA and total polyphenols is a rather unilateral approach, providing limited information, whereas the simultaneous variation of concentrations within predetermined ranges may be more illustrative of the kind of interactions. This is because it has been demonstrated that the relevant amounts of AA and polyphenols in a mixture may significantly affect the overall antioxidant effect (Choueiri *et al.,* 2012).

On this ground, a response surface design was deployed to evaluate interactions between OLL extract and AA. Evaluation of term contribution by performing ANOVA showed that C_{TP} and C_{AA} and their quadratic terms exerted statistically significant effects on the A_{AR} of the mixtures. However, cross terms were non-significant in this regard ($p > 0.05$) and thus they were omitted from the models (mathematical equations), which are given in their final form in **Table 18**. The use of the desirability function (**Fig. 27**) enabled the determination of the settings recommended to achieve A_{AR} maximisation. Under these C_{TP} and C_{AA} combinations, maximum A_{AR} was estimated to be 168.98±10.43 and 143.39±11.18 μmoL DPPH g^{-1} dw, for the extracts obtained with DES/m-β-CD and DES, respectively. As can be seen in **Fig. 28**, the interaction pattern with AA was identical, but the difference of 15% in performance was a further confirmation that the OLL extract could act as a better radical scavenger in the absence of m-β-CD.

Table 18: Polynomial equations and statistical parameters describing the effect of the independent variables on the response (A_{AR}) for all OLL/AA mixtures tested.

Antioxidant test	2nd order polynomial equations	R^2	p
Without m-β-CD	$88.36 + 38.96C_{TP} - 35.96C_{AA} - 9.72C_{TP}^2 + 15.18C_{AA}^2$	1.00	< 0.0001
With m-β-CD	$76.49 + 32.13C_{TP} - 32.04C_{AA} - 8.64C_{TP}^2 + 12.84C_{AA}^2$	0.99	0.0002

Fig. 27: Prediction profiler displaying the overall desirability of the model developed, after implementing response surface methodology.

Fig. 28: 3D plots illustrating the effect of simultaneous variation of C_{TP} and C_{AA} on the A_{AR} in the absence (left plot) and presence (right plot) of m-β-CD.
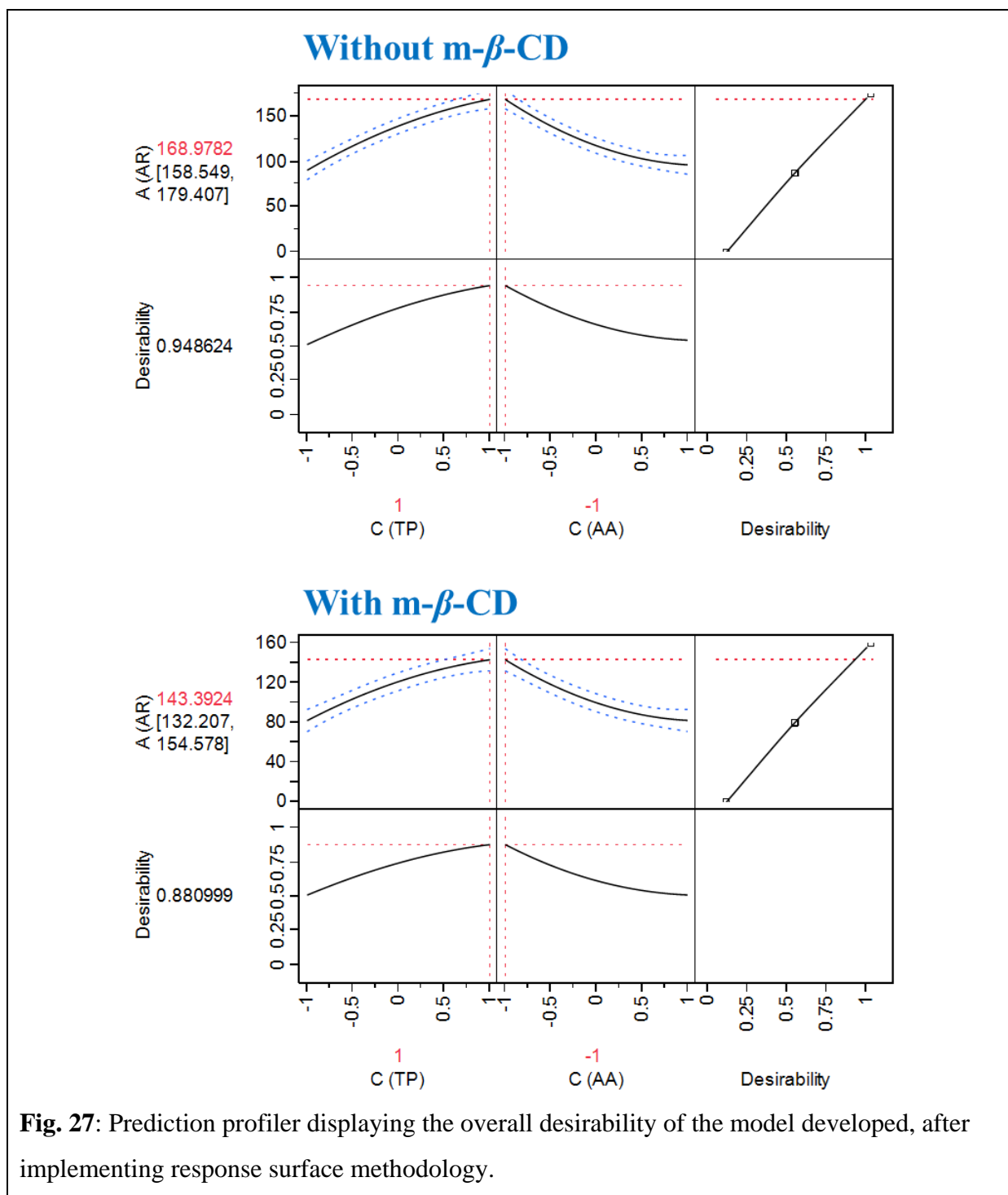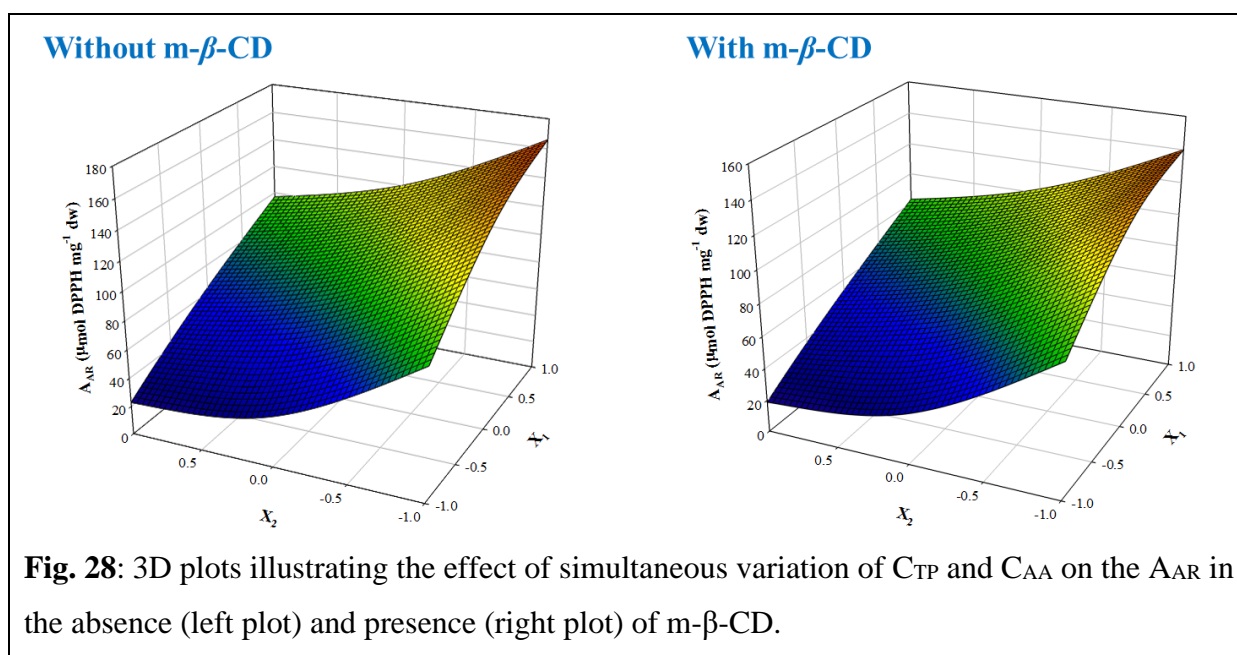
The optimal estimated ratio of C_{TP}/C_{AA} to attain maximum A_{AR} was in both cases 7/1 (**Table 19**), which clearly indicated that switching of C_{AA} to higher levels would not provide higher A_{AR}. This particular antiradical behaviour of the OLL extract/AA would most probably be ascribed to the nature of the major radical scavengers occurring in OLL extracts. In previous studies pertaining to A_{AR} of mixtures of polyphenol-containing extracts with AA, it was shown that grape stem extracts displayed the highest performance when combined with AA at a C_{TP}/C_{AA} ratio of 1/1 (Karvela *et al.,* 2012), but for grape seed extracts optimal ratio of 0.82/1 was also determined (Karvela *et al.,* 2013). These findings suggested that the nature of principal antioxidant polyphenols in an extract might greatly define the antiradical effects. Investigations with pure polyphenols including quercetin, hesperetin and ferulic acid, revealed that interactions with AA at molar ratio 1:1 yielded antagonism (Aoun and Makris, 2012), a behaviour that was confirmed by a following detailed study on reducing power, employing response surface methodology (Aoun and Makris, 2013). In this study, however, it was shown that maximum response in hesperetin/AA mixtures was achieved at a molar ratio of 5.2/1, which clearly indicated that the nature of polyphenolic antioxidant may define the molar ratio that could yield maximum antioxidant effect, in combination with AA.

Table 19: Optimal, predicted concentration ratios and theoretically calculated maximum response for all mixtures tested, obtained from the implementation of design.

Antioxidant test	Maximum predicted response	Optimal ratio
Without m-β-CD	168.98±10.43	70/10
With m-β-CD	143.39±11.18	70/10

5.4. Conclusions

The examination of the effect of m-β-CD on the radical scavenging ability of OLL polyphenols was approached first by a kinetic assay to determine stoichiometry and second by a response surface methodology to investigate interactions with the natural antioxidant ascorbic acid. In spite of the references reporting on the enhancement of the antioxidant activity of polyphenols in the presence of various cyclodextrins, this study demonstrated that m-β-CD may lower the radical efficiency of OLL polyphenols. Similar outcome was observed in the interactions of OLL polyphenols with ascorbic acid, which was a further concurrence of the hindering effect of m-β-CD. Based on the evidence emerged by this investigation, it was postulated that the orientation of oleuropein, the most abundant OLL antioxidant, inside the m-β-CD cavity, as well as the interactions of the encapsulated polyphenols with m-β-CD may be responsible for the phenomena observed. It is proposed that further studies with different β-cyclodextrin derivatives are required to fully clarify the mechanism of suppression (or enhancement) of the antiradical activity of polyphenol-containing extracts.

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Chapter 6:
General conclusions

A newly synthesided deep eutectic solvent (DES) composed of two biomolecules, glycerol and glycine, was tested for its effectiveness in extracting polyphenolic antioxidants from olive leaves (OLL). Following a first stage of parameter optimisation, this particular DES was demonstrated to be highly efficient, giving significantly increased polyphenol yield compared with conventional bio-solvents, such as aqueous ethanol and water. The DES extract also displayed stronger antioxidant effects.

Further studies demonstrated for the first time that methyl-β-cyclodextrin (m-β-CD) can be very effectively combined with this DES, boosting polyphenol extraction from OLL. The investigations performed showed that yield in total polyphenols (Y_{TP}) and yield in total flavonoids (Y_{TFn}) achieved with DES/m-β-CD were significantly higher compared with those attained with aqueous ethanol. In addition, the DES/m-β-CD extract possessed improved antioxidant properties, and this might not be simply attributed to the higher polyphenol concentration, as interactions with m-β-CD could have an important contribution. Liquid chromatography-diode array-mass spectrometry (LC-DAD-MS) analysis revealed that DES/m-β-CD and DES extracts had identical polyphenolic profile, suggesting that m-β-CD posed no issue of selectivity, but it acted solely towards boosting the extraction yield. Contrary to that, the aqueous ethanolic extract had poorer composition, revealing the effectiveness of the DES/m-β-CD as an extraction medium. Then, the study was still in progress to ascertain the validity of the model proposed in scale-up procedures, which is anticipated to further illustrate its applicability for industrial use.

OLL extracts, obtained with either DES/m-β-CD or only DES were shown to suffer alterations regarding their polyphenolic profile, when stored for a period of 20 days at 50 °C. These alterations were mainly characterised by degradation of some glycosides and the formation of a yellow pigments as a result of polyphenol oxidation. It was therefore postulated that the decline in reducing power (P_R) seen in the stored OLL extracts could be ascribed to the

oxidation of some OLL constituents. However, the kinetic study demonstrated that the incorporation of m-β-CD in the extraction solvent may result in notable retardation of the P_R decay. This finding highlighted the importance of such additives in stabilising polyphenol-containing extracts in DES.

However, with regard to the antioxidant effects of the extract obtained with DES/m-β-CD and in spite of the references reporting on the enhancement of the antioxidant activity of polyphenols in the presence of various cyclodextrins, this study demonstrated that m-β-CD may lower the radical efficiency of OLL polyphenols. Similar outcome was observed in the interactions of OLL polyphenols with ascorbic acid, which was a further concurrence of the hindering effect of m-β-CD. Based on the evidence emerged by this investigation, it was postulated that the interactions of the encapsulated polyphenols with m-β-CD may be responsible for the phenomena observed.

The DES study of this investigation is proposed as an eco-friendly means of boosting polyphenol recovery through solid-liquid extraction and this may also pave the way for more effective valorisation of other phytochemicals, from agri-food waste biomass. Since the solvent is composed of non-toxic substances naturally occurring in foods, the extracts may be directly used as bioactive constituents in food/cosmetic/pharmaceutical formulations, where this would be convenient, thus negating the need of downstream processing for solvent removal. This would eventually lead in lower cost and energy consumption, in line with a sustainable process. Further studies are required to clarify the exact mechanism of protection, which could lead in more efficient utilisation of m-β-CD in formulations related with food, pharmaceutical and cosmetic products. Further more it is proposed that additional studies with different β-cyclodextrin derivatives are required to fully clarify the mechanism of suppression (or enhancement) of the antiradical activity of polyphenol-containing extracts.

Appendices

A. Curriculum vitae

Mr. Vassilis Athanasiadis has the Bachelor's degree (BSc) in Food Technology since 2010, also has the Master's degree (MSc) in Applied Public Health and Environmental Hygiene with focus on Food and Water Quality and Safety and Public Health since 2013, and he is PhD candidate in Food Science and Nutrition. He is an Academic Research Fellow at the Departments of Food Technology and Nutrition and Dietetics of University of Applied Sciences (T.E.I.) of Thessaly. He has experience in recovery, separation and identification of natural product extracts and determination of their antioxidant activity. He has also experience in modern analytical techniques (chromatography, spectrophotometry, etc.). He participated in 7 national research programs. He has published 10 research articles in scientific journals (with more than 110 citations), more than 22 presentations in international and national conferences, and he is a reviewer in 8 international scientific journals (with more than 20 research articles). He is also a member of Food InnovaLab research laboratory (at T.E.I. of Thessaly), and of Hellenic Association of Food Technologists.

