Design optimization study for oleuropein content and antioxidant activity of olive leaves extracts with environmentally friendly extraction techniques

Xynos Nikos*, Papaefstathiou Georgios, Psychis Marios, Argyropoulou Aikaterini, Aligiannis Nektarios, Gikas Evangelos, Skaltsounis Alexios-Leandros.

Department of Pharmacognosy and Natural Products Chemistry, Faculty of Pharmacy, University of Athens, Panepistimiopolis Zografou, 15771, Athens, Greece

***nikolasx79@gmail.com, Fax: 00302107274594**

Olea europaea (Oleaceae) leaves contain active metabolites, such as iridoids (oleuropein, 11-demethyloleuropein, ligstroside etc), flavonoids, triterpenes and phenolic compounds [1-4]. The present study is an effort to optimize their extraction procedure, producing high yield extracts, rich in oleuropein with high radical scavenging activity. 'Green' technologies [Pressurised Liquid Extraction (PLE) and Supercritical Fluid Extraction (SFE)] with environmentally friendly solvents like CO_2, Water and Ethanol were applied. The leaves were subjected to three extractions processes: i) solely SFE (using ethanol as co-solvent), ii) solely PLE and iii) SFE and PLE, sequentially. The effect of variant parameters was studied through carefully designed optimization process. Validation of the optimum extraction procedure was done with comparison of the extracts yield, quantitative determination of oleuropein with HPLC and evaluation of their scavenging activity in a DPPH assay. SFE using ethanol as co-solvent resulted to the extraction of oleuropein. SFE usage culminated in the removal of the non polar molecules (terpenes, fatty acids, waxes etc.) from the leaves, resulting in the enrichment of the extract in more polar compounds after the subsequent application of polar solvents in PLE, compared to the direct use of PLE. The SFE-PLE process gave higher amount of oleuropein, compared with PLE. All the samples demonstrated very good antioxidant activity in the DPPH assay. As a conclusion, a successive extraction of the leaves with SFE and PLE seems to be the optimal process given the aforementioned goal.

1. Introduction

Olea europaea is a species of the *Oleaceae* family, native to the coastal areas of the eastern Mediterranean Basin (the adjoining coastal areas of southeastern Europe. western Asia and northern Africa) as well as northern Iran at the south end of the Caspian Sea.

It is well known that its leaves contain many active metabolites, such as iridoids (oleuropein which is the main constituent, 11-demethyloleuropein, ligstroside etc.), flavonoids (luteolin and its glucosides, apigenin, rutin, and diosmetin), triterpenes (oleanolic acid, maslenic acid etc.) and phenolic compounds (hydroxytyrosol, tyrosol, caffeic acid etc.) [1-6]. Most of these compounds possess significant biological activities and have been used in traditional medicine for the prevention of

hypertension, arrhythmia and intestinal muscle spasms and for their diuretic properties [7, 8].
Percolation, maceration, hydrodistillation and Soxhlet extraction are probably the most established extraction techniques worldwide. The reasons for this universal adoption are simplicity and low cost. Nowadays their application is slowly starting to decline. More efficient, safer and cost effective techniques have come to the fore. Stricter regulations, increased consumer needs, product safety and demand for better product properties/characteristics led to the discovery of new and innovative technologies. The main advantages that characterize them as environmentally friendly are low solvent consumption and disposal, absence of residual toxic solvents from the final extracts and reduction of process time [10, 11, 12].
Probably the most applied "green" technologies are supercritical fluid extraction (SFE), and pressurized liquid extraction. The first one utilizes CO_2 in a supercritical state (P>71 bar and T> 31.1 ºC) as a -quite non-polar- solvent that is recycled and can be mixed with an appropriate co-solvent (EtOH usually) in order to increase polarity and solvent power. CO_2 (+ EtOH) are fed with a constant flow rate to the extractor. A decompression valve ensures that in the separators compartment the extract is no more soluble in CO_2 and it is thus collected as the latter is being recycled. This technique is mainly used for the recovery of non-polar to medium polarity compounds.
Pressurized liquid extraction (PLE) is a technique which uses classical solvents and performs a fully automated extraction under standard pressure and various controllable parameters like temperature, static extraction time, cycles of extraction, duration of purging the extraction cell at the end of each extraction etc. The filtration of the final extract is being performed automatically during its collection. Water and ethanol can be efficiently used in pressurized liquid extractions for the recovery of polar to medium polarity compounds, thus maintaining the environmentally-friendly properties.
Generally, "green" extraction technologies can, ideally, be combined in order to provide high-added value extracts. In the present study we aimed to develop olive leaves extracts with specific desired properties: high oleuropein content and/or high antioxidant activity, combined with maximum yield of extraction. For this purpose trials were performed with two different extraction methods (SFE, PLE) and a combination of them. Regarding PLE, a design optimization study was planned in order to maximize the desired properties for the final extracts.

