Optimization of a green extraction method for the recovery of polyphenols from olive leaf using cyclodextrins and glycerin as co-solvents

Ioannis Mourtzinis¹ · Eleni Anastasopoulou¹ · Athanasios Petrou¹ ·
Spyros Grigorakis² · Dimitris Makris³ · Costas G. Biliaderis¹

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Abstract Olive leaf, an agricultural by-product, was studied for the valorization of its biophenols using green extraction techniques; i.e. non-toxic and eco-friendly extraction solvents were used, involving water and glycerol. 2-hydroxypropyl- β -cyclodextrin (CD), was also employed as an enhancer of the extraction, since cyclodextrins (CD's) are known to improve the extractability of olive leaf polyphenols by forming water soluble inclusion complexes. The process was optimized by implementing a central composite (Box-Behnken) experimental design and response surface methodology, taking into consideration the following independent variables: glycerol concentration (C_{gl}), CD concentration (C_{CD}) and temperature (T). The evaluation of the extraction model was based on two responses: the total polyphenol yield (Y_{TP}) and the antiradical activity (A_{AR}). Optimum values for the extraction process were obtained at 60% (w/v) glycerol content, $T = 60^\circ\text{C}$ and 7% (w/v) CD content. LC–MS analysis was also applied in order to characterize the polyphenolic composition of

extracts containing cyclodextrins. The main polyphenols present were oleuropein and oleuropein derivatives. Olive leaf aqueous extracts containing glycerol and cyclodextrins may be used as raw materials/ingredients for several end-users in the food, cosmetic and pharmaceutical industries.

Keywords Cyclodextrins · Polyphenols · Inclusion complex formation · LC–MS analysis · Extraction

Introduction

Olive leaves (*Olea europaea*), by-products of olive farming, is a rich and cheap source of polyphenols that could be extracted and valorized for the development of products that promote health and wellbeing (Rahmanian et al. 2015). The most important class of phenolics in olive leaves are secoiridoids. Oleuropein, the main secoiridoid, is a complex phenol present in large quantities in olive tree leaves as well as in low concentrations in olive oil and is responsible for the bitter taste and pungent aroma of olive oil. Olive leaves also contain several flavonoids (apigenin, kaempferol, luteolin) as well as simple phenolic compounds such as caffeic acid, tyrosol, hydroxytyrosol (Talhaoui et al. 2014). Studies have shown that oleuropein is an important biophenol, as it acts as an antitumor compound and also exhibits anti-ischemic, antioxidative, hypolipidemic, antiviral, antimicrobial, antiatherogenic, cardioprotective, antihypertensive and anti-inflammatory properties (Visioli and Galli 1998; Singh et al. 2008).

The exploitation of plant by-products by the food industry is gaining ground and presents indisputable advantages, as it is not only a low-cost source of functional ingredients but also constitutes an environmentally

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✉ Costas G. Biliaderis
biliader@agro.auth.gr

¹ Department of Food Science and Technology, Faculty of Agriculture, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

² Food Quality and Chemistry of Natural Products, Mediterranean Agronomic Institute of Chania (M.A.I.Ch.), 73100 Chania, Greece

³ School of Environment, University of the Aegean, Mitr. Ioakim Street, Myrina, 81400 Lemnos, Greece

friendly processing route, since it greatly simplifies waste disposal management strategies (Agourram et al. 2013; El-Baroty et al. 2014). Agro-wastes rich in phytochemicals have been already used as a natural source of antioxidants and antimicrobials in food products (Martins et al. 2014; Shah et al. 2014). Moreover, consumers tend to be rather skeptical lately towards chemical additives since consciousness has risen about toxicity and long-term detrimental health effects (Shah et al. 2014).

Phytochemicals from wastes and by-products generated during olive oil production processes have been obtained by conventional methods of extraction and also non-conventional methodologies involving the use of ultrasounds, microwaves, sub- and supercritical fluid extractions, pressurized liquid extraction, pulsed electric fields and high voltage electrical discharges, and targeting for high extraction yield, low cost and selectivity of extraction (Ahmad-Qasem et al. 2013; Rosello-Soto et al. 2015; Xynos et al. 2012).