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B. Doctoral thesis projects

i. Published work in international scientific journals with referees

1. Highly efficient extraction of antioxidant polyphenols from *Olea europaea* leaves using an eco-friendly glycerol/glycine deep eutectic solvent

   V. Athanasiadis, S. Grigorakis, S. Lalas and D.P. Makris

   Journal Name: *Waste and Biomass Valorization*

   Publication Date: Jun 22, 2017

   DOI: 10.1007/s12649-017-9997-7

   URL: https://link.springer.com/article/10.1007/s12649-017-9997-7

2. Methyl-β-cyclodextrin as a booster for the extraction for *Olea europaea* leaf polyphenols with a bio-based deep eutectic solvent

   V. Athanasiadis, S. Grigorakis, S. Lalas and D.P. Makris

   Journal Name: *Biomass Conversion and Biorefinery*

   Publication Date: Aug 24, 2017

   DOI: 10.1007/s13399-017-0283-5

   URL: https://link.springer.com/article/10.1007/s13399-017-0283-5

3. Stability effects of methyl-β-cyclodextrin on *Olea europaea* leaf extracts in a natural deep eutectic solvent

   V. Athanasiadis, S. Grigorakis, S. Lalas and D.P. Makris

   Journal Name: *European Food Research and Technology*

   Publication Date: submitted

   DOI:

   URL:

4. Effect of methyl-β-cyclodextrin on radical scavenging kinetics of olive leaf extracts and interactions with ascorbic acid

   V. Athanasiadis, S. Lalas and D.P. Makris

   Journal Name: *ChemEngineering*

   Publication Date: Sep 11, 2017

   DOI: 10.3390/chemengineering1010006

   URL: http://www.mdpi.com/2305-7084/1/1/6

ii. Work presentations in conferences

1. Eco-friendly glycerol/glycine low-transition temperature mixture (LTTM) as an effective solvent for the extraction of antioxidant polyphenols from *Olea europaea* leaves

   V. Athanasiadis, S. Grigorakis, S. Lalas and D.P. Makris

   More Info: *7th National Conference - Trends in the Field of Lipids*, Greek Lipid Forum, Thessaloniki, Greece

   Event date: Oct 5, 2017

   URL: https://greeklipidforum7thconference.wordpress.com

2. Highly efficient extraction polyphenols from olive leaves (*Olea europaea*) with a deep eutectic solvent using methyl-β-cyclodextrin

   V. Athanasiadis, S. Grigorakis, S. Lalas and D.P. Makris

   More Info: *Food Industry & Environment,* Chem-EcoLink 2017, Paiania, Greece

   Event date: Nov 26, 2017

   URL1: http://www.chem-ecolink.gr

   URL2: http://www.petet.org.gr/pages/?p=4500

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ORIGINAL PAPER

Highly Efficient Extraction of Antioxidant Polyphenols from *Olea europaea* Leaves Using an Eco-friendly Glycerol/Glycine Deep Eutectic Solvent

Vassilis Athanasiadis[1] · Spyros Grigorakis[2] · Stavros Lalas[3] · Dimitris P. Makris[1]

Abstract A number of biological activities have been attributed to polyphenolic substances occurring in the leaves of the olive tree (*Olea europaea*), an olive oil industry waste material, and for this reason several extraction processes have been developed for the efficient recovery of these phytochemicals. The study presented herein describes for the first time the use of an eco-friendly deep eutectic solvent (DES) as a highly effective solvent for the extraction of polyphenols from olive tree leaves. Initially, a central composite design was implemented to optimise solvent composition and liquid-to-solid ratio and then a kinetic assay was performed to investigate diffusivity (D_e) and temperature effects. The maximum total polyphenol yield was 106.25 mg gallic acid equivalents per g dry weight, achieved at 70 °C. This value was by 18% higher than that achieved with 60% aqueous methanol. Furthermore, the DES extract exhibited significantly higher antiradical activity and reducing power. Temperature increase up to 70 °C was shown to boost D_e and the activation energy determined for the process was 29.55 kJ mol^{-1}. Characterisation of the extract by means of liquid chromatography–diode array–mass spectrometry showed oleuropein and certain flavone glycosides to be the major polyphenolic constituents.

List of Symbols

A_{AR}	Antiradical activity (µmol DPPH g^{-1})
C_{DES}	DES concentration (%, w/v)
D_e	Diffusivity (m s^{-1}).
E_a	Activation energy (kJ mol^{-1})
H	Initial extraction rate (mg g^{-1} min^{-1})
k	Second-order extraction rate constant (g mg^{-1} min^{-1})
P_R	Reducing power (µmol AAE g^{-1})
$R_{L/S}$	Liquid-to-solid ratio (mL g^{-1})
t	Time (min)
T	Temperature (°C or K)
Y_{TFn}	Yield in total flavonoids (mg RtE g^{-1})
Y_{TP}	Yield in total polyphenols (mg GAE g^{-1})
$Y_{TP(s)}$	Yield in total polyphenols at saturation (mg GAE g^{-1})

AAE	Ascorbic acid equivalents
DES	Deep eutectic solvents
DPPH	2,2-diphenyl-1-picrylhydrazyl radical
GAE	Gallic acid equivalents
MW	Molecular weight
OLL	Olive tree leaves
RtE	Rutin equivalents
TPTZ	2,4,6-tripyridyl-*s*-triazine

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Introduction

The generation of food waste biomass encompasses a spectrum of activities, such as agricultural practices, industrial manufacturing and household consumption and contemporary environmental concepts, such as the circular economy, are regarded as the driving force for the implementation of "zero waste policies". The high volume of waste deriving from the food industry raises serious concerns, pertaining to both economic and environmental aspects, but a large amount of this residual biomass has a significant potential used as raw material for the production of novel commodities and specialty feedstock through biorefining procedures [1].

The recovery of precious secondary metabolites from waste plant tissues is an attractive prospect in this regard, since many parts of plant material rejected during fruit and vegetable processing, such as peels, stems, seeds and leaves, bear a considerable load of such phytochemicals, including polyphenols. This particular class of compounds embraces numerous chemical structures of variable biological properties and functionalities, which are related with antioxidant and antimicrobial activity, as well as chemoprotective activity against cancer and other degenerative diseases [2]. Olive leaves (OLL) are a waste material of the olive oil industry and they are rejected during olive fruit cleaning. OLL contain relatively high amounts of polyphenols compared with other food wastes [3] and possess a peculiar polyphenolic profile, composed mainly of oleuropein, a major bioactive phenolic, but also several flavone glycosides [4, 5]. OLL polyphenols have been a subject of extensive and thorough studies, owed to their pharmacological potency [6] and for this reason OLL polyphenol recovery has been a primal research field [7].

A crucial step in the recovery process is the molecule extraction, and conventional solvent extraction is the tool of preference, implemented on industrial scale to extract bioactive compounds from plant matrices [8]. However, food industries are facing the challenge to move towards sustainable process strategies, to maximize valorization of wastes for higher profitability and reduced environmental aggravation [9]. In this direction, emerging technologies are increasingly adopted by food industries, on the recognition of their potential to recover high-added value compounds effectively and in a sustainable framework. With regard to extraction on industrial scale, conventional methodologies have some major drawbacks, such as low extract recovery and long extraction duration and intensive heating, resulting in high energy consumption. Additionally, due to toxicity and the increasing prices of fossil resources, replacement of traditional solvents is a primary objective. Thus green extraction processes are being oriented toward extraction intensification, increased mass and heat transfer,

reduced equipment size, and reduction of processing steps [10].

Deep eutectic solvents (DES) are novel liquids, composed of inexpensive, recyclable and non-toxic materials, which can be natural substances (e.g. sugars, organic acids and salts etc.) [11]. DES synthesis is eco-friendly, facile and straightforward and properties such as low vapour pressure, absence of flammability and water miscibility make DES ideal solvents for a range of sustainable and environmentally benign applications. Recently, the use of such solvents for the extraction of natural products has been attracting interest, because of their unique potency that allows for extraction yields higher than those achieved with conventional solvents [3]. Considering the above concepts and perspectives, the present examination was carried out to test a newly synthesized, eco-compatible DES, with regard to its potential in recovering bio-active polyphenolic phytochemicals from OLL. The approach employed included first optimization of basic extraction parameters and then a kinetic assay to assess the effect of temperature on the extraction process. The results were evaluated by comparison with other environmentally benign solvents, and characterization of the antioxidant activity and polyphenolic profile of the extracts.

Materials and Methods

Chemicals

Solvents used for liquid chromatography-mass spectrometry were HPLC grade. Glycerol and aluminium chloride were from Fisher Scientific (NJ, USA). Ferric chloride hexahydrate was from Acros Organics (Geel, Belgium). Gallic acid, glycine, Folin–Ciocalteu reagent, 2,2-diphenyl-*1*-picrylhydrazyl (DPPH), ascorbic acid, rutin (quercetin 3-*O*-rutinoside) and 2,4,6-tripyridyl-*s*-triazine (TPTZ) were from Sigma-Aldrich (Steinheim, Germany). Sodium acetate trihydrate was from Penta (Prague, Czeck Republic).

Preparation of the DES

DES preparation was carried out according to a previously reported methodology [3]. Briefly, glycerol (HBD) was mixed with glycine (HBA) and water at a molar ratio 7:1:3. The mixture was heated in a stoppered glass vial at 80–90 °C for approximately 90 min, under stirring, until a perfectly transparent liquid was formed. The DES was stored in screw-cap glass vial, in the dark, at room temperature. The DES was periodically inspected for the appearance of crystals, within a period of several weeks.

Plant Material

Olive leaves (OLL) were from the *olea europaea* variety Agrielia Kalamon, collected from an olive tree plantation located at Avlida (Evia, Central Greece). Sampling and OLL handling was performed as described in an earlier study [12].

Experimental Design

A central composite design with one central point was deployed [13] to test the effect of concentration of the DES (C_{DES}) and the liquid-to-solid ratio ($R_{L/S}$) on the total polyphenol yield (Y_{TP}). For this scope, appropriate amounts of dry OLL powder were mixed with 25 mL of aqueous DES and stirred at 600 rpm for 120 min, at 50 °C. C_{DES} and $R_{L/S}$ used were predetermined by the experimental design (Table 1). The response (Y_{TP}) at each design point was measured and recorded (Table 2).

Kinetics and Temperature Assay

Amount of 1.56 g of dry OLL powder was mixed with 50 mL of solvent, [$C_{DES} = 80\%$ (w/v), $R_{L/S} = 32$ mL g^{-1}] and extractions were performed as described above, within a temperature range of 40–70 °C. Sampling was accomplished at regular intervals (5–280 min) to measure Y_{TP}. The second-order model, as previously described [14], was obtained by plotting $1/Y_{TP}$ as a function of t (Fig. 1). Then kinetic parameters including second-order extraction rate constant (k), initial extraction rate (h) and extraction yield at saturation ($Y_{TP(s)}$) were determined. Likewise, the effect of temperature was estimated by calculating the activation energy (E_a), according to a previous study [15]. Diffusivity was estimated as described in details elsewhere [16].

Spectrophotometric Determinations

Total polyphenol yield (Y_{TP}) was determined using the Folin–Ciocalteu reagent and expressed as mg gallic acid equivalents (GAE) per g of dry material [16]. Total flavonoid yield was determined using the $AlCl_3$ reagent as described previously [15] and expressed as mg rutin equivalents (RtE) per g of dry material. Ferric reducing

Table 2 Measured and predicted responses for all design points considered for the experimental design

Design point	Independent variables		Response (Y_{TP}, mg GAE g^{-1} dw)	
	C_{DES} (X_1)	R_{mol} (X_2)	Measured	Predicted
1	−1	−1	82.33	81.23
2	−1	1	80.86	80.13
3	1	−1	91.29	91.67
4	1	1	89.45	90.21
5	−1	0	80.78	82.61
6	1	0	94.01	92.87
7	0	−1	78.02	78.74
8	0	1	77.49	77.46
9	0	0	79.36	80.02

power (P_R) was estimated using the TPTZ methodology [16]. The antiradical activity (A_{AR}) was measured with the DPPH probe, as described elsewhere [17]. P_R and A_{AR} were expressed as µmol ascorbic acid equivalents (AAE) and µmol DPPH per g of dry material, respectively.

Qualitative Liquid Chromatography–Diode Array–Mass Spectrometry (LC–DAD–MS)

A previously described methodology [16] was employed to tentatively characterise the principal polyphenolic metabolites, with some modifications. Briefly, the
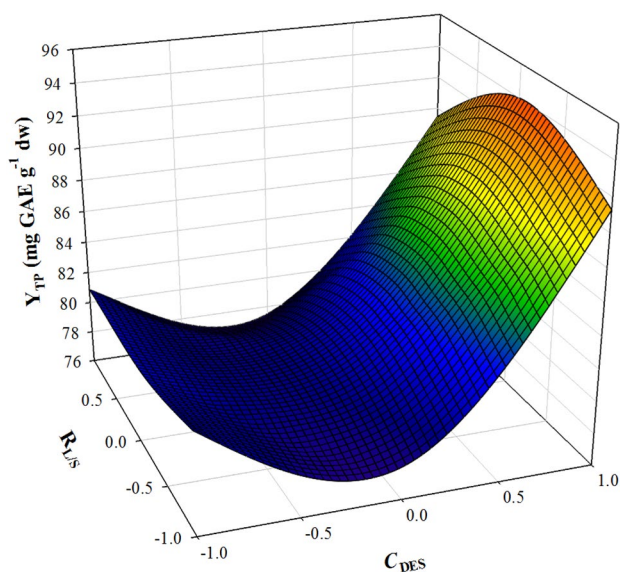
Fig. 1 3D plot displaying the effect of simultaneous variation of C_{DES} and $R_{L/S}$ on Y_{TP}. OLL Extractions were carried out at 50 °C, under continuous stirring at 600 rpm, for 120 min

Table 1 Coded and actual levels of the independent variables used for the central composite design

Independent variables	Code units	Coded variable level		
		−1	0	1
C_{DES} (w/v, %)	X_1	50	65	80
$R_{L/S}$	X_2	20	35	50

equipment used was a Finnigan MAT Spectra System P4000 pump, coupled with a UV6000LP diode array detector and a Finnigan AQA mass spectrometer. A Fortis RP-18 column, 150×2.1 mm, 3 μm, was used, at 40 °C. Analyses were performed with electrospray ionization (ESI) in positive ion mode, with acquisition set at 5 and 50 eV, capillary voltage 4 kV, source voltage 25 V, detector voltage 650 V and probe temperature 350 °C. Spectra scans were performed within a *m/z* range of 100 to 1000 amu. Eluent (A) and eluent (B) were 1% acetic acid and methanol, respectively. The flow rate was 0.2 mL min^{-1}, and the elution programme used was as follows: 0–2 min, 10% B; 2–40 min, 100% B; 45 min, 100% B.