2. Materials and methods

Plant material:

Olea europea leaves were collected in 2009 at the region of Attica and dried in a well ventilated area and shady place. The leaves were grinded and separated by a 3 mm sieve.

Supercritical fluid extraction (SFE):

The Supercritical device flow diagram used for these experiments is shown in Figure 1. This experimental apparatus (SFE-1-2 No 4218, SEPAREX F 54250 CHAMPIGNEULLES) is a semi – pilot scale devise and it is designed to allow the study of a wide range of conditions. It consists of a CO_2 tank, a liquid CO_2 pump (that can deliver up to 10 kg/hr), 2 extraction vessels (1L and 2L respectively) which are

both connected directly and parallel between them, 3 separators (with 200 mL capacity each), a co-solvent pump (with 24 mL/min maximum flow rate) and a cooling system.

Table 1. Specification of SFE-1-2 No 4218 apparatus.

Maximum operation pressure	350 bar
Temperature heating range	24 – 200 ^{o}C
Extractors x2	1 L and 2 L
Separators x3	200 mL each
CO_2 Pump flow rate	2,5 – 10 kg/h
Co-solvent Pump flow rate	2,5 – 10 kg/h
Cooling system	-25 to 40 ^{o}C
Building material	316 L stainless steel PTFE

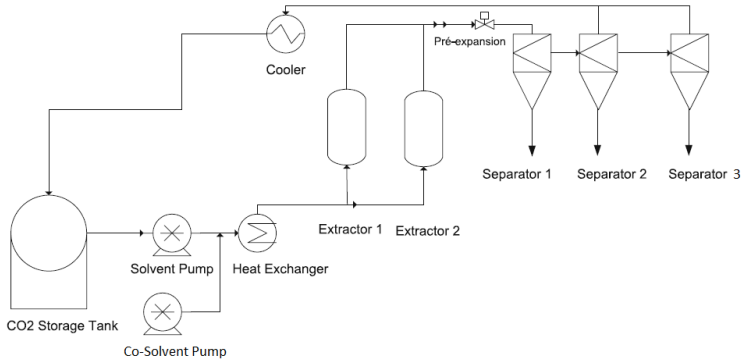


Figure 1: SFE flow diagram

Pressurized liquid extraction (PLE):

An Accelerated solvent extraction (ASE) 300 System with 33 mL stainless steel ASE vessels was used for the pressurized liquid extraction. Specifically 7.0 g of grinded olive leaves powder were placed into the extraction cells. The extraction cells were then placed into the carousel and the samples were extracted under specified condition. The pressure applied was kept at ~104 bar. The extracts were then evaporated to dryness using a rotary evaporator at 45 °C and/or lyophilisation.

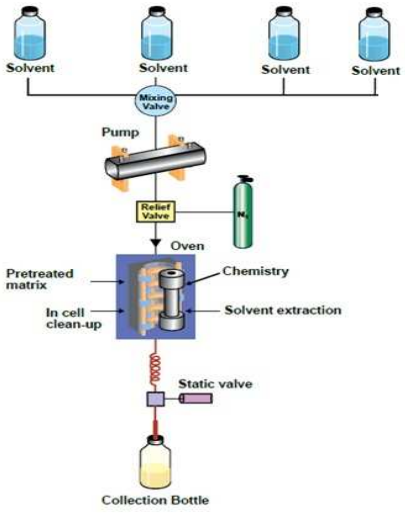


Figure 2: PLE flow diagram

HPLC analysis:

The quantitative determination of oleuropein was performed in a HPLC-DAD system. A Thermo-Finnigan equipment coupled with a PDA Spectra UV6000LP, P4000 Pump, AS3000 Autosampler and SN4000 Controller. A gradient method with two solvents (A. H_2O + 1% Acetic Acid and B. MeOH) was used. The flow rate was set at 1 ml/min and the elution program followed was: 0-2 linear gradient to 5% B; 2-10 min linear gradient to 25% B; 10-20 min linear gradient to 40% B; 20-30 min linear gradient to 50% B; 30-34 min 50% B isocratic; 34-45 min linear gradient to 90% B; 45-50 90% B isocratic; 50-60 min linear gradient to 100% B; 60-65 min 100% B isocratic. For the detection of oleuropein the PDA was set at 248 nm and the column used was RP C_{18} Supelco (250 x 4.6 mm, i.d., 5.0 μm).

DPPH assay:

The DPPH assay was based on the publication of Lee et al. (1998), with modifications. Test reaction mixtures were prepared by adding 10 μL of plant extract (diluted in DMSO) with 190 μL of DPPH solution (12.4 mg DPPH in 100 mL of ethanol). The resulting mixture was incubated for 30 min at 25^{o}C and the absorbance was measured at 517 nm [A_{517} (sample)]. For the preparation of the control, 10 μL of DMSO were added to 190 μL of DPPH solution. After incubation for 30 min at 25^{o}C the absorbance was measured at 517 nm [A_{517} (control)].

The inhibition of the DPPH radical was calculated:

$$\% \text{ Inhibition} = \{[A_{517} \text{ (control)} - A_{517} \text{ (sample)}] / A_{517} \text{ (control)}\} * 100$$

The IC_{50} was defined as the concentration of the plant extract required to inhibit 50% of the DPPH free radical.

Experimental design:

In the present work, a full-set of optimization procedure has been employed and applied with the use of Design Expert®. Due to the number of parameters involved in PLE extractions, each one of them reporting different behavior on the response of interest, initially a screening experimental design approach has been performed in order to decide on the most influential parameters, in terms of yield, oleuropein content and radical scavenging activity of the dry extract. Our aim was to minimize the number of experiments needed to optimize the responses under evaluation. A Plackett-Burman factorial design was applied. These designs are used to explore n-dimension experimental space using n+1 experiments. These experiments are regularly used when the number of parameters potentially contributing to the model are more than seven. The main characteristic of such designs is that the main effects are orthogonal between them and they are only partially aliased with higher order interactions, which differentiated them from the resolution three-fractional factorial designs (main effects are aliased with two-factor interactions). In the present case, 11 factors have been employed leading to a total of 12 experiments (n+1). Each factor's values (low and high) are equally distributed throughout all the experiments, namely in 6 (+1) and in 6 (-1). Each main effect can be determined by the following equation:

$$Effect = 1/6\,[\Sigma_{(y+1\ level)} - \Sigma_{(y-1\ level)}]$$

The Plackett-Burman screening procedure will indicate the main factors influencing the measured responses. These factors in turn will be used as input for constructing a model for optimization of the process in respect of the aforementioned responses, employing Response Surface Methodology (RSM).
The central composite design (CCD) approach has been used for modeling the responses generated by the Plackett-Burman design. Such an experiment employees the standard 2k factorial points originating from the center, along with 2k axially-spaced points. A variant of CCD is the circumscribed design in which the axial points are chosen such that they allow rotatability, which ensures that the variance of the model prediction is constant at all points equidistant from the design center. The center points could be replicated allowing an estimation of the experimental error (noise). Such a model permits the numerical optimization in order to find the desired solutions of the optimization procedure. Each parameter is estimated in five levels, namely ±1, ±α, and the center point.