Recently the principles of green engineering and green chemistry have been introduced in various extraction schemes with an aim to promote sustainable extraction processes, basically by using non-petroleum derived solvents and renewable plant residues as raw materials (Li et al. 2013). Glycerol a bio product of bio diesel industry could be considered as an ideal green non-conventional solvent for the extraction of polyphenols as it is non-toxic, non-flammable and has a high boiling point. Recent studies demonstrated that, aqueous glycerol mixtures might constitute a very suitable extraction medium for polyphenol recovery, since they possess a relatively low dielectric constant, which has been claimed to be a key characteristic regarding their solvency towards polyphenols that are otherwise sparingly soluble in pure water (Shehata et al. 2015). Cyclodextrins, a group of cyclic oligosaccharides (non-reducing molecules) which have the structure of a truncated cone (torus-like structures) and can act as host molecules in forming inclusion complexes with polyphenols (Đorđević et al. 2014; Pinho et al. 2014, 2015). Aqueous solutions of cyclodextrins can be considered as alternative, green solvents, since upon formation of complexes between the hydrophobic CD cavities and non-polar compounds (e.g. polyphenols), a reduction of the system's energy can be achieved (Chemat et al. 2012; Đorđević et al. 2014).

The formation of inclusion complexes of olive leaf polyphenols with β -cyclodextrin has been already confirmed with differential scanning calorimetry and nuclear magnetic resonance spectroscopy (Mourtzinou et al. 2007). Molecular encapsulation of olive leaf phenolic compounds into β -cyclodextrin could result in increased aqueous solubility due to changes in partitioning of the

compounds in the oil/water binary system (Rodis et al. 2002), improved protection against oxidation during storage, and, possibly, a greater bioavailability of the extractants (guest compounds). Encapsulated forms of olive leaf extract, that retain the properties of bioactive ingredients have been already tested for the replacement of synthetic antioxidants (Mohammadi et al. 2016).

The aim of this study was to perform an optimization of an extraction process for efficient recovery of polyphenols from olive leaves, using water/glycerol/2-hydroxypropyl- β -cyclodextrin ternary mixtures. The optimization was based on respond surface methodology that has been already used in order to optimize extraction of antioxidants (Singh et al. 2012); a Box-Behnken experimental design was adopted, and the responses measured were the total polyphenol yield (Y_{TP}) and the antiradical activity (A_{AR}) of the extracts.

Materials and methods

Chemicals and reagents

Folin-Ciocalteu phenol reagent was from Fluka (Sigma-Aldrich: Steinheim, Germany). 2-Hydroxypropyl- β -cyclodextrin (CD, average MW ~ 1460), gallic acid, trolox and the 2,2-diphenyl-picrylhydrazyl (DPPH) stable radical were procured from Sigma Chemical Co. (St. Louis, MO, USA.). Glycerol ($>99\%$) was procured from Fisher Scientific (New Jersey, NJ, USA).

Plant material

Olive leaves (*Olea europaea*), without apparent damages and infections, were bought from a local supermarket (Thessaloniki, Greece). Leaves were dried at $65\text{ }^{\circ}\text{C}$ for 48 h and grounded using a domestic blender (Bosch MMB 11R) and a cyclone sample mill (UDY, Fort Collins, USA), giving a powder with a particle size less than 0.3 mm. Dried and grounded olive leaves were stored at $-20\text{ }^{\circ}\text{C}$ until further use.

Extraction procedure

An amount of plant tissue fine powder was mixed with solvent (liquid-to-solid ratio 50 mL g^{-1}), composed of varying concentrations of water (40–100% w/v), glycerol (0–60% w/v) and CD (1–13% w/v) in a stoppered glass bottle. The material was subjected to extraction under continuous stirring at 600 rpm for 180 min in a water bath at three different temperatures ($40, 60, \text{ and } 80 \pm 1\text{ }^{\circ}\text{C}$). Following extraction, the dispersions were centrifuged in a

table centrifuge (Hermle Z300 K, Wehingen, Germany) at 5000 rpm for 10 min. The clear supernatant was stored at -20°C until used for further analysis.

Determination of total polyphenol yield (Y_{TP}) and antiradical activity (A_{AR})

The total polyphenol yield in the extracts (Y_{TP}) was determined according to a well-established protocol (Karakashov et al. 2015) using the Folin-Ciocalteu methodology; the results (Y_{TP}) were expressed as mg gallic acid equivalents (GAE) per g of dry olive leaf weight.

For A_{AR} determination, a previously described protocol was essentially applied (Bassil et al. 2005). Briefly, an aliquot of 0.025 mL of the sample was added to 0.975 mL DPPH solution (100 μM in MeOH), and the absorbance was read at $t = 0$ and $t = 30$ min. TroloxTM equivalents (mM TRE) were determined from linear regression, after plotting $\% \Delta A_{515}$ of known solutions of troloxTM against concentration, where

$$\% \Delta A_{515} = \frac{A_{515}^{t=0} - A_{515}^{t=30}}{A_{515}^{t=0}} \times 100 \quad (1)$$

Results were expressed as μmol TRE per g of dry olive leaf weight.