Statistics

Extractions were repeated at least twice and all determinations at least in triplicate. Values reported are averages and standard deviations. Linear and non-linear regressions were performed with SigmaPlot® 12.0. Central composite experimental design and all associated statistics were performed with JMP® 10.

Results and Discussion

DES Synthesis and Optimisation of Solvent Composition

In an earlier investigation [18], DES composed of L-lactic acid and glycine at a molar ratio 3:1 was shown to be unstable, yielding a plastic solid. This mixture was successfully tailored by adding three moles of water, to give a stable DES, which was found to be a very efficient solvent for the extraction of polyphenolic antioxidants from various medicinal herbs, compared with a few other L-lactic acid-based DES that contained organic salts as HBAs. This finding evidenced that glycine might be a HBA with a high potency in forming DES with enhanced extraction efficiency. In this context, attempts were undertaken to combine glycerol (HBD) with glycine, in the view of synthesising an efficient and more cost-effective solvent, which could have an important prospect for polyphenol recovery. To this purpose, glycerol was combined with glycine starting from a molar ratio 1:1, but up to ratio 6:1 all mixtures were unstable, giving crystallisation. A ratio of 7:1 was of improved stability as the crystallisation observed was limited, but stability was assured through tailoring with 3 moles of water. Thus the final composition of the DES was glycerol:glycine:water (7:1:3). This DES was used for further examination.

The next step was the optimisation of the extraction process with respect to DES concentration (C_{DES}) and

the liquid-to-solid ratio ($R_{L/S}$), two critical parameters that largely define extraction yield [16]. For this scope, a central composite design with one central point was used and the extraction efficiency was estimated by determining the total polyphenol yield (Y_{TP}). The effect of the simultaneous variation of the independent variables (C_{DES} and $R_{L/S}$) was illustrated in the form of a 3D plot (Fig. 1). Variations in $R_{L/S}$ provoked rather weak changes in Y_{TP}, but switching of C_{DES} had a pronounced impact in this regard. This is in line with the outcome of recent studies, which demonstrated that water content between 10 and 50% may greatly enhance the extraction performance of various DES [19]. This is because mixing the DES with water regulates properties such as polarity and viscosity, which are directly related with the extraction capacity of a solvent [20]. Depending on the polyphenols to be extracted, the ideal water content may vary up to approximately 64% (v/v) [21].

To assess quantitatively the combined effect of both independent variables, the desirability function was used (Fig. 2). It was found that only C_{DES} and its quadratic term were significant ($p < 0.005$) and therefore removal of the non-significant terms gave the following model:

Y_{TP} (mg GA Eg^{-1} dw)

$$= 80.02 + 5.26X_1 + 7.71X_1^2 \ (R^2 = 0.97, \ p = 0.0043)$$

Using the fitted model, the optimal condition was found to be $C_{DES} = 80\%$ (w/v). Although $R_{L/S}$ exerted a non-significant effect on the model, the optimal value

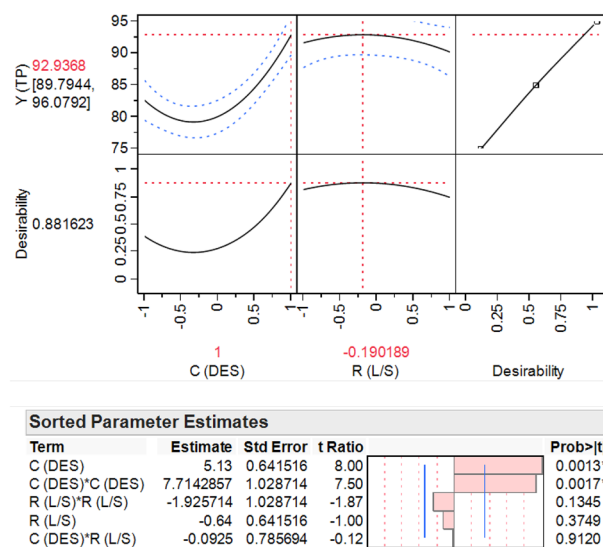| Sorted Parameter Estimates					
Term	Estimate	Std Error	t Ratio		Prob>|t|
C (DES)	5.13	0.641516	8.00		0.0013*
C (DES)*C (DES)	7.7142857	1.028714	7.50		0.0017*
R (L/S)*R (L/S)	-1.925714	1.028714	-1.87		0.1345
R (L/S)	-0.64	0.641516	-1.00		0.3749
C (DES)*R (L/S)	-0.0925	0.785694	-0.12		0.9120

Fig. 2 The use of desirability function to predict the optimal combination of C_{DES} and $R_{L/S}$ to achieve maximum Y_{TP}, under the conditions employed. The *inset table* illustrates the significance of each C_{DES} and $R_{L/S}$, as well as their quadratic and cross terms, to the model

determined (32 mL g^{-1}) was adopted, because it was within the range reported for polyphenol extraction with DES [21]. Under these conditions, the estimated maximum Y_{TP} was 92.94 ± 3.15 mg GAE g^{-1} dw.

Extraction Kinetics: Effect of temperature

In order to further evaluate the extraction capacity of the solvent, the optimal conditions ($C_{DES} = 80\%$ (w/v), $R_{L/S} = 32$ mL g^{-1}) were used to trace extraction kinetics,
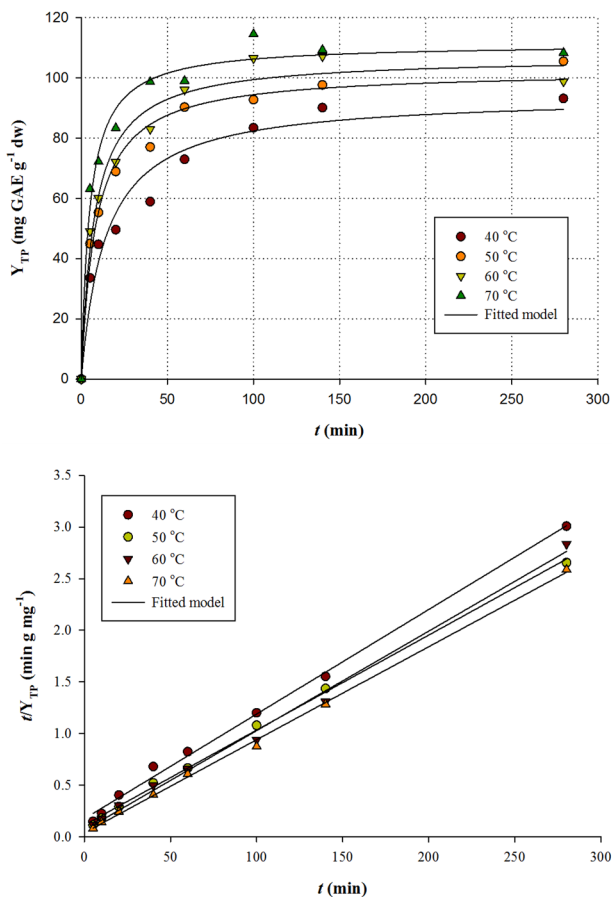

Fig. 3 Time course of Y_{TP} (*upper plot*) during extraction of OLL with the DES, under optimal conditions [$C_{DES} = 80\%$ (w/v) and $R_{L/S} = 32$ mL g^{-1}]. The *lower plot* shows the second-order kinetics

with the aim to estimating some basic kinetic parameters and the effect of temperature. The model best fitted to the data generated was second-order kinetics (Fig. 3), as previously shown for OLL extraction with aqueous glycerol and aqueous ethanol [22], but also with various glycerol-based DES for polyphenol extraction from *Satureja thymbra* [21]. It can be seen in Table 3 that an increase in T from 40 to 70 °C revealed kinetics obeying the Arrhenius model, giving $E_a = 29.55$ kJ mol^{-1}. This value was higher than that determined for polyphenol extraction from red grape pomace with aqueous glycerol (13.94 kJ mol^{-1}) [23], but lower than those reported for polyphenol extraction with aqueous glycerol from onion solid wastes (31.52 kJ mol^{-1}) [24] and *Artemisia* (37.64 kJ mol^{-1}) [17]. However, similar data for polyphenol extraction with DES are not available in the literature.

The effect of temperature on diffusivity (D_e) followed a similar trend, but a significant increase, approximately by fivefold, was recorded when the temperature was raised from 60 to 70 °C. Such a steep change might be associated with a sharp decrease in viscosity, since according to Stokes–Einstein equation, diffusion (D) is reciprocally related with viscosity [25]. It has been proposed that aqueous DES solutions could be disintegrated through hydrogen bond rupture, by the energy provided through temperature increase, hence abolishing their unique properties [21]. Such a theory could explain this unusual behaviour, yet it is to be proven. It should be emphasised that such a phenomenon has not been described in studies on polyphenol extraction with conventional solvents, where diffusivity increases in response to raising the temperature were much smoother [17, 23, 24]. On the other hand, if DES disintegration occurred, then a drop in its extraction capacity would have been observed, leading to lower Y_{TP} and Y_{TFn}, but this was not the case. Therefore, the steep increase in D_e recorded at 70 °C might be attributed to a weakening of hydrogen bonds between the HBD and HBA, and not complete rupture.

Comparison with Conventional Eco-friendly Solvents

To bring out the extraction capacity of the DES used, extraction of OLL was carried out under optimised

Table 3 Parameters of second-order kinetics, determined for the extraction of TP from OLL, using 80% (w/v) aqueous DES

T (°C)	Kinetic parameters				
	k ($\times 10^{-3}$) (g mg^{-1} min^{-1})	h (mg g^{-1} min^{-1})	$Y_{TP(s)}$ (mg GAE g^{-1})	D_e (m s^{-1}) $\times 10^{-12}$	E_a (kJ mol^{-1})
40	0.750	6.63	94.04	0.127	29.55
50	1.157	12.12	102.36	0.138	
60	1.240	14.16	106.87	0.205	
70	1.864	23.10	111.33	1.041	

Extractions were performed under continuous stirring at 600 rpm and $R_{L/S} = 32$ mL g^{-1}

conditions, at 70 °C, and the results were compared with those obtained using distilled water and 60% ethanol (AE), but also 60% methanol (AM), which was used as a positive control. The results showed that extraction with the DES afforded almost 18% higher Y_{TP} than AE, over than 24% higher than AM and approximately 29% higher than water (Fig. 4, upper plot). To further illustrate the higher capacity of the DES, Y_{TFn} was also determined. In the same fashion, extraction with the DES gave 9.6, 15.3 and 43.8% higher Y_{TFn}, compared with AE, AM and water, respectively (Fig. 4, lower plot). Regarding A_{AR}, extracts with AM and AE had comparable levels of 656.0 and 643.8 µmol DPPH g^{-1} dw, but the DES extract exhibited $A_{AR} = 1097.8$ µmol DPPH g^{-1} dw (Fig. 5, upper plot). Likewise, AM and AE extracts had P_R values of 381.6 and 394.9 µmol AAE g^{-1} dw, but P_R for the DES extract was 445.1 µmol AAE g^{-1} dw (Fig. 5, lower plot). These findings clearly demonstrated that the DES used was far more efficient than AE
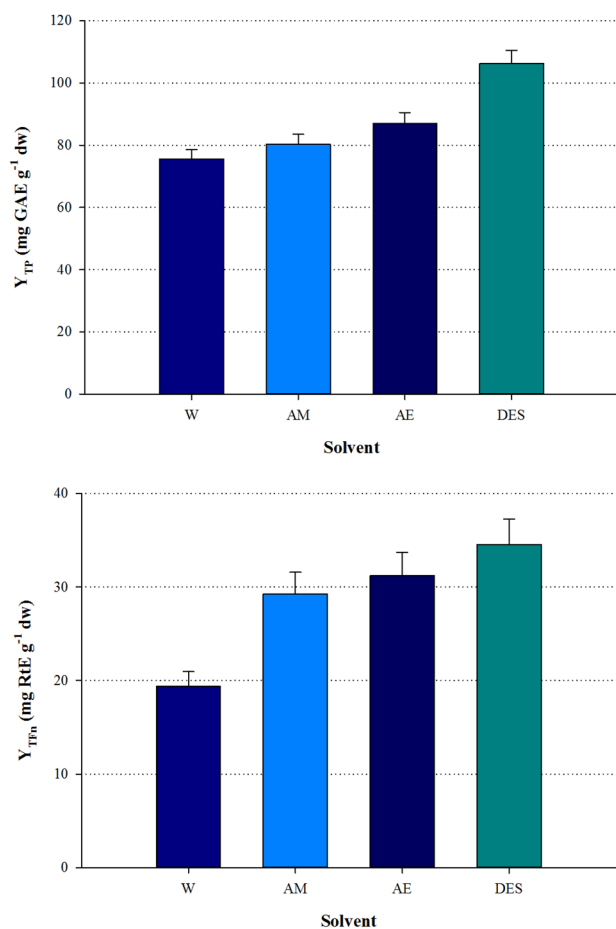
Fig. 4 Comparative diagram for Y_{TP} (upper plot) and Y_{TFn} (lower plot), obtained using 80% (w/v) DES, 60% aqueous methanol (AM), 60% aqueous ethanol (AE) and distilled water (W). Extractions were carried out at 70 °C, at $R_{L/S} = 32$ mL g^{-1}, under continuous stirring at 600 rpm, for 280 min
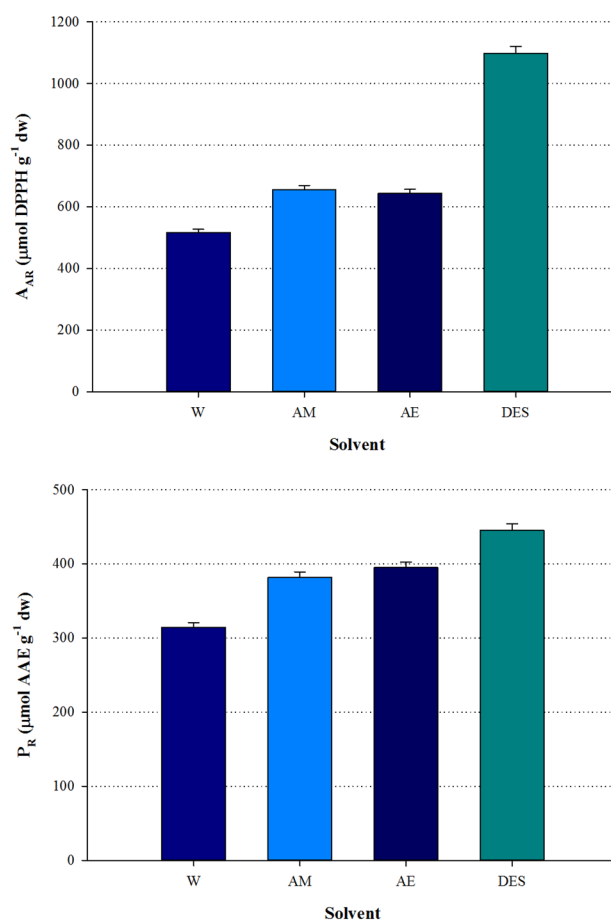
Fig. 5 Comparative diagram for A_{AR} (*upper plot*) and P_R (*lower plot*), determined for OLL extracts obtained using 80% (w/v) DES, 60% aqueous methanol (AM), 60% aqueous ethanol (AE) and distilled water (W). Extractions were carried out at 70 °C, at $R_{L/S} = 32$ mL g^{-1}, under continuous stirring at 600 rpm, for 280 min

and water, yielding extracts with higher total polyphenol and total flavonoid content, but also with superior antioxidant properties.