3. Results

I. Optimization of the PLE process

Eleven parameters have been considered in the screening design (low and high value for each). All these are summarized in the following table, along with the designed series of experiments and the results for the various responses:

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9	Factor 10	Factor 11	Response 1	Response 2	Response 3	
Run	**EtOH**	**Temp.**	**Static time**	**Cycles**	**Flush**	**Purge**	**Pre-heat**	**Cell Vol.**	**Dummy1**	**Dummy2**	**Dummy3**	**Yield**	**Oleuropein**	**DPPH**	**Recovery**
	%	°C	min	No	%	sec	sec	ml				%	%	IC50 (μg/ml)	
1	100	190	25	1	40	60	180	33	1	1	-1	50,7	10,6	131,0	7,4
2	0	40	5	1	40	60	60	100	-1	-1	-1	26,6	11,2	157,5	4,0
3	100	40	5	1	100	60	180	100	-1	1	1	24,3	14,3	125,9	4,6
4	0	40	25	1	100	180	60	100	1	1	-1	29,9	10,7	126,0	4,4
5	100	40	25	3	40	180	180	100	-1	-1	-1	31,4	15,7	118,4	6,6
6	0	190	25	3	40	60	60	100	-1	1	1	53,0	11,4	159,1	8,2
7	100	40	25	3	100	60	60	33	1	-1	1	33,4	18,4	151,3	8,2
8	0	190	25	1	100	180	180	33	-1	-1	1	53,3	10,0	138,5	7,2
9	100	190	5	3	100	180	60	33	-1	1	-1	51,3	12,0	138,2	8,3
10	100	190	5	1	40	180	60	100	1	-1	1	46,0	9,3	122,5	6,3
11	0	40	5	3	40	180	180	33	1	1	1	30,3	11,7	146,9	4,8
12	0	190	5	3	100	60	180	100	1	-1	-1	49,9	10,7	144,7	7,2

*g of oleuropein extacted from 100g of olive leaves

Three responses have been taken under consideration, namely yield (%), oleuropein content (%) and radical scavenging activity (IC_{50} at DPPH test). In order to identify the main effects the Pareto chart for the influence on the studied parameters has been used. The size of effects of each parameter is proportional to the height of the bar. As shown, only 3 parameters are statistically significant at a level of p=0.05: Temperature, EtOH (%), Extraction cycles and those three have been used to construct the model. In this case the rest of the parameters have been used to estimate the experimental error.

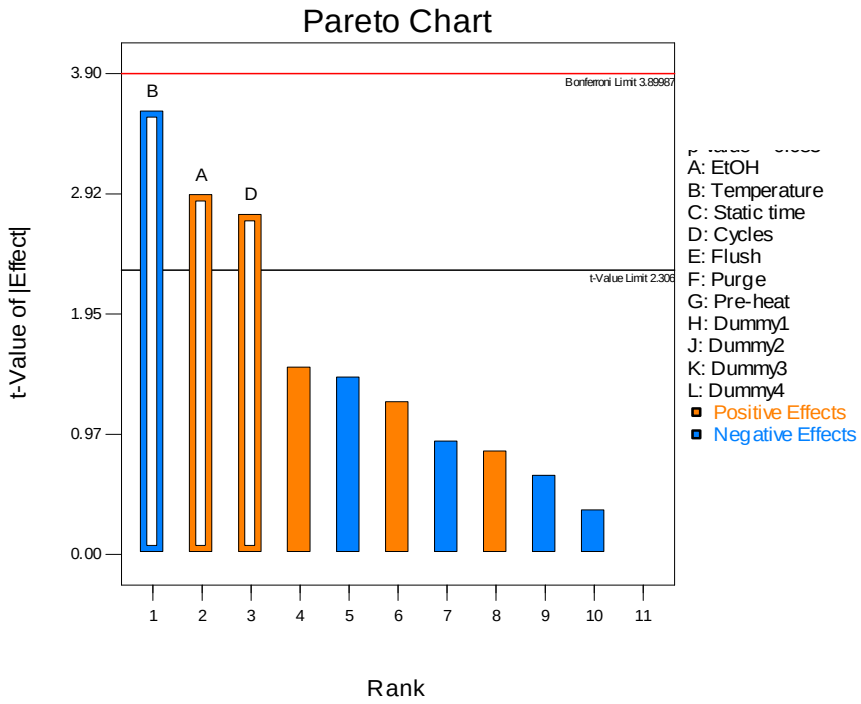


Figure 3: Pareto chart for oleuropein analysis

The ANOVA table confirms the significance of the model as well as of its individual factor at the level of p=0.05. The normal probability test does not indicate the presence of any outliers.