Experimental design

A 2^3 -full factorial, central composite design was used to identify the relationship between the response functions and process variables, as well as to determine those conditions that optimized extraction. The three independent variables or factors studied were 2-hydroxypropyl- β -cyclodextrin concentration (C_{CD}), varying between 1 and 13% (w/v), glycerol concentration (C_{gl}), varying between 0 and 60% (w/v) and temperature (T), varying between 40 and 80 $^{\circ}\text{C}$. Value ranges were chosen on the basis of preliminary experimentation and previous studies (Kyriakidou et al. 2016). The independent variables were coded at three levels, -1 , 0 and 1 (1, 7, 13 w/v for C_{CD} , 0, 30, 60 w/v for C_{gl} and 40, 60, 80 $^{\circ}\text{C}$ for T) according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i}, \quad x_i = 1, 2, 3 \quad (2)$$

where x_i and X_i are dimensionless, and the actual value 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. The extraction yield (Y_{TP}) and the antiradical activity (A_{AR}) were chosen as the dependent variables or responses because of their well-known dependencies on the extraction process conditions.

The data obtained were subjected to regression analysis using least square methodology to obtain equations that described the response values as a function of the independent variables (mathematical models). Analysis of variance (ANOVA) was used to assess the statistical significance of the model. Insignificant dependent terms ($p > 0.05$) were omitted from the regression models derived, through a “backward elimination” process. Contour plots were subsequently obtained using the fitted model.

Liquid chromatography–mass spectrometry

A previously used method was employed for LC–MS (Apostolakis et al. 2014); a Finnigan MAT Spectra System P4000 pump was used coupled with a UV6000LP diode array detector and a Finnigan AQA mass spectrometer. Analyses were carried out on a Superspher RP-18, 125×2 mm, 4 μm , column (Macherey–Nagel, Germany), coupled with a guard column packed with the same material, and maintained at 40 $^{\circ}\text{C}$. Analyses were carried out by employing electrospray ionisation (ESI) at the positive ion mode, with acquisition set at 12 and 50 eV, capillary voltage 4 kV, source voltage 4.9 kV, detector voltage 650 V and probe temperature 400 $^{\circ}\text{C}$. Eluent (A) and eluent (B) were 2.5% acetic acid and methanol, respectively. The flow rate was 0.33 mL/min, the sample concentration was 5 mg GAE g^{-1} dw and the elution programme used was as follows: 0–5 min, 0% B; 5–30 min, 100% B; 30–35 min, 100% B.

Statistical analysis

Extractions were repeated twice and all analytical determinations were carried out at least in triplicate. The values obtained were averaged. The experiment design and response surface statistics were performed with JMPTM 10.

Results and discussion

Optimization of the extraction was carried out by evaluating the effect of the three selected independent variables; i.e. CD concentration (C_{CD}), glycerol concentration (C_{gl}) and temperature (T). Time of the extraction, is also a crucial parameter during a solid/liquid extraction and it has been optimized previously for olive leaf extracts (Apostolakis et al. 2014). The values of the responses (Y_{TP} and A_{AR}) obtained experimentally were analyzed by multiple regression and after removal of the non-significant factors ($p > 0.05$). The significance of model fitting was assessed using the square coefficient of correlation (R^2), which was over than 0.93 ($p < 0.05$). This outcome clearly pointed to

a statistically significant match between observed and predicted responses, and that the models can predict the optimal experimental conditions with high reliability. The values of the independent process variables (X_1 :CD, X_2 : C_{gl} and X_3 :T) considered, as well as measured and predicted values for all responses, are presented in Table 1.

The polynomial equation of the Y_{TP} variable showed that there was a clear positive effect of C_{gl} and T and also significant interaction effects between the two variables. The influence of C_{CD} was clearly shown in the polynomial equation of A_{AR} . On the contrary, the quadratic term of C_{CD} had a negative impact in both cases of the dependent variables. In most plant extracts the values of Y_{TP} and A_{AR} are directly proportional; however, different patterns have been reported and are attributed to synergism and/or antagonism among constituents present in the extracts (Makris et al. 2007; Karvela et al. 2012). The evolution of Y_{TP} and A_{AR} as a function of simultaneous variation in the process variables are illustrated in the form of contour plots. As can be seen, maximization of Y_{TP} levels was observed when the glycerol content varied within 40–60%, whereas CD content and temperature showed optimum values for the extraction of olive leaf polyphenols at 7% (w/v) and 60 °C, respectively (Fig. 1a). A similar pattern was also noted with A_{AR} (Fig. 1b), confirming optimum values for the extraction process at 60% (w/v) glycerol content, T = 60 °C and 7% (w/v) CD content. The reason that the polyphenolic content decreased above 70 °C may