At this point, it should be emphasised that the antioxidant activity seen in the OLL extracts has been proposed to be the integration of interactions amongst the polyphenolic constituents, rather than that of the individual substances [26]. Such a claim has been demonstrated by studies that assessed the antiradical behaviour of mixtures of pure antioxidants and extracts [27]. However, compounds such as hydroxytyrosol and luteolin 7-*O*-glucoside were shown to be major radical scavengers in OLL extracts [28] and it is likely that their levels in OLL extracts may affect significantly their antioxidant potency. However, since the polyphenolic composition and hence the antioxidant activity of OLL is subject to seasonal variations [28] and age [29], then methodologies that maximise extraction yield, as the one proposed herein, could assure extracts with high antioxidant activity.

Table 4 UV–Vis and mass spectrometric data of the major polyphenols detected in the OLL extracts, obtained with 80% (w/v) DES, at 70 °C

No.	Rt (min)	UV–Vis	$[M+H]^+$ (m/z)	Other ions (m/z)	Tentative identity
1	14.24	288, 322(s)	391	413 $[M+Na]^+$, 261	Oleoside
2	18.80	288, 332	391	413 $[M+Na]^+$, 229	Oleoside
3	22.86	248, 348	765	579, 503, 233	Luteolin derivative
4	23.36	266, 340	611	449, 287	Luteolin di-glycoside
5	24.96	264, 318, 368	603	475, 303	Quercetin derivative
6	25.90	248, 280(s), 356	595	617$[M+Na]^+$, 449, 287	Luteolin rutinoside
7	26.39	248, 354	595	617$[M+Na]^+$, 449, 287	Luteolin rutinoside
8	26.69	252, 280	541	563, 361, 137	Oleuropein isomer
9	27.30	244, 340	579	271	Apigenin rutinoside
10	28.38	252, 280	541	563, 385, 345	Oleuropein

Characterisation of Principal Polyphenols

The DES extract that displayed the highest Y_{TP} was analysed by LC–DAD–MS to tentatively characterise its polyphenolic profile. At this point it should be stressed that although olive leaf extracts from various Greek native varieties have been examined [30], data on the particular variety Agrielia Kalamon used in this study, to the best of our knowledge, are inexistent. In total, ten compounds could be identified including secoiridoid, flavone and flavonol derivatives (Table 4). Critical comparison with previous studies on olive leaf extract composition [12, 22, 31] showed the presence of two oleoside derivatives (compounds 1 and 2) and a quercetin derivative with $m/z = 603$ (compound 5), which was heretofore unreported. Other common representatives, including oleuropein (compound 10), a luteolin di-glycoside (compound 4), which most probably corresponds to luteolin 4′,7-O-di-glucoside, two luteolin rutinosides (compounds 6 and 7) and an apigenin rutinoside (compound 9) were also present. On the other hand, no rutin (quercetin 3-O-rutinoside) or verbascoside, which are commonly encountered constituents [32], were detected.

Conclusions

In this investigation, a newly synthesised DES composed of two biomolecules, glycerol and glycine, was tested for its effectiveness in extracting polyphenolic antioxidants from OLL. Following a first stage of parameter optimisation, this particular DES was demonstrated to be highly efficient, giving significantly increased polyphenol yield compared with conventional bio-solvents, such as aqueous ethanol and water. The DES extract also displayed stronger antioxidant effects. Thus this solvent is proposed as an eco-friendly means of boosting polyphenol recovery through solid–liquid extraction and this may also pave the way for more effective valorisation of other phytochemicals, from agri-food waste biomass. Since the solvent is composed of non-toxic substances naturally occurring in foods, the extracts may be directly used as bioactive constituents in food/cosmetic/pharmaceutical formulations, where this would be convenient, thus negating the need of downstream processing for solvent removal. This would eventually lead in lower cost and energy consumption, in line with a sustainable process. Currently, work is in progress to test a broader range of glycine-based DES, in an effort to further improve polyphenol extraction.

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CrossMark

Methyl β-cyclodextrin as a booster for the extraction for *Olea europaea* leaf polyphenols with a bio-based deep eutectic solvent

Vassilis Athanasiadis[1,2] · Spyros Grigorakis[3] · Stavros Lalas[2] · Dimitris P. Makris[1]

Abstract A novel deep eutectic solvent (DES) with optimised composition was used to evaluate the effect of methyl β-cyclodextrin (CD) on the efficiency of polyphenol extraction from *Olea europaea* leaves (OLLs). The process developed was based on a 2^3 full-factorial design and response surface methodology to assess the simultaneous effect of CD concentration (C_{CD}), liquid-to-solid ratio ($R_{L/S}$) and temperature (T). Under optimised conditions (C_{CD} = 9%, $R_{L/S}$ = 40 mL g^{-1}, T = 51 °C), the yield in total polyphenols (Y_{TP}) was 116.65 ± 3.60 mg gallic acid equivalents per g dry weight. This value was significantly higher than that determined for the extraction performed with 60% aqueous ethanol. The extraction kinetics also showed that the extraction rate was slowed down in the presence of CD, yet the higher extraction capacity of the DES/CD medium was confirmed. Characterisation of the extracts obtained with DES/CD and DES by means of liquid chromatography-mass spectrometry demonstrated that there was no selective extraction of any particular polyphenol, suggesting that CD acted merely as an extraction booster.

Keywords Antioxidants · Deep eutectic solvents · Methyl β-cyclodextrin · *Olea europaea* · Polyphenols

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Nomenclature

A_{AR}	Antiradical activity (μmol DPPH g^{-1})
D_e	Diffusivity (m^2 s^{-1})
h	Initial extraction rate (mg g^{-1} min^{-1})
k	Second-order extraction rate constant (g mg^{-1} min^{-1})
P_R	Reducing power (μmol AAE g^{-1})
$R_{L/S}$	Liquid-to-solid ratio (mL g^{-1})
t	Time (min)
T	Temperature (°C)
Y_{TFn}	Yield in total flavonoids (mg RtE g^{-1})
Y_{TP}	Yield in total polyphenols (mg GAE g^{-1})
$Y_{TP(s)}$	Yield in total polyphenols at saturation (mg GAE g^{-1})

Abbreviations

AAEs	Ascorbic acid equivalents
CD	Methyl β-cyclodextrin
DESs	Deep eutectic solvents
DPPH	2,2-Diphenyl-1-picrylhydrazyl radical
GAEs	Gallic acid equivalents
OLLs	*Olea europaea* leaves
RtE	Rutin equivalents
TPTZ	2,4,6-Tripyridyl-*s*-triazine

1 Introduction

Nowadays, it is regarded imminent to emerging circular economy that the waste biomass deriving from the agricultural and food sector should not be simply treated as waste, but rather as a bioresource with high potential in value-added substances, chemicals and fuels. Agro-industrial activity accounts for the production of a large volume of wastes, including processing residues, such as leaves, branches, peels, roots, stems and seeds. This biomass is usually undervalorised, in spite its richness in

an array of high value-added substances. A highly regarded class of phytochemicals of broad occurrence in agri-food wastes is polyphenols, embracing a bewildering diversity of structures [1]. A plethora of these compounds exhibits biological properties of particular interest, including antiinflammatory and chemopreventive activities [2], and therefore, they are considered to have an undisputed perspective as bioactive agents in food, pharmaceutical and cosmetic industries.

Mediterranean countries are facing pressure to deal with problems associated with agri-food wastes, typical to their native principal crops, such as olives. Olive industry wastes not properly treated are a primal environmental issue for olive-producing countries, and therefore, strategies aiming at valorising olive wastes, especially those allowing a sustainable recovery of valuable natural components, are gaining acceptance. Olive leaves, a regular olive-processing residue, may bear a high load of polyphenols [3] with potential pharmacological applications [4]; hence, various extraction methodologies have been developed for their effective recovery. However, green procedures for effective olive leaf polyphenol retrieval are rather limited [5], but the use of eco-friendly extraction techniques may represent unprecedented opportunities to face such a challenge in a sustainable manner [6].

A selective solvent with a high solubility of the target compounds is a salient feature of processes destined to achieve a high yield of target compounds in a short process time. Most of the extraction procedures are based on regulating solvent properties to increase solubility of the solute molecules by modifying solvent polarity. Apart from its physical–chemical capacity in dissolving the target compound(s), the toxicity of a solvent and the environmental impact by its use should also be considered. In this view, deep eutectic solvents (DESs), which are composed of inexpensive, recyclable and non-toxic natural substances (e.g. sugars, organic acids and salts), appear as the most promising prospect. This is because characteristics including low vapour pressure, absence of flammability and water miscibility make DES ideal solvents for a range of sustainable and eco-friendly applications [7].

Cyclodextrins are natural cyclic oligosaccharides originating from enzymic cleavage of starch, and they comprise of 6, 7 or 8 glucose units linked by $\alpha(1 \rightarrow 4)$ glycosidic bond. Cyclodextrin structure has a truncated cone shape, with a hydrophobic cavity inside and a hydrophilic external surface; therefore, cyclodextrins can form inclusion complexes with sparingly water-soluble molecules (such as polyphenols), increasing their solubility [8]. Furthermore, cyclodextrin encapsulation of polyphenols contributes in higher stability and controlled release [9]. Recent studies showed that combining 2-hydroxypropyl β-cyclodextrin with aqueous glycerol may effectively increase yield of polyphenol extraction [10, 11]. Similar results were reported for water extraction of apple flavonols, using various cyclodextrins [12]. In the light of

such evidence, the study carried out aimed at testing the efficiency of polyphenol extraction from OLL, using a combination of methyl β-cyclodextrin (CD) with a glycerol-based DES [13]. The process was first optimised by implementing a central composite design and performance assessment was based on kinetics and extract characterisation, using representative antioxidant tests and liquid chromatography-mass spectrometry.

2 Materials and methods

2.1 Chemicals and reagents

HPLC-grade solvents were used for liquid chromatography. Folin-Ciocalteu reagent was from Fluka (Steinheim, Germany). Ferric chloride hexahydrate and ascorbic acid were from Acros Organics (Geel, Belgium). Glycerol and aluminium chloride were from Fisher Scientific (NJ, USA). Sodium acetate was from Penta (Prague, Czech Republic). Methyl β-cyclodextrin, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), rutin (quercetin 3-O-rutinoside), glycine and 2,4,6-tripyridyl-s-triazine (TPTZ) were from Sigma-Aldrich (Steinheim, Germany).

2.2 Plant material and DES synthesis

Details regarding collection and handling of *Olea europaea* leaves (OLLs) have been analytically described elsewhere [14]. The material used for extraction was dried OLL powder, with an average mean particle diameter of approximately 0.5 mm. For DES synthesis, the optimised conditions were used [13]. Briefly, the DES was prepared using glycerol as the hydrogen bond donor (HBD) and glycine as the hydrogen bond acceptor (HBA), at a molar ratio HBD:HBA:water of 7:1:3. Aqueous solution 80% (w/v) of this DES was employed for all extractions performed.

2.3 Batch extraction process

A suitable amount of OLL powder was mixed with the DES and CD in a 50-mL glass vial, and extractions were carried out under continuous stirring on a magnetic stirrer, at 600 rpm, for 180 min. The amount of OLL powders, the concentration of CD and the temperature of the extraction were defined by the experimental design (Table 1). After the completion of each extraction, samples were centrifuged at 20,000×g and the clear supernatant was used for all analysis, after dilution 1:20 with water.

2.4 Experimental design

A 2^3 full-factorial design (Box-Behnken) was used, as described previously [15], with the yield in total

Table 1 Actual values and coded levels of the independent variables used for the 2^3 full-factorial design

Independent variables	Code units	Coded variable level		
		−1	0	1
C_{CD} (% w/v)	X_1	1	5	9
$R_{L/S}$ (mL g^{-1})	X_2	10	25	40
T (°C)	X_3	40	50	60

Table 2 Measured and predicted value of Y_{TP}, determined for individual design points

Design point	Independent variables			Response (Y_{TP}; mg GAE g^{-1} dw)	
	X_1	X_2	X_3	Measured	Predicted
1	−1	−1	−1	74.67	75.17
2	−1	−1	1	87.20	86.88
3	−1	1	−1	104.94	105.56
4	−1	1	1	112.30	112.99
5	1	−1	−1	86.46	86.20
6	1	−1	1	91.99	91.81
7	1	1	−1	112.57	113.33
8	1	1	1	114.72	114.66
9	−1	0	0	103.27	101.78
10	1	0	0	108.38	108.13
11	0	−1	0	86.10	86.36
12	0	1	0	114.98	112.98
13	0	0	−1	99.42	97.80
14	0	0	1	104.44	104.31
15	0	0	0	102.68	103.68
16	0	0	0	101.18	103.68

polyphenols (Y_{TP}) as the response. The three independent variables considered were the concentration of CD (C_{CD}) (X_1, varying between 1 and 9%, w/v), liquid-to-solid ratio ($R_{L/S}$) (X_2, varying between 10 and 40 mL g^{-1}) and temperature (T) (X_3, varying between 40 and 60 °C). The range used for each variable was chosen on the basis of previous findings [8] and preliminary experimentation. Each variable was coded at three levels, −1, 0 and 1 (Table 1), according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i}, i = 1, 2, 3 \tag{1}$$

The terms x_i and X_i represent the dimensionless and the actual values of the independent variable i, X_0 is the actual value of the independent variable i at the central point and ΔX_i is the step change of X_i corresponding to a unit variation of the dimensionless value. For each design point, the response was determined and recorded (Table 2). Data from the experimental design were subjected to regression analysis using least squares regression methodology to obtain the parameters of the mathematical models. Analysis of variance (ANOVA) was used to assess the significance of the model. 3D plots were obtained using the fitted model.

2.5 Kinetic and diffusivity (D_e) determination

Extractions were carried out as described above, under optimised conditions, and sampling was accomplished at predetermined intervals (5–180 min) to calculate Y_{TP}. Then, Y_{TP} was plotted against time (t) and model fitting was performed with non-linear regression. The second-order model was obtained after plotting t/Y_{TP} as a function of t, according to the following equation [16]:

$$\frac{t}{Y_{TP(t)}} = \frac{1}{k Y_{TP(s)}^2} + \frac{t}{Y_{TP(s)}} \tag{2}$$

The initial extraction rate (h) and the yield in total polyphenols at saturation ($Y_{TP(s)}$) were determined graphically, from the slope ($1/Y_{TP(s)}$) and the intercept ($1/h$), respectively. The second-order extraction rate constant (k) was then calculated:

$$h = k Y_{TP(s)}^2 \tag{3}$$

Diffusivity (D_e) was determined using the linearised expression of Fick's second law for non-steady state, as described previously [17]:

$$1 - \frac{Y_{TP(t)}}{Y_{TP(s)}} = \frac{6}{\pi^2} e^{-\frac{D_e \pi^2 t}{r^2}} \tag{4}$$

D_e calculation was based on the line with the shallow slope (slope = $\frac{D_e \pi^2}{r^2}$), obtained by plotting $\ln\left(\frac{Y_{TP(s)}}{Y_{TP(s)} - Y_{TP(t)}}\right)$ against t, where r is the radius of the solid particles.