Concerning the other two measured responses (yield and radical scavenging activity), the same analysis has been applied in order to decide on the most influential parameters. The Pareto chart for the influence on the studied parameters has been used once again. Summarized below are the most influential parameters for each response:

- EtOH (%), temperature and extraction cycles seem to influence more the oleuropein content.
- Temperature, static time and cycles of extraction seem to influence more the extraction yield.
- EtOH (%), seems to be the only main influential parameter on the DPPH activity of the extracts.

As the main objective of the project was to optimize the oleuropein content of the dry extract but secondarily the yield and radical scavenging activity, we considered as most important parameters of extraction the following: EtOH (%), temperature and extraction cycles. The CCD applied for the optimization for two numerical [EtOH (%), and Temperature both varied over 5 levels] and one categorical (Cycles of extraction, varied over 2 levels) factors, comprised 22 experiments under the conditions summarized in the table below:

	Factor 1	Factor 2	Factor 3	Response 1	Response 2		Response 3
Run	**A:Temperature**	**B:EtOH**	**C:Cycles**	**Yield**	**OE**	**Recovery***	**DPPH**
	°C	%	No	%	%		IC50 (μg/ml)
1	115,0	0,0	3	32,9	16	5,25	141,6
2	115,0	50,0	3	36,7	21	7,70	127,6
3	115,0	50,0	3	27,9	21,3	5,95	124,7
4	115,0	50,0	3	30	18,1	5,44	124,9
5	40,0	50,0	1	37,1	15,6	5,78	138,0
6	168,0	85,4	1	42,9	20,7	8,89	133,4
7	62,0	85,4	1	16,7	20,8	3,47	105,6
8	115,0	50,0	1	23,8	22,9	2,16	131,3
9	115,0	50,0	1	23,9	22,8	5,45	127,7
10	62,0	14,6	1	14,7	18,4	2,70	167,6
11	168,0	14,6	3	28,3	13,5	3,81	132,9
12	168,0	14,6	1	37,3	20,6	7,69	150,8
13	62,0	85,4	3	19,7	19,7	3,88	133,5
14	190,0	50,0	1	21,6	31,8	6,86	128,7
15	168,0	85,4	3	48,3	19	9,18	127,4
16	115,0	50,0	1	23,85	26,55	6,33	141,2
17	115,0	100,0	1	25,4	15,5	3,95	144,7
18	62,0	14,6	3	18,4	17,5	3,22	157,7
19	190,0	50,0	3	53,9	12,7	6,83	138,2
20	115,0	0,0	1	21,6	11,3	2,43	157,7
21	40,0	50,0	3	14,6	16,8	2,45	140,1
22	115,0	100,0	3	27,4	17,3	4,75	135,2

*g of oleuropein extracted from 100g of olive leaves

The measured responses were three: yield (%), oleuropein (%) and IC_{50} for DPPH. Four models were considered, namely linear, linear + two-factor interactions, linear + two-factor interactions + quadratic terms, linear + two-factor interactions + quadratic terms + cubic terms.

It was found that, concerning the extraction yield, the model was linear with temperature being the only significant term (model p-value=0.0022). The respective equation for three cycles is:

*Yield =12.00111+ (0.14255*Temperature) + (0.046838*EtOH)*

The normal probability plot shows no outliers.

For the oleuropein content the model was linear + two-factor interactions + quadratic terms with significant terms the cycles, the interaction between temperature and cycles and quadratic term of EtOH (model p-value=0,0420). The respective equation for three cycles is:

*OE=10.13108+(0.069715*Temperature)+(0.28972*EtOH)+(6.66667E-005*Temperature * EtOH)-(4.25185E-004* Temperature^2)-(2.63667E-003* EtOH^2)*