be attributed to the degradation or modification of polyphenols above this temperature (possible covalent interactions with other constituents in the plant cell matrices) (Volf et al. 2014). According to Attya et al. (2010), at temperature 80 °C the concentration of oleuropein was reduced to half after 6 h, whereas it was no longer present after 3 h heating at 230 °C. Regarding the glycerol content impact, a recent study showed similar results, where by increasing the amount of glycerol in the extraction solvent there was an increase in the polyphenolic content up to a maximum glycerol level of 90% (w/v) (Shehata et al. 2015). In the present study, due to the parallel usage of cyclodextrin, glycerol cannot be used in amounts higher than 60% as the extraction solvent mixture becomes very viscous and a certain amount of cyclodextrin becomes non-dissolvable. Overall, it appears that glycerol can be effective as an alternative green solvent for the extraction of polyphenols with improved efficiency than water when methanol or ethanol have to be avoided.

The optimization of CD content in the extraction medium is also crucial as the total amount of ingredients (of varying composition) that can form inclusion complexes with CD and thereby influence positively the extraction process cannot be estimated theoretically. Ligand inclusion in the CD cavity is a stoichiometric phenomenon and molecular inclusion between oleuropein and CD has a stoichiometry of 2:1 (Mourtzinou et al. 2007). On the other hand, other ingredients of the olive leaf extract may

Table 1 Measured and predicted value of Y_{TP} and A_{AR} , determined for individual design points, for the extractions performed with water/glycerol mixtures

Design point	Independent variables			Response (Y_{TP} , mg GAE g ⁻¹ dw)		Response (A_{AR} , μ molTRE g ⁻¹ dw)	
	X_1	X_2	X_3	Measured	Predicted	Measured	Predicted
1	-1	-1	-1	9.69	7.61	222.55	216.75
2	-1	-1	1	18.96	18.37	202.14	201.98
3	-1	1	-1	14.42	17.52	251.23	238.82
4	-1	1	1	56.78	55.10	311.49	316.14
5	1	-1	-1	19.51	20.74	235.67	231.96
6	1	-1	1	20.6	17.05	207	220.35
7	1	1	-1	22.05	22.19	276.5	277.60
8	1	1	1	43.69	45.317	351.35	358.09
9	-1	0	0	22.24	23.49	183.18	196.90
10	1	0	0	24.6	25.16	242.96	225.47
11	0	-1	0	30.24	35.23	276.5	272.82
12	0	1	0	57.51	54.32	352.81	352.72
13	0	0	-1	23.15	20.76	249.28	270.09
14	0	0	1	33.51	37.70	327.53	302.95
15	0	0	0	40.42	36.42	269.7	276.38
16	0	0	0	36.05	36.42	275.53	276.38

Fig. 1 Contour plots illustrating the effect of the independent variables examined on the **a** Y_{TP} and **b** A_{AR} . The *upper left, upper right and lower plots* show the effect of simultaneous variation of C_{gl} and C_{CD} , T and C_{CD} and T and C_{gl} , respectively

compete or promote inclusion complex formation between CD and the olive leaf main bioactives. In the present study, the HP- β -CD was selected in order to study the CD impact, as the β -CD, the most common cyclodextrin used in foods can be dissolved in water up to a limited concentration of 16 mM.

The mathematical models enable the determination of the optimal set of conditions and the maximum predicted Y_{TP} and A_{AR} values. Such determination was based on the simultaneous maximization of the desirability function,

which provided the optimal values for all process variables considered (Fig. 2). The maximum desirability (0.99) was thus achieved with $C_{CD} = 7\%$ (w/v), $C_{gl} = 60\%$ (w/v) and $T = 60^\circ\text{C}$. Under these conditions, the maximum values estimated were $Y_{TP} = 54.33 \text{ mg GAE g}^{-1} \text{ dw}$ and $A_{AR} = 352.72 \text{ μmol TRE g}^{-1} \text{ dw}$. The Y_{TP} value is significantly higher than those reported for olive leaf extracts obtained with water/glycerol mixtures without the inclusion of cyclodextrin as a co-solvent (Apostolakis et al. 2014). The addition of cyclodextrin in the extraction solvent

Fig. 2 Prediction profiler displaying the overall desirability of the model, following adjustment of the independent variables at their optimal values

Fig. 3 Polyphenolic profile of the extract obtained with 7% w/v CD content, 60% (w/v) glycerol content and temperature at 60 °C. The eluted compounds were monitored at 260 nm