2.6 Determinations

Total polyphenol yield (Y_{TP}) was determined using the Folin-Ciocalteu reagent and expressed as mg gallic acid equivalents (GAEs) per g of dried material [18]. Total flavonoid yield was determined using the AlCl$_3$ reagent as described previously [19] and expressed as mg rutin equivalents (RtE) per g of dried material. Ferric reducing power (P_R) was estimated using the TPTZ methodology [18]. The antiradical activity (A_{AR}) was measured with the DPPH probe. P_R and A_{AR} were expressed

as μmol ascorbic acid equivalents (AAEs) and μmol DPPH per g of dried material, respectively.

2.7 Qualitative liquid chromatography-diode array-mass spectrometry

The equipment used was a Finnigan MAT Spectra System P4000 pump, coupled with a UV6000LP diode array detector and a Finnigan AQA mass spectrometer. A Fortis RP 18 column, 150 × 2.1 mm, 3 μm, was used, at 40 °C. Analyses were carried out as described elsewhere [17].

2.8 Statistical analyses

All extractions were carried out twice and all determinations in triplicate. Values reported are averages. Response surface design and associated statistics were performed with JMP™ 10. Kinetics was estimated by non-linear regression between Y_{TP} and t, using SigmaPlot™ 12.0, at least at a 95% significance level.

3 Results and discussion

3.1 Extraction optimisation

The objective of the optimisation process was to assess simultaneously the effect of C_{CD}, $R_{L/S}$ and T on Y_{TP}. To do so, a 2^3 full-factorial central composite design with two central points (point #15 and #16; Table 2) was deployed and the trend in Y_{TP} variation as a function of parallel changes in all three process (independent) variables was recorded in the form of 3D plots (Fig. 1). The ANOVA test indicated that all process variables considered (C_{CD}, $R_{L/S}$ and T) exerted a statistically significant effect on Y_{TP}, but the terms T^2, C_{CD}^2, $R_{L/S} \cdot T$ and $R_{L/S} \cdot C_{CD}$ were non-significant. Thus, the model obtained after omitting the non-significant terms was as follows:

$$Y_{TP}(\text{mg GAE g}^{-1} \text{ dw}) = 103.68 + 3.17C_{CD} + 13.31R_{L/S}$$
$$+ 3.26T - 1.53C_{CD} \cdot T - 4.01R_{L/S}^2$$

$$(R^2 = 0.99, p < 0.0001)$$

With the use of the desirability function (Fig. 2), the recommended settings to achieve the highest Y_{TP} were estimated to be $C_{CD} = 9\%$ (w/v), $R_{L/S} = 40$ mL g^{-1} and $T = 51$ °C. Under these conditions, the predicted Y_{TP} was 116.65 ± 3.60 mg GAE g^{-1} dw.

The optimal $R_{L/S}$ found was close to 36.2 mL g^{-1} reported for polyphenol extraction from *Satureja thymbra*, using a DES composed of glycerol and sodium acetate (3:1) [20], and to
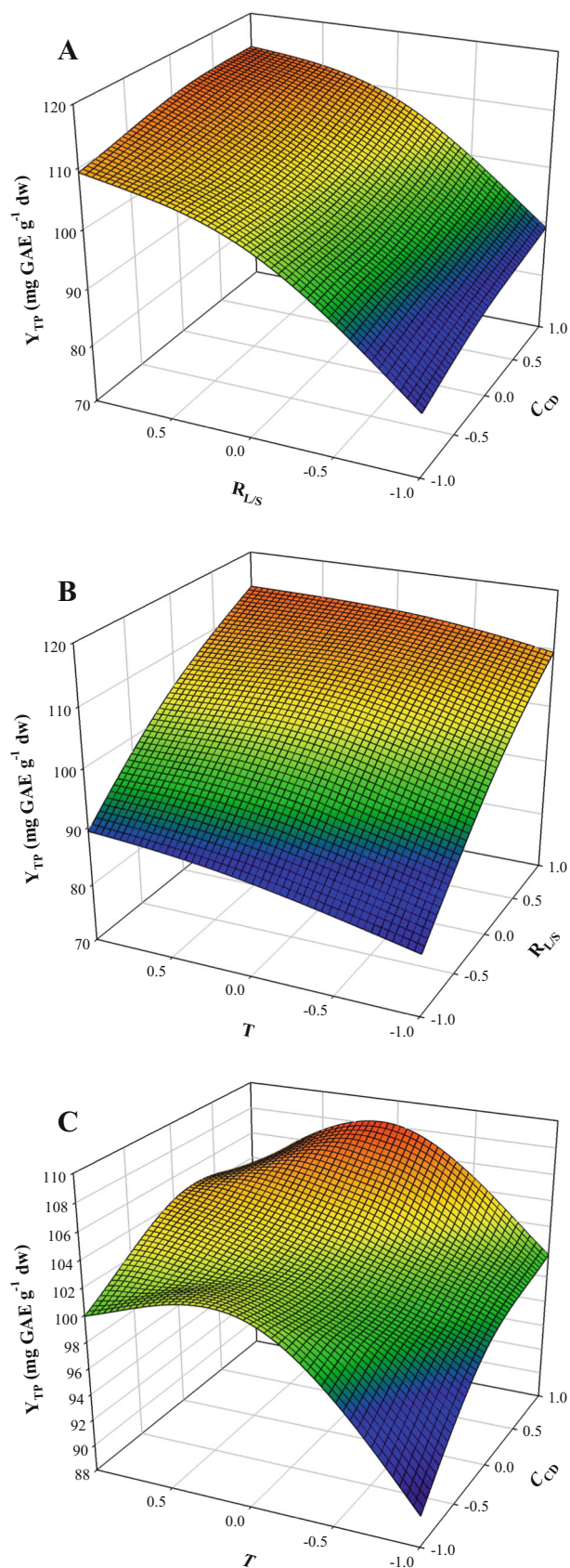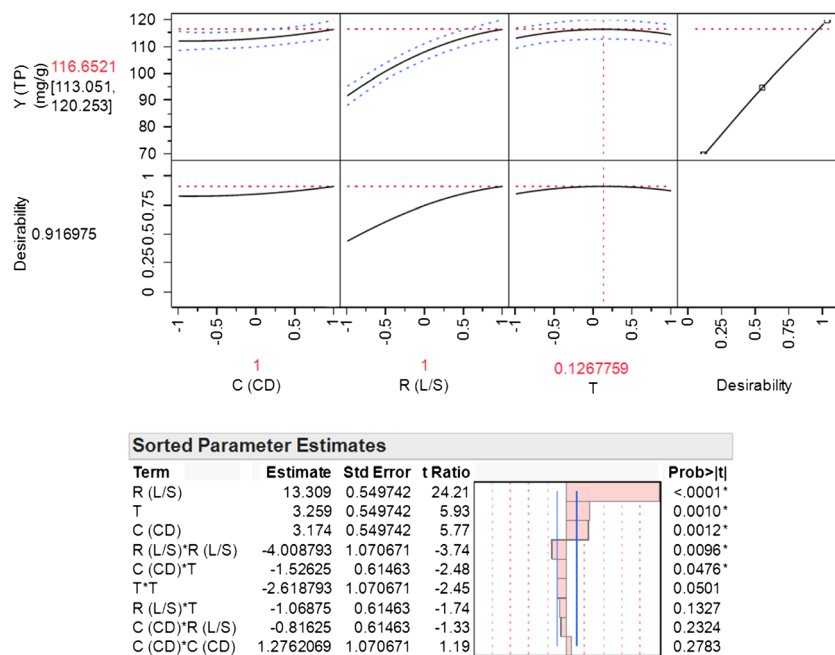

Fig. 1 3D plots showing the effect of simultaneous variation of C_{CD}, $R_{L/S}$ and T on the Y_{TP}

Fig. 2 Prediction profiler displaying the overall desirability of the model, along with the optimal C_{CD}, $R_{L/S}$ and T values. Extractions were carried out under stirring (600 rpm), at 50 °C, for 180 min

Sorted Parameter Estimates

Term	Estimate	Std Error	t Ratio		Prob>|t|
R (L/S)	13.309	0.549742	24.21		<.0001*
T	3.259	0.549742	5.93		0.0010*
C (CD)	3.174	0.549742	5.77		0.0012*
R (L/S)*R (L/S)	-4.008793	1.070671	-3.74		0.0096*
C (CD)*T	-1.52625	0.61463	-2.48		0.0476*
T*T	-2.618793	1.070671	-2.45		0.0501
R (L/S)*T	-1.06875	0.61463	-1.74		0.1327
C (CD)*R (L/S)	-0.81625	0.61463	-1.33		0.2324
C (CD)*C (CD)	1.2762069	1.070671	1.19		0.2783

33.3 mL g^{-1}, determined as optimal for the extraction of polyphenols from *Carthamus tinctorius* L. using several choline chloride-based DES [21]. This is a corroboration that, unlike polyphenol extraction with conventional solvents that may display optimal $R_{L/S}$ of 90–120 mL g^{-1} [22, 23], polyphenol extraction with DES may be effectively performed using lower solvent volume. Such a feature is highly desirable, entailing the use of smaller equipment and lower cost. This prospect, so far disregarded, is particularly essential, highlighting another aspect of the use of DES as efficient solvents.

Likewise, the optimal T was 51 °C, suggesting that higher T did not favour polyphenol extraction. This outcome is in absolute accordance with a previous study, which demonstrated that yield of polyphenol extraction from *Moringa oleifera* leaves with a glycerol-based DES dropped, when T was raised from 50 to 80 °C [24]. This is also in line with recent data, which showed that yield of polyphenol extraction from red grape pomace with certain DES declined upon switching T from 60 to 80 °C [25]. Such a trend was also seen in polyphenol extraction from *Equisetum palustre* L. using choline chloride-based DES [26]. This particular behaviour could be attributed to the nature of the DES, whose structure depends on the number and the strength of hydrogen bonds between the HBD and the HBA. The increase in extraction temperature may initially favour polyphenol extraction due to higher diffusivity, which stems from a decrease in viscosity [27]. However, further temperature increase may endow the system with energy sufficient to provoke hydrogen bond rupture, and thus, the extraction efficiency of the DES could be abrogated [20].

With reference to the effect of C_{CD}, the optimal value determined by the model was 9% (w/v), which is fairly close to 7% (w/v) required for polyphenol extraction from olive leaves with aqueous glycerol [11]. However, 13% (w/v) 2-hydroxypropyl β-cyclodextrin was determined as optimal for polyphenol extraction from oak acorn with aqueous glycerol [10]. Moreover, recovery of polyphenols from grape pomace was shown to be optimal using 1% (w/v) β-cyclodextrin in either 80% aqueous ethanol or water, whereas recoveries declined at higher β-cyclodextrin concentration [28]. Ligand inclusion in the CD cavity is a stoichiometric phenomenon, and usually, only one molecule may be incorporated into the cavity so as to become entrapped. Thus, it would normally be expected increased extraction yield as a response to raising C_{CD}, as previously demonstrated for β-cyclodextrin-aided polyphenol extraction [29].

Such a phenomenon may be attributed to the specific polyphenol/CD interactions. In an aqueous medium, the driving force that enables polyphenol-CD complex formation is the displacement of water molecules outside the CD cavity and retention of the polyphenol within through apolar-apolar association [30]. CD would increase solubility of the less polar polyphenols through entrapment, and the eventual result seen could represent the integration of such an effect. On the other hand, polyphenols may behave as HBDs and interact with glycine of the DES, and a sort of antagonism may develop, most probably because CD might weaken polyphenol-glycine interactions. This could happen because polyphenol/CD complex formation involves strong hydrogen bond formation [31]. Yet, since combinations of DES with CDs have never been previously reported, such an assumption is to be elucidated by profounder examinations.

3.2 Extraction kinetics

To further clarify the effect of the combination of DES/CD on the polyphenol extraction from OLL, a kinetic investigation was undertaken. The kinetic model implemented (Fig. 3) allowed for the determination of certain parameters that permitted a comparative evaluation. It can be seen in Table 3 that k and h for the extractions carried out with DES and DES/CD were significantly lower ($p < 0.05$) compared with the corresponding determined for the aqueous ethanol extraction, indicating a much slower extraction rate. By contrast, differences in D_e were not so pronounced, yet extraction with aqueous ethanol displayed the highest value. This outcome was most probably attributed to the higher viscosity of the DES system, which might have resulted in low diffusivity, as observed in studies where viscous solvents, such as aqueous glycerol, were compared with water [27] or aqueous ethanol [18, 32].

Extraction with the DES/CD afforded $Y_{TP(s)}$ = 116.58 mg GAE g^{-1} dw, which matched exactly the value predicted by the response surface model. This was a sound confirmation for the model validity. The $Y_{TP(s)}$ attained with DES/CD was by 5.7% higher than that achieved with the DES and 17.8% higher than that with aqueous ethanol. The difference in $Y_{TP(s)}$ between DES/CD and DES was not significant ($p > 0.05$), but CD enhanced significantly $Y_{TP(s)}$ of the DES/CD compared with the aqueous ethanol ($p < 0.05$). This finding pointed clearly to a boosting of the extraction yield triggered by the addition of the CD in the DES, evidencing its improved effectiveness. It is to be noted that the use of various cyclodextrins, such as β-cyclodextrin [29, 33] or 2-hydroxypropyl β-cyclodextrin [10, 11], in combination with aqueous solvents resulted in increased extraction yields, suggesting that cyclodextrins can effectively enhance extraction efficiency. However, such an effect is for the first time reported for combination of DES and CD.

3.3 Model evaluation and efficiency testing

To check the applicability of the model established through the response surface methodology, extraction was repeated under optimal conditions and afforded 113.21 ± 3.02 mg GAE g^{-1} dw. This value was virtually equal to the maximum value predicted by the model (116.65 ± 3.60 mg GAE g^{-1} dw). Moreover, to bring out clearly the efficiency of the DES/CD to extract OLL polyphenols, extractions were carried out with DES and 60% (v/v) aqueous ethanol, under the same conditions, and the extracts were analysed, in addition to Y_{TP}, for Y_{TFn}, A_{AR} and P_R. The results confirmed the outcome of the kinetic study, since the Y_{TP} attained with the DES/CD was by 5.3 and 19.3% higher than that reached with the DES and 60% (v/v) aqueous ethanol, respectively (Fig. 4, upper plot). Additionally, the extract obtained with the DES/CD displayed 16.0 and 4.5% higher Y_{TFn}, compared with the DES and 60% (v/v) aqueous ethanol, respectively (Fig. 4, lower plot).