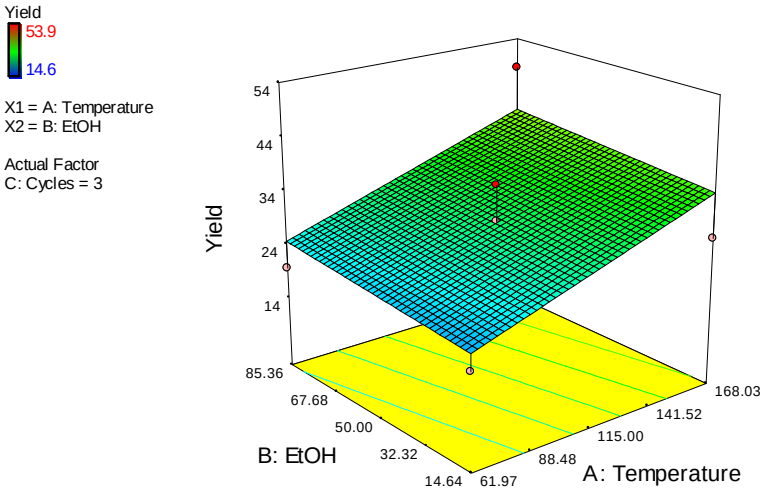
Similarly in this case no outliers have been detected.

Finally, for the DPPH IC_{50} the model was again linear + two-factor interactions + quadratic terms with significant terms the EtOH, the interaction between the temperature and EtOH as well as the quadratic term of EtOH (model p-value=0,0137). The respective equation for three cycles is :

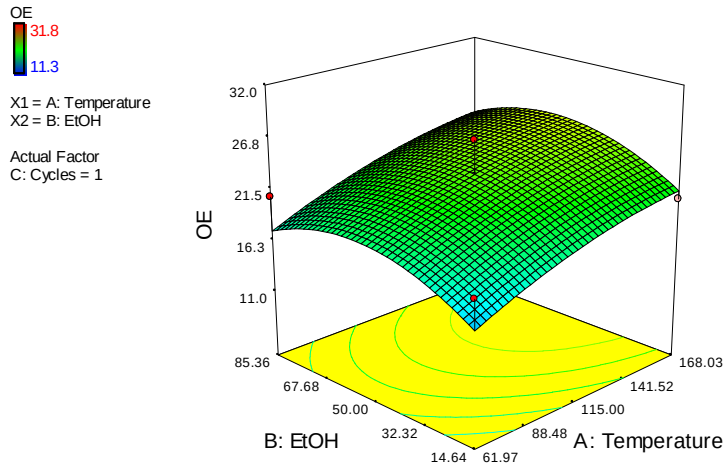
*DPPH IC_{50}=195.55030-(0.52411*Temperature)-(1.19346*EtOH)+(4.21850E-003*Temperature * EtOH)+(1.01731E-003*Temperature^2)+(5.71896E-003* EtOH^2)*

For all models the predicted studentized residual values did not exceed the value of three, which signifies that the models are reliable.

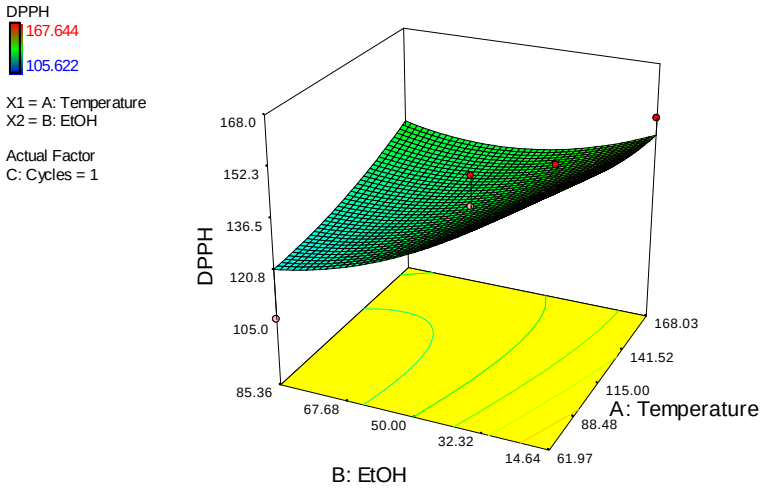
The response surface for the three measured responses are shown in the 3D diagrams below, along with the best two solutions proposed for optimized response (predicted value) each time:



Solution	Temperature °C	EtOH%	Cycles	Predicted Yield%
1	190.00	100.00	3	43.8
2	190.00	85.30	3	43.1



Solution	Temperature°C	EtOH %	Cycles	Predicted Oleuropein %
1	190.00	56.96	1	26.1
2	81.76	56.29	3	21.1