Table 2 UV-vis and mass spectral characteristics of the main phytochemicals detected in the optimally obtained olive leaf extract

Peak	Rt (min)	λ_{max} (nm)	[M + H] ⁺	Other ions (<i>m/z</i>)	Compound
1	19.83	244, 274, 336	611	287 [M – 2 glucosyl units + H] ⁺	Luteolin diglucoside
2	21.10	252, 264, 348	449	287 [M – glucosyl unit + H] ⁺	Luteolin glucoside
3	23.47	254, 356	611	303 [M – rutinosyl unit + H] ⁺	Rutin (quercetin 3- <i>O</i> -rutinoside)
4	24.56	248, 280	541	563 [M + Na] ⁺ , 361 [M – glucosyl unit + H] ⁺ , 137 [hydroxytyrosyl unit] ⁺	Oleuropein isomer
5	26.08	252, 350	579	433 [M – rhamnosyl unit + H] ⁺ , 271 [M – rutinosyl unit + H] ⁺	Apigenin rutinoside
6	26.63	252, 350	433	271	Apigenin rhamnoside
7	27.29	268, 344	449	287 [M – glucosyl unit + H] ⁺	Luteolin glucoside
8	29.94	248, 280	541	563 [M + Na] ⁺ , 361 [M – glucosyl unit + H] ⁺ , 137 [hydroxytyrosyl unit] ⁺	Oleuropein
9	30.60	268, 344	449	287 [M – glucosyl unit + H] ⁺	Luteolin glucoside
10	32.24	252, 264, 352	625	287	Luteolin derivative
11	36.92	254, 264, 352	617	287	Luteolin derivative

has a similar effect in the extraction efficiency to that noted by increasing the extraction time for 2 h (Apostolakis et al. 2014).

The proposed methodology could be described as simultaneous extraction and encapsulation of olive leaf polyphenols. The formation of inclusion complexes between polyphenols and cyclodextrins, known as molecular encapsulation, does provide certain advantages over other conventional solvent-extraction processes, such as increased water solubility, protection against oxidation and protection against decomposition caused by heat or light. The formation of inclusion complexes have been also applied to isolated and individual molecules (Mourtzinis et al. 2007). Overall, the addition of cyclodextrin in the extraction media can lead to increased extraction efficiency, possibly due to inclusion complex formation.

Phytochemical profiling

The polyphenolic composition of olive leaf is affected by many factors as seasoning age, climatic conditions, and genetic factors (Ranalli et al. 2006). Moreover, the extraction conditions (temperature, time, solvent, drying of the leaves, particle size of the material) also affect the polyphenolic content of the final extract. Recently, olive leaf extracts were used in order to study the effect of the polyphenols on human health and especially chronic diseases (Lockyer et al. 2016). A prerequisite to such investigations is the usage of well characterized extracts in terms of polyphenolic composition and may often require profiling the entire bioactive ingredient pool.

In general, cyclodextrins seem to modify the solubility of bioactive ingredients in aqueous solutions leading to increased extractability of biophenols. Moreover, less soluble or even non-polar ingredients can be solubilized in aqueous media. In order to examine the extraction selectivity of the mixed solvent system used, water/glycerol/2-hydroxypropyl- β -cyclodextrin, LC–MS analysis of the extract was also performed. In Fig. 3 the polyphenolic profile of olive leaf extract obtained with 7% w/v CD concentration is given. The LC–MS analysis revealed the existence of at least 11 main constituents in the extracts. The compounds that were identified were typical of olive leaf phenolic constituents, including luteolin glucosides, luteolin derivatives, apigenin glucosides, apigenin, rutin and oleuropein (Table 2). Cyclodextrin inclusion into the solvent medium seems to enhance the solubility of the main polyphenols without altering the phenolic profile of a typical olive leaf extract in polar solvents (Apostolakis et al. 2014; Mylonaki et al. 2008).

Conclusion

A novel approach for more efficient extraction of polyphenols from olive leaves, leading to eco-friendly extracts and processes, is presented in this study. The findings indicate that it is possible to develop convenient extraction techniques of bioactive plant polyphenols with the adoption of green-extraction techniques and thus minimize the use of petrochemicals. Liquid extracts of plant polyphenols, involving aqueous media fortified with eco-friendly compatible co-solvents (e.g. glycerol,

cyclodextrins), to further enhance extractability of olive leaf phenolics could become attractive and safe alternative solvents of these compounds to fortify food products or to use as nutritional supplements for enhancement of the antioxidant and antimicrobial potency of a daily diet. However, all these materials (extractants) should be tested for their stability upon storage to maximize their effectiveness when incorporated in a real food matrix.

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