However, the determination of the antioxidant activity demonstrated that the DES/CD extract was not the most potent in scavenging radicals, having an A_{AR} value that was by 4.3% lower than that seen with the DES extract (Fig. 5, upper plot). Although the difference in A_{AR} was not statistically different ($p < 0.05$), both DES/CD and DES extract exhibited by 40–43% higher A_{AR} that the aqueous ethanol extract. Similarly, the DES extract had by 5.4 and 28.5% higher P_R compared with the DES/CD and aqueous ethanol extract, respectively. These data suggested that CD did not impact the antioxidant behaviour of the DES extract, yet both DES/CD

Fig. 3 Second-order kinetic models for the extraction of TP from OLL with the DES + CD, DES and 60% (v/v) aqueous ethanol. Extractions were carried out under continuous stirring at 600 rpm, at optimal C_{CD}, $R_{L/S}$ and T, for 180 min
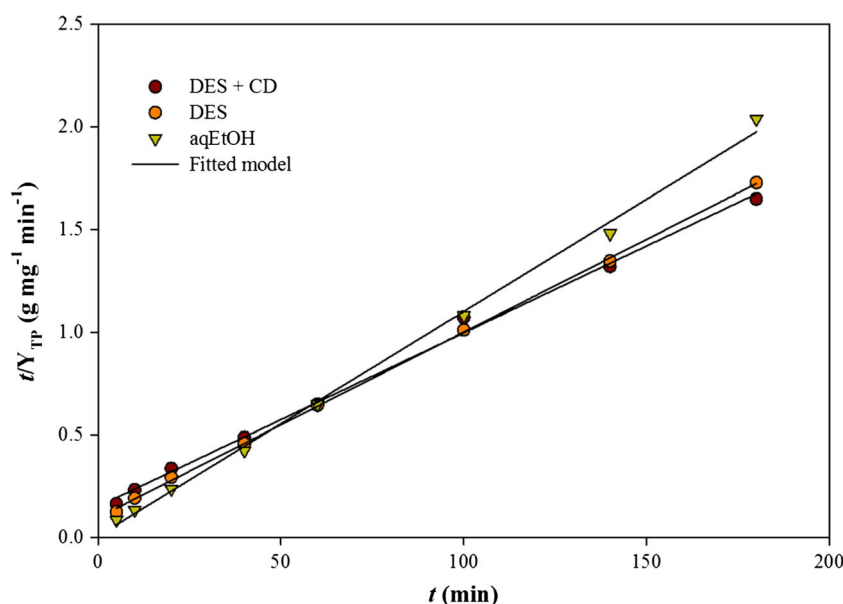
Table 3 Parameters of second-order kinetics, determined for the extraction of TP from OLL, under optimised conditions

Solvent	Kinetic parameters			
	k ($\times 10^{-3}$) (g mg^{-1} min^{-1})	h (mg g^{-1} min^{-1})	D_e ($\times 10^{-12}$) (m s^{-1})	$Y_{TP(s)}$ (mg GAE g^{-1})
DES + CD	0.502	6.82	6.06	116.58
DES	0.867	10.46	5.42	109.93
60% EtOH	3.576	32.82	7.44	95.81

and DES extracts possessed considerably stronger antioxidant capacity compared with the aqueous ethanol.

It has been shown that inclusion complexes of 2-hydroxypropyl β-cyclodextrin with polyphenols such as chlorogenic acid [34] and quercetin [31], but also β-cyclodextrin complexes with rosmarinic acid [35], quercetin and rutin [36], displayed more powerful antioxidant activity compared with the non-encapsulated polyphenols. This phenomenon
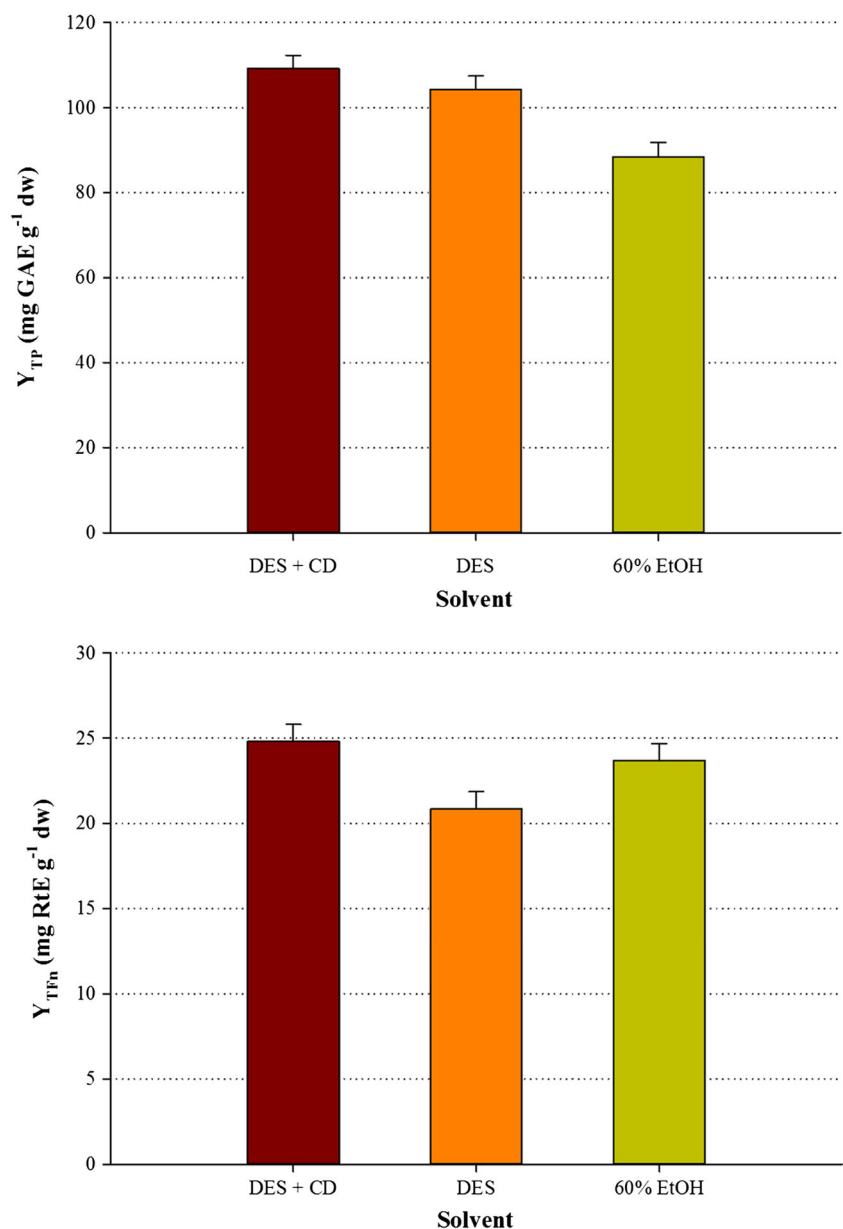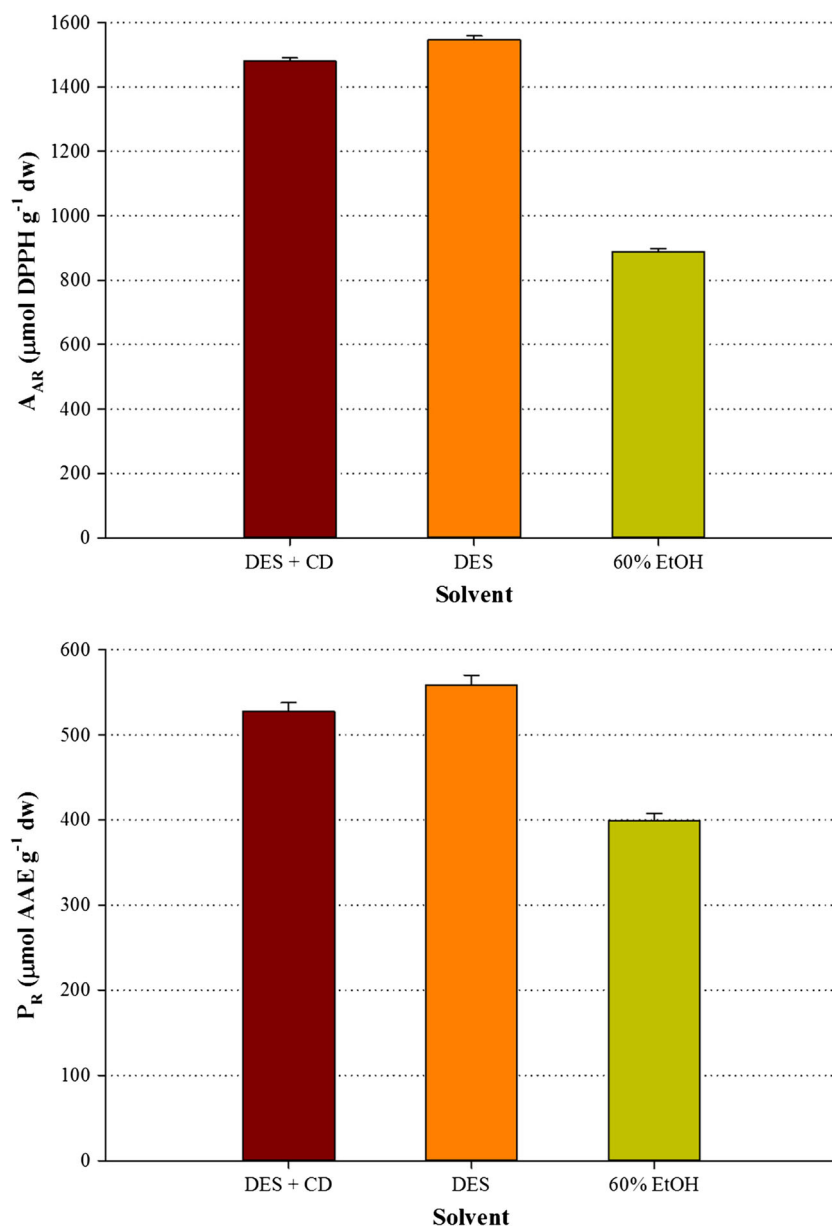

Fig. 4 Comparative diagram for Y_{TP} (upper plot) and Y_{TFn} (lower plot), obtained using DES + CD, DES and 60% aqueous ethanol (AE). Extractions were carried out under continuous stirring at 600 rpm, at optimal C_{CD}, $R_{L/S}$ and T, for 180 min

has also been demonstrated for chlorogenate-rich coffee extracts [37]. Nevertheless, the inclusion complex cyclodextrin/polyphenol is better stabilised with molecules having higher hydrophobicity [38]. Therefore, not all polyphenols present in the DES extract would form equally stable complexes and this might compromise the antioxidant capacity.

Another factor that could contribute towards such an effect is probably the orientation of the encapsulated molecule in the CD cavity. For OLL, it has been demonstrated that complex of β-cyclodextrin with oleuropein, the major OLL polyphenol, involves deep insertion of the dihydroxyphenethyl moiety inside the cavity from its secondary side [39]. Such a fact would

Table 4 UV-Vis and mass spectral data for the major polyphenolic phytochemicals tentatively identified in OLL extracts obtained under optimal conditions, using DES/CD and DES

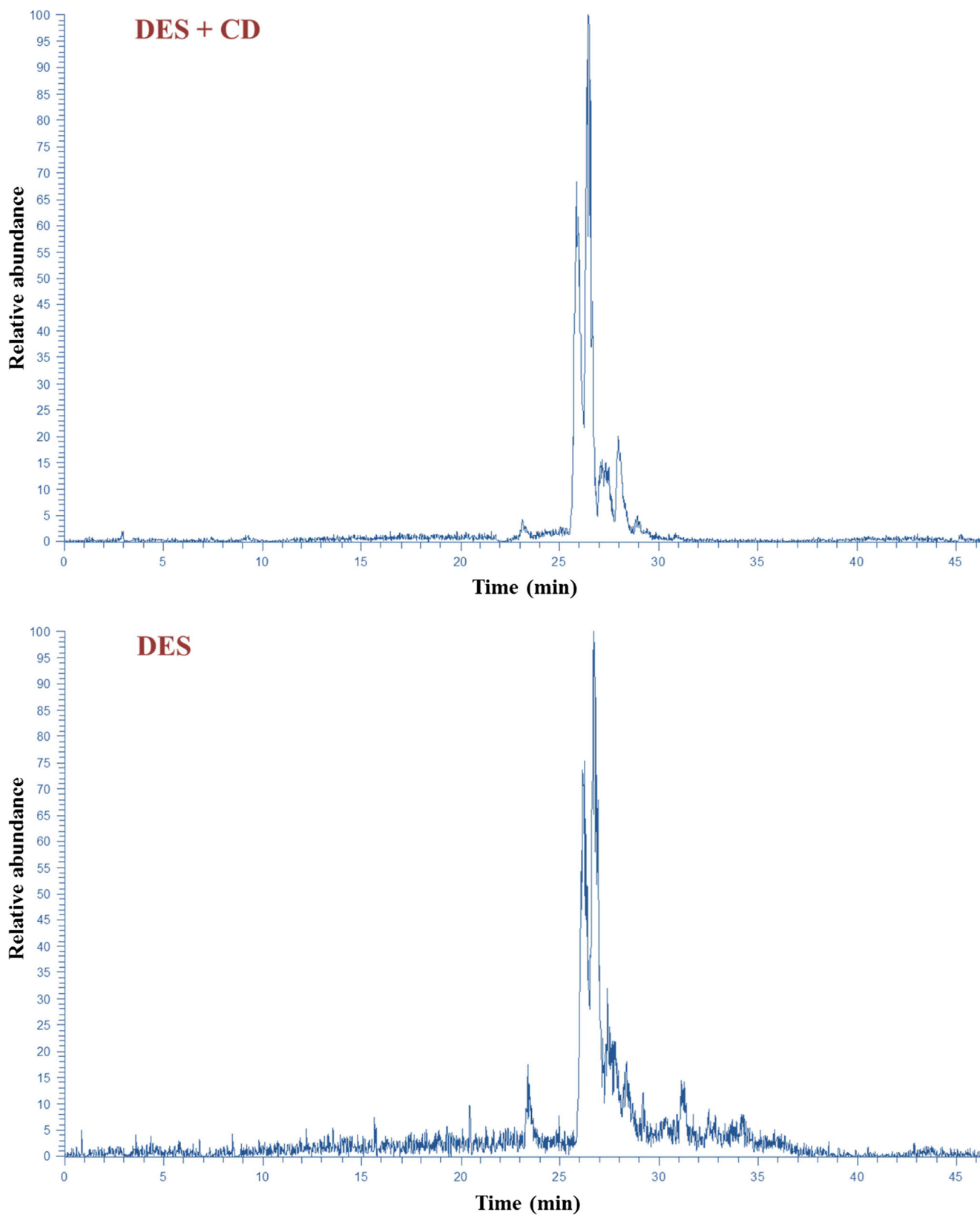| Peak	Rt (min)	$[M + H]^+$ (m/z)	Other ions (m/z)	Tentative identity
1	25.85	449	287	Luteolin glucoside
2	26.46	541	563 $[M + Na]^+$, 361, 137	Oleuropein
3	27.14	579	601 $[M + Na]^+$, 271	Apigenin rutinoside
4	27.96	449	471 $[M + Na]^+$, 418, 287	Luteolin glucoside

Fig. 6 Total ion chromatograms showing the principal polyphenols detected in the extracts obtained with DES + CD (upper figure) and DES (lower figure)

pose constrains with regard to oxidant/antioxidant interactions arising from steric effects, thus resulting in reduced antioxidant activity. In fact, in a more recent study, an increase from 7 to 13% (w/v) in 2-hydroxypropyl β-cyclodextrin concentration for OLL polyphenol extraction with aqueous glycerol did not result in significantly higher A_{AR}, but rather, a slight decline was observed [11]. This was probably the reason why the DES/CD extract did not express more powerful antioxidant properties. Therefore, the antioxidant effects observed in DES/CD extracts may not simply reflect the higher polyphenol concentration, but interactions associated with polyphenol/CD complexes as well. However, such a hypothesis remains to be elucidated.