Solution	Temperature °C	EtOH %	Cycles	Predicted DPPH (IC_{50})
1	40.00	100.00	1	118.8
2	175.32	39.69	3	125.9

In the next step the numerical method has been applied for the simultaneous optimization of all three responses, maximizing the yield and oleuropein content and minimizing the DPPH IC_{50}. The lower and upper limits for each of the aforementioned parameters were:

- Yield : 30% and 55%
- Oleuropein content : 11.3% and 31.8%
- DPPH IC_{50} : 90 μg/ml and 130 μg/ml

After applying this methodology, two solutions have been found, along with the predicted values for the measurable responses:

Solution	Temperature	EtOH	Cycles	Yield	OE	Recovery*	DPPH
1	173.10	47.6	3	38.9	17.8	6,92	126.2
2	139.23	74.9	1	30.9	24.4	7,54	129.5

*g of oleuropein extracted from 100g of olive leaves

II. SFE of olive leaves

Supercritical CO_2 extraction was performed in the following conditions:

- P = 300 bar
- T = 50ºC
- EtOH was used as co-solvent

It was designed in two stages: In a first step, up to Ratio 120 ($Ratio = \frac{Mass\ of\ CO2(kg)}{Mass\ of\ olive\ leaves\ (kg)}$) , the flow rate of EtOH was 5% (w/w) to CO_2. Consequently, up to Ratio 290 the flow rate of EtOH was 20% (w/w) to CO_2. The results for this experiment are summarized in the following table:

SAMPLE	DETAILS	% Yield	% Oleuropein	Recovery*	DPPH IC50 (μg/ml)
SFE 1	CO2+5%EtOH	14,7	0,58	0,09	752,1
SFE 2	CO2+20%EtOH	17,0	30,00	5,04	113,9

*g of oleuropein extracted from 100g of olive leaves

III. SFE-PLE of olive leaves

The experimental procedure set up for the optimal recovery of oleuropein from the olive leaves was designed as follows:

- In the first step olive leaves were extracted with supercritical CO_2 + 5% EtOH for the removal of undesirable compounds, such as chlorophylls, waxes etc.
- In the second step, olive leaves' residue was submitted to PLE under the conditions that were found to be optimal for the recovery of oleuropein from the olive leaves, as depicted by the previously mentioned design optimization study :

Solution	Temperature°C	EtOH %	Cycles
1	190.00	56.96	1
2	81.76	56.29	3

The results of the two extractions are summarized in the table below:

SAMPLE	% Yield	% Oleuropein	Recovery*	DPPH IC50 (μg/ml)
SFE-ASE 1	50,1	20,4	10,21	135,7
SFE-ASE 2	36,8	24,0	8,84	119,9

*g of oleuropein extracted from 100g of olive leaves

IV. Conclusions

It is evident that, depending on the more desirable characteristics of an extract from olive leaves, various methodologies can be proposed in terms of extraction procedures, using only environmentally friendly technologies. Three different basic schemes have been investigated: i) PLE, ii) SFE and iii) SFE-PLE. The responses that were evaluated were the dry yield of the extraction, the oleuropein content of the dry extract and the inhibition concentration (IC_{50}) for the DPPH free radical.

As for the IC_{50}, even though variations have been noted among the obtained extracts, all of them exhibited good antioxidant activity and the ratio of max. to min. values among the extracts did not vary much. Concerning the yield and the oleuropein content, those two responses in combination determine the "recovery" of oleuropein from the olive leaves. It represents the grams of oleuropein extracted from 100 grams of olive leaves. This was the comparative value of choice in order to assess the different extracts and the procedures employed. The comparison shows that among the three procedures for extracting oleuropein, the recovery was clearly better when SFE and PLE were used sequentially reaching

10.21%. This was better than SFE solely (5.04%) and ASE solely (9.18%). Moreover, the physicochemical characteristics of the SFE - PLE extract were advantageous regarding further processing, e.g. cyclodextrin complexation and formulation due to its better solubility in water than the SFE extract, which additionally contains chlorophylls. As a next step, optimization of the parameters of the SFE-PLE procedure for best recovery and antioxidant activity must be envisaged.

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