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

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Introduction

Olea europaea (Oleaceae) leaves contain bioactive metabolites, such as iridoids, flavonoids, triterpenes and phenolic compounds [1-6]. The present study is an effort to optimize their extraction procedure and obtaining high yield extracts, rich in oleuropein with high radical scavenging activity. 'Green' technologies [Pressurised Liquid Extraction (PLE) and Supercritical Fluid Extraction (SFE)] and environmentally friendly solvents like CO_2, water and ethanol were used. These technologies were applied as such, as well as in combination in order to optimize certain characteristics of the extract. 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, content of oleuropein with HPLC and radical scavenging activity in a DPPH assay.

Plant material and extraction apparatuses

Olea europea leaves were collected in 2009 from the region of Attica and dried in