3.4 Extract characterisation

Characterisation was performed by analysing the DES/CD and DES extracts with liquid chromatography-diode array-mass spectrometry (LC-DAD-MS) to investigate whether CD acted selectively towards particular OLL polyphenols. To this purpose, major constituents previously detected in OLL extracts [13] were tentatively identified and chosen as indicators (Table 4). These substances were two luteolin glucosides (peak #1 and #4), an apigenin rutinoside (peak #3) and the secoiridoid derivative oleuropein (peak #2). As can be seen in Fig. 6, the chromatographic profiles of DES/CD and DES extracts were virtually identical, which clearly showed that the DES containing CD did not selectively extract any specific phytochemical. This finding indicated that CD acted merely as an extraction booster and did not affect the composition of the extract.

4 Conclusions

In this study, it was demonstrated for the first time that CD can be very effectively combined with a DES, boosting polyphenol extraction from OLL. The investigations performed showed that Y_{TP} and Y_{TFn} achieved with DES/CD were significantly higher compared with those attained with aqueous ethanol. In addition, the DES/CD extract possessed improved antioxidant properties, and this might not be simply attributed to the higher polyphenol concentration, as interactions with CD could have an important contribution. LC-DAD-MS analysis revealed that DES/CD and DES extracts had identical polyphenolic profile, suggesting that CD posed no issue of selectivity, but it acted solely towards boosting the extraction yield. Contrary to that, the aqueous ethanolic extract had poorer composition, revealing the effectiveness of the DES/CD as an extraction medium. Currently, work is in progress to ascertain the validity of the model proposed in scale-up procedures, which is anticipated to further illustrate its applicability for industrial use.

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Effect of Methyl *β*-cyclodextrin on Radical Scavenging Kinetics of Olive Leaf Extracts and Interactions with Ascorbic Acid

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Abstract: Olive leaf (OLL) extracts contain a high load of antioxidant polyphenols with significant pharmacological potency. In this study, the use of a novel natural deep eutectic solvent enabled the effective extraction of OLL polyphenols and their testing as radical scavengers, in the presence or absence of methyl *β*-cyclodextrin (m-*β*-CD), using descriptive kinetics. Testing was extended to include interactions with ascorbic acid—a natural powerful antioxidant—by implementing a response surface methodology. The kinetic study showed that m-*β*-CD may hinder the radical scavenging effect of OLL extracts, yielding lower stoichiometry upon reaction with the radical probe 2,2-diphenyl-1-picrylhydrazy (DPPH). The extension of the reaction time to determine the total stoichiometry confirmed this effect. As a further concurrence, the interactions of OLL extracts with ascorbic acid showed lower radical scavenging performance in the presence of m-*β*-CD. These results were discussed on the grounds of the role that m-*β*-CD may play in similar systems.

Keywords: antioxidants; deep eutectic solvents; methyl *β*-cyclodextrin; olive leaf extracts; polyphenols

1. Introduction

In recent years, there has been a growing demand for natural antioxidants to both replace synthetic ones and also to act as functional additives that could provide biological systems with protection against harmful free radicals. Plant-derived antioxidant polyphenols are becoming increasingly important in this respect, as numerous of these substances have been shown to possess a very high capacity for quenching free radicals [1]. This has stimulated a broad spectrum of studies regarding the use of plant extracts as rich sources of natural antioxidants. Olive leaves (OLL), which represent a major proportion of the waste generated during the production of olive oil, have attracted a great deal of interest because they may bear an important load of polyphenolic phytochemicals, which may possess beneficial biological properties [2].

Although antioxidant activity may be effectively estimated in plant extracts—with several tests developed for such a purpose [3]—a few studies have investigated in detail the rate of antiradical reactions, which might represent the rate at which antioxidants react with free radicals. Reaction kinetics information complements that of antiradical activity and may be of value for characterizing a potential antioxidant source. 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) is widely used for quickly assessing the ability of antioxidants to transfer labile H atoms to radicals, based on the theory that a hydrogen donor is an antioxidant. This reaction is stoichiometric with respect to the number of hydrogen atoms absorbed. Therefore, the antioxidant effect can be easily evaluated by following the decrease of UV absorption at 515 nm [4].

On the basis of this theoretical background, this examination was carried out to assess the antiradical behaviour of OLL extracts obtained using a novel methodology that involved extraction with a combination of a deep eutectic solvent (DES) with methyl *β*-cyclodextrin (m-*β*-CD) as an extraction booster [5]. OLL extracts were also generated without m-*β*-CD, in order to evaluate the effect of m-*β*-CD on the antiradical potency of the extracts. The investigations included a kinetic assay and also interactions with ascorbic acid (AA), after implementing a response surface methodology.

2. Materials and Methods

2.1. Chemicals

Anhydrous sodium carbonate came from Carlo Erba Reactifs (Val de Reuil, France). Methyl *β*-cyclodextrin was obtained from Acros Organics (Geel, Belgium). Folin-Ciocalteu reagent was from Fluka (Steinheim, Germany). Glycerol (99%), 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid and ascorbic acid were from Sigma-Aldrich (Steinheim, Germany). Glycine (99.5%) was from NeoLab Migge Laborbedarf-Vertiebs (Heildelberg, Germany).

2.2. Preparation of the Deep Eutectic Solvent (DES)

The Deep Eutectic Solvent (DES) used was synthesised according to the optimised conditions described previously in [6]. Briefly, glycerol (HBD) was mixed with an appropriate amount of glycine (HBA) and water to give a molar ratio of HBD:HBA:water of 7:1:3, and the mixture was mildly heated under stirring until the formation of a transparent liquid. An aqueous solution of 80% (w/v) of this DES was used for the extractions.

2.3. Plant Material

The material used for all extractions was dried *Olea europaea* leaf (OLL) powder, with an average particle diameter of 0.5 mm. Details concerning the variety and methodology of leaf collection and the handling of the plant material have been analytically given elsewhere [6].

2.4. Batch Extraction Procedure and Sample Handling

Polyphenol-containing extracts were prepared from OLL by implementing the optimized methodology previously developed in [5]. Briefly, 2.5 g of dried OLL was mixed with 100 mL of 80% (w/v) aqueous DES containing 9% (w/v) methyl *β*-cyclodextrin (m-*β*-CD) and extractions were carried out at 70 °C, under continuous stirring at 600 rpm for 280 min. Extractions without m-*β*-CD were also performed under identical conditions. Samples were centrifuged in a table centrifuge (Hermle, Wehingen, Germany) at $10,000 \times g$ for 10 min, and the clear extract was used for all assays.

2.5. Total Polyphenol Determination

Total polyphenols were determined with the Folin-Ciocalteu reagent, following a previously published protocol [7]. Results were expressed as mg gallic acid equivalents per L of extract, using a gallic acid calibration curve (30–600 mg L^{-1}).

2.6. Kinetic Assay

The ability of the extracts to transfer H-atoms to DPPH was assessed by measuring changes in the absorbance at 515 nm (A_{515}). Typically, 0.975 mL of freshly prepared DPPH solution in methanol (100 μM), was mixed in a spectrometer cell with 0.025 mL of OLL extract. The decay in A_{515} was monitored over a period of 2 min to determine rate constant (k_1). The assay was extended up to 21 min for the determination of total stoichiometries (n_t).

2.7. Interaction with Ascorbic Acid

The methodology implemented was a 3×3 central composite design, aimed at investigating the interactions between OLL extracts and ascorbic acid and to clarify the effect of m-β-CD. Thus the two independent variables chosen were the total polyphenol concentration of the extracts (C_{TP}, mg GAE L^{-1}), termed as X_1, and the ascorbic acid concentration (C_{AA}, mg L^{-1}), termed as X_2. A central composite experimental design was used with two central points and both independent variables were coded between -1 (lower limit) and $+1$ (upper limit), using the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i} , i = 1, 2 \tag{1}$$

Terms x_i and X_i represent the dimensionless and the actual value of the independent variable *i*, respectively. X_0 is the actual value of the independent variable *i* at the central point of the design, and ΔX_i the step change of X_i, which corresponds to a unit change of the dimensionless value (Table 1). The choice for the range of values for both variables was based on preliminary runs, but also on published information [8]. The response considered was the antiradical activity (A_{AR}). ANOVA was performed to estimate model significance, the significance for each polynomial coefficient, and determine the overall coefficient R^2 for the mathematical model. Statistically non-significant dependent terms ($p > 0.05$) were removed from the equations, which were visualised in the form of 3D plots. The models were validated by performing experiments under the predicted optimal conditions, and comparing for each model the predicted values with the actual (measured) ones. For each design point, measured and predicted response values were recorded (Table 2). For each design point, C_{TP} and C_{AA} were fixed as dictated by the experimental design. A_{AR} (μmol DPPH g^{-1} dry OLL weight) was determined as described elsewhere [9].

Table 1. Actual values and coded levels of the independent variables used for the experimental design.

Independent Variables	Code Units	Coded Variable Level		
		-1	0	1
C_{TP} (mg·L^{-1})	X_1	10	40	70
C_{AA} (mg·L^{-1})	X_2	10	40	70

Table 2. Measured and predicted A_{AR} values of OLL extract and AA mixtures, determined for individual design points.

Design Point	Independent Variables		Response (A_{AR}, μmol DPPH g^{-1} dw)			
			Without m-β-CD		m-β-CD	
	X_1	X_2	Measured	Predicted	Measured	Predicted
1	-1	-1	91.04	90.58	82.13	82.06
2	-1	1	22.85	19.14	18.87	15.04
3	1	-1	166.02	168.98	140.08	143.39
4	1	1	96.88	96.59	82.68	82.23
5	-1	0	35.5	39.68	31.81	35.71
6	1	0	120.27	117.60	102.84	99.98
7	0	-1	141.99	139.50	124.61	121.37
8	0	1	63.58	67.58	53.00	57.28
9	0	0	89.11	88.36	78.18	76.49
10	0	0	89.11	88.36	75.83	76.49

2.8. Statistics

Curve-fittings of absorbance vs. time were carried out by non-linear regression. The kinetic model was obtained by performing linear regression. All analyses were carried out at least at a 95% significance level, using SigmaPlot™ 12. The experimental design for the response surface methodology and all associated statistics was accomplished with JMP™ 10.

3. Results and Discussion

3.1. Reaction Stoichiometries and the Effect of m-β-Cyclodextrin

The H-transfer reactions from polyphenols to DPPH can be very effectively assessed by monitoring the decay of A_{515}, using as molar absorptivity ε = 11,240 M^{-1} cm^{-1} and considering the purity of the reagent. The decay in A_{515} is initiated following addition of the antioxidant(s) to the DPPH solution [10] and potent antioxidants may provoke a rapid decay over 1–2 min, as a result of the transfer of H-atoms of the antioxidant that possess low C-H bond dissociation enthalpies (fast step). This step is followed by a much slower decline in A_{515}, which corresponds to the donation by the antioxidant(s) of the residual H-atoms (slow step) [11,12].

A simple hypothesis considers that an antioxidant AH bears n independent antioxidant subunits, which may all transfer a single H atom to DPPH with the same second-order rate constant k [4]. Such a background can be described as follows:

$$A = \varepsilon[\text{DPPH}] \tag{2}$$

$$R = -\frac{d}{dt}[\text{AH}] = -\frac{d}{dt}[\text{DPPH}] = k[\text{AH}][\text{DPPH}] \tag{3}$$

As mentioned above, the initial (fast) step of the reaction actually represents the donation of the most readily abstracted H-atoms from the antioxidant. Hence the initial reaction rate R_0 could be given as:

$$R_0 = k_1 c c_0 \tag{4}$$

where c is the initial antioxidant concentration, c_0 is the initial DPPH concentration and k_1 the reaction rate constant of the first abstracted H-atom. Therefore k would be $\frac{k_1}{n}$. Based on Beer-Lambert's law, the [DPPH] that reacts with the first H-atom may be represented as $A_0 - A_f$, where A_0 and A_f correspond to the initial and final A_{515}. Thus by replacing [DPPH] with $A_0 - A_f$, the Equation (3) can be transformed after integration, as follows:

$$\ln\left(\frac{1 - \frac{A_f}{A}}{1 - \frac{A_f}{A_0}}\right) = \frac{k_1 c}{\frac{A_0}{A_f} - 1} \tag{5}$$

The slope of the straight line obtained after plotting $\ln\left(\frac{1 - \frac{A_f}{A}}{1 - \frac{A_f}{A_0}}\right)$ as a function of time t, equals k_1.

On such a theoretical basis, OLL extracts obtained with or without m-β-CD were assayed with the aim to clarifying the role of m-β-CD on the antiradical effects exerted by OLL polyphenols. To this purpose, the extracts generated were adjusted at a final C_{TP} of 0.1 g L^{-1} and reaction with DPPH was monitored up to 2 minutes (Figure 1, upper plot). Determination of k_1 was performed by tracing the second order kinetics (Figure 1, lower plot) and gave values of 1.925 and 2.221 M^{-1} s^{-1}, for the extract obtained with DES/m-β-CD and DES, respectively. The slower reaction rate of the extract obtained with DES/m-β-CD compared with that obtained only with DES could not be interpreted as weaker antiradical activity, but only as a measure of the radical scavenging rate. This is because several polyphenolic antioxidants were shown to respond differently in kinetic and stoichiometric assays based on reaction with DPPH [13].
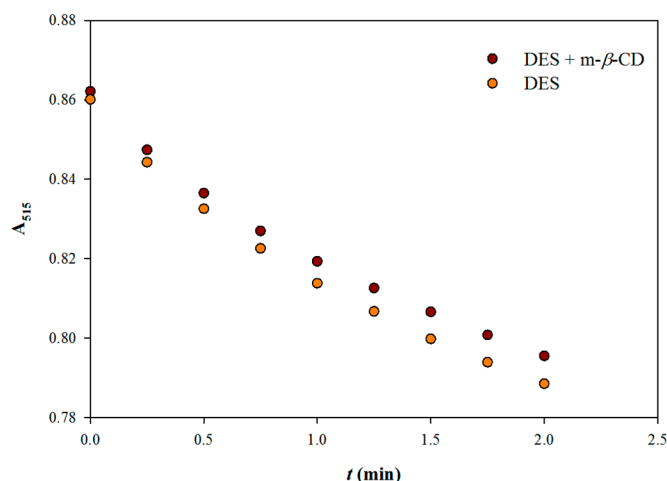
(a)
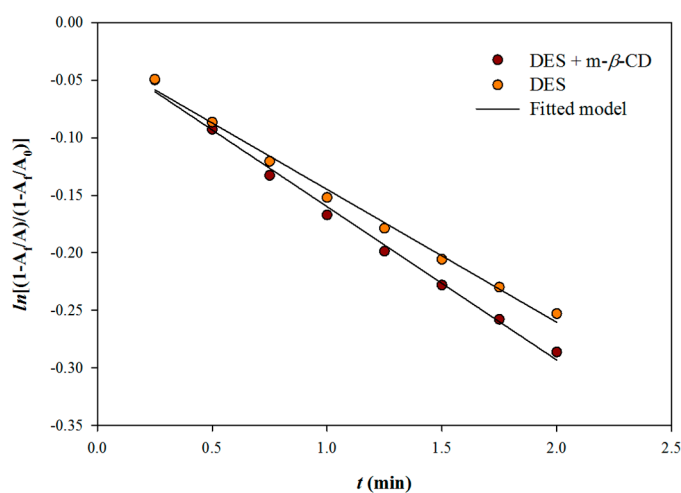
(b)

Figure 1. Fast reaction kinetics recorded upon mixing OLL extract with DPPH. C_{TP} was adjusted to 0.1 g L^{-1}, c_0 was 100 μmol L^{-1}. (**a**) Time course of A$_{515}$ decrease within the first two minutes; (**b**) Second-order kinetics.

Thus in order to have a more integrated picture, the total stoichiometries (n_t) were also determined by extending the reaction of each extract with DPPH, up to 21 min (Figure 2), using the following equation:

$$n_t = \frac{A_0 - A_f}{\varepsilon \, C_{TP}}$$ (6)

where C_{TP} is the total polyphenol concentration of the extracts, which as mentioned above was adjusted to 0.1 g GAE L^{-1}. Determination of n_t for the DES/m-β-CD and DES extracts gave corresponding values of 1.05×10^{-4} and 1.92×10^{-4} mol g^{-1}, indicating higher stoichiometry for the extract in the absence of m-β-CD.
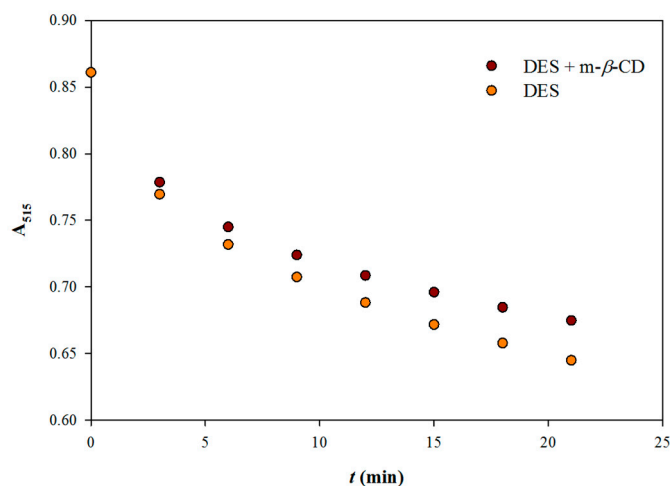
Figure 2. Extended reaction kinetics recorded upon mixing OLL extract with DPPH. C_{TP} was adjusted to 0.1 g L^{-1}, c_0 was 100 μmol L^{-1}.

Considering both k_1 and n_t, it could be argued that the extract obtained only with DES displayed superior radical scavenging potency. This finding contrasted previous ones, which demonstrated that polyphenol-containing extracts obtained with the aid of various cyclodextrins, such as Melissa officinalis leaf extract [14] and pomegranate fruit extract [15] exhibited increased antiradical activity. Likewise, simple phenolics such as rosmarinic acid [16], chlorogenic acid [17] and *trans*-resveratrol [18], and quercetin and glycosides therof [19], showed improved antioxidant properties when they were encapsulated in cyclodextrins. However, a detailed study on the inclusion complexes of tea catechins suggested that the nature of the polyphenol, as well as the orientation of the encapsulated molecule inside the cyclodextrin cavity, may affect antioxidant potency either negatively or positively [20].

Hydrophobicity would be an issue in this regard, because cyclodextrin/polyphenol inclusion complexes may be better stabilized with molecules having higher hydrophobicity [21]. On the other hand, hydrogen bonding could also greatly affect the antioxidant behaviour of the complexed polyphenols, because if there is extended intermolecular hydrogen bond development between the encapsulated and the host molecule, then radical scavenging is abrogated [22]. Such a claim was made for the apparent null effect of hydroxypropyl β-CD on caffeic acid antioxidant potency [23], where intramolecular hydrogen bond between the hydroxyl groups of the *o*-diphenol moiety would not allow for intermolecular interactions. On the basis of the above concepts, it could be supported that there might be a slower reaction for the OLL extract with DPPH in the presence of m-β-CD. This phenomenon might be ascribed to the inclusion of OLL polyphenols inside the m-β-CD cavity, which could slow down H-atom transfer to DPPH due to steric effects. Such a hypothesis would be concurred by the fact that complexation of oleuropein, the most abundant polyphenolic antioxidant in OLL, most probably involves deep insertion of the dihydroxyphenethyl moiety inside the cavity from its secondary side, as demonstrated for OLL interactions with β-cyclodextrin (β-CD) [21]. The formation of similar inclusion complexes with of β-CD has also been shown for chlorogenic acid [24].

3.2. Interactions with Ascorbic Acid

In an earlier study, interactions of polyphenol-containing extract with ascorbic acid (AA) were very effectively examined using response surface methodology [8]. It was proposed that by combining fixed amounts of AA and total polyphenols is a rather unilateral approach, providing limited information, whereas the simultaneous variation of concentrations within predetermined ranges may be more illustrative of the kind of interactions. This is because it has been demonstrated that the relevant amounts of AA and polyphenols in a mixture may significantly affect the overall antioxidant effect [25].

On these grounds, a response surface design was deployed to evaluate interactions between OLL extract and AA. Evaluation of term contribution by performing ANOVA showed that C_{TP} and C_{AA} and their quadratic terms exerted statistically significant effects on the A_{AR} of the mixtures. However, cross terms were non-significant in this regard ($p > 0.05$) and thus they were omitted from the models (mathematical equations), which are presented in their final form in Table 3. The use of the desirability function (Figure 3) enabled the determination of the settings recommended to achieve A_{AR} maximisation. Under these C_{TP} and C_{AA} combinations, maximum A_{AR} was estimated to be 168.98 $\pm$ 10.43 and 143.39 $\pm$ 11.18 µmol DPPH g^{-1} dw, for the extracts obtained with DES/m-β-CD and DES, respectively. As can be seen in Figure 4, the interaction pattern with AA was identical, but the difference of 15% in performance was a further confirmation that the OLL extract could act as a better radical scavenger in the absence of m-β-CD.

Table 3. Polynomial equations and statistical parameters describing the effect of the independent variables on the response (A_{AR}) for all OLL/AA mixtures tested.

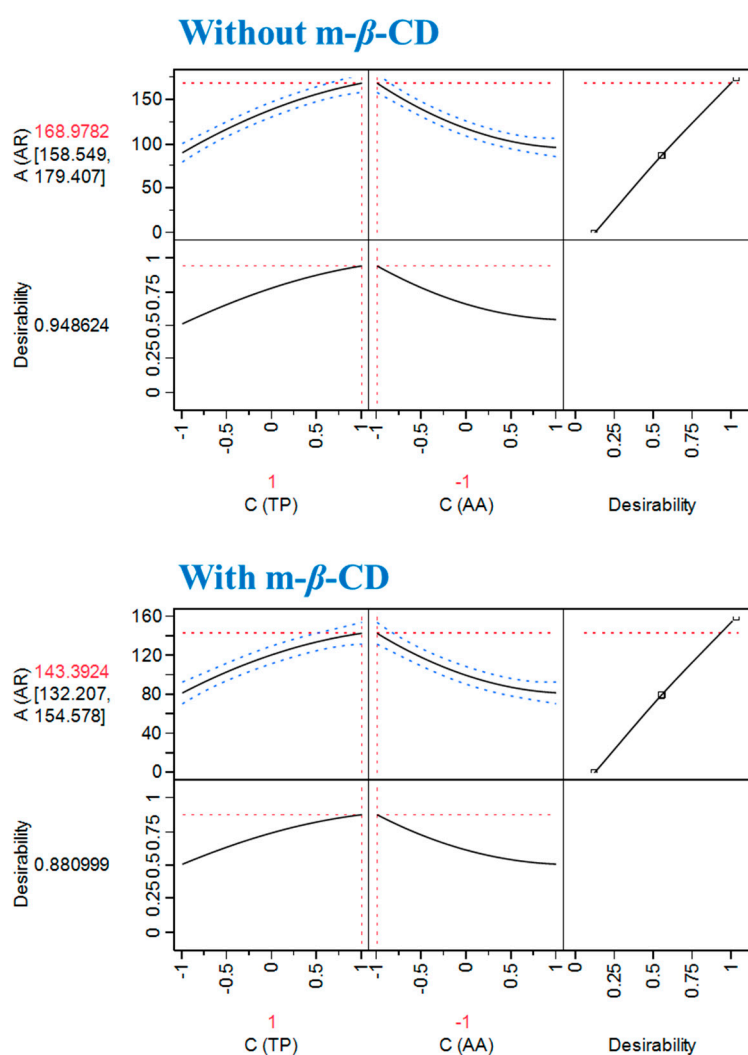| Antioxidant Test	2nd Order Polynomial Equations	R^2	p
Without m-β-CD	$88.36 + 38.96C_{TP} - 35.96C_{AA} - 9.72C_{TP}^2 + 15.18C_{AA}^2$	1.00	<0.0001
With m-β-CD	$76.49 + 32.13C_{TP} - 32.04C_{AA} - 8.64C_{TP}^2 + 12.84C_{AA}^2$	0.99	0.0002

Figure 3. Prediction profiler displaying the overall desirability of the model developed, after implementing response surface methodology.
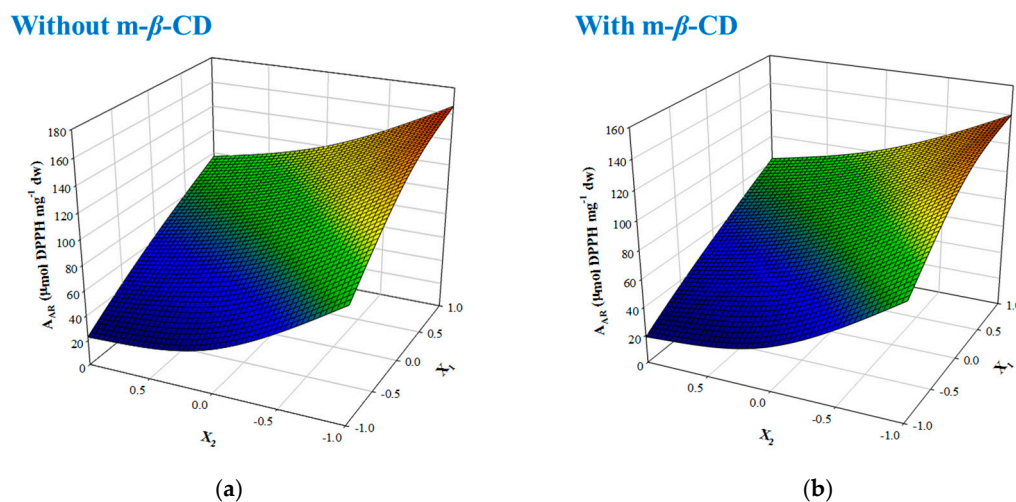
Without m-*β*-CD　　　　　　　　　　　　　　　**With m-*β*-CD**

(**a**)　　　　　　　　　　　　　　　　　　　　　(**b**)

Figure 4. 3D plots illustrating the effect of simultaneous variation of C_{TP} and C_{AA} on the A_{AR} in the (**a**) absence and (**b**) presence of m-*β*-CD.

The optimal estimated ratio of C_{TP}/C_{AA} to attain maximum A_{AR} was in both cases 7/1 (Table 4), which clearly indicated that the switching of C_{AA} to higher levels would not provide higher A_{AR}. This particular antiradical behaviour of the OLL extract/AA would most probably be ascribed to the nature of the major radical scavengers occurring in OLL extracts. In previous studies pertaining to A_{AR} of mixtures of polyphenol-containing extracts with AA, it was shown that grape stem extracts displayed the highest performance when combined with AA at a C_{TP}/C_{AA} ratio of 1/1 [8], but for grape seed extracts optimal ratio of 0.82/1 was also determined [26]. These findings suggested that the nature of principal antioxidant polyphenols in an extract might greatly define the antiradical effects. Investigations with pure polyphenols including quercetin, hesperetin and ferulic acid, revealed that interactions with AA at molar ratio 1:1 yielded antagonism [27], a behaviour that was confirmed by a following detailed study on reducing power, employing response surface methodology [28]. In this study, however, it was shown that the maximum response in hesperetin/AA mixtures was achieved at a molar ratio of 5.2/1, which clearly indicated that the nature of polyphenolic antioxidant may define the molar ratio that could yield maximum antioxidant effect, in combination with AA. Furthermore, other authors supported that compounds that are co-extracted with polyphenols from OLL could also interfere [29], but such an effect remains to be elucidated.

Table 4. Optimal, predicted concentration ratios and theoretically calculated maximum response for all mixtures tested, obtained from the implementation of design.

Antioxidant Test	Maximum Predicted Response	Optimal Ratio
Without m-*β*-CD	168.98 ± 10.43	70/10
With m-*β*-CD	143.39 ± 11.18	70/10

4. Conclusions

The examination of the effect of m-*β*-CD on the radical scavenging ability of OLL polyphenols was approached first by a kinetic assay to determine stoichiometry and second by a response surface methodology to investigate interactions with the natural antioxidant ascorbic acid. In spite of the references reporting on the enhancement of the antioxidant activity of polyphenols in the presence of various cyclodextrins, this study demonstrated that m-*β*-CD may lower the radical efficiency of OLL polyphenols. A similar outcome was observed in the interactions between OLL polyphenols and ascorbic acid, which was a further concurrence of the hindering effect of m-*β*-CD. Based on the evidence found by this investigation, it was postulated that the orientation of oleuropein—the

most abundant OLL antioxidant—inside the m-β-CD cavity, as well as the interactions between the encapsulated polyphenols and m-β-CD may be responsible for the phenomena observed. It is proposed that further studies with different β-cyclodextrin derivatives are required to fully clarify the mechanism of suppression (or enhancement) of the antiradical activity of polyphenol-containing extracts.

Author Contributions: Vassilis Athanasiadis carried out the experimental work; Stavros Lalas and Dimitris P. Makris conceived the theoretical background, handled and analysed the results and wrote the paper.

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

Nomenclature

A_0	initial absorbance at 515 nm
A_t	absorbance at 515 nm at any time t
A_{AR}	antiradical activity (μmol DPPH g^{-1} dry weight)
c_1	initial antioxidant concentration (mol L^{-1})
c_0	initial DPPH concentration (mol L^{-1})
C_{AA}	ascorbic acid concentration (mg L^{-1})
C_{TP}	total polyphenol concentration (mg L^{-1})
ε	molar absorptivity (M^{-1} cm^{-1})
k	second-order rate constant (M^{-1} s^{-1})
k_1	second-order rate constant of the first abstracted H-atom (M^{-1} s^{-1})
n_t	total stoichiometry (dimensionless)
R_0	initial reaction rate (M^{-1} s^{-1})
t	time (min)

Abbreviations

AA	ascorbic acid
DES	deep eutectic solvent
DPPH	2,2-diphenyl-1-picrylhydrazyl radical
HBA	hydrogen bond acceptor
HBD	hydrogen bond donor
m-β-CD	methyl β-cyclodextrin
OLL	olive leaves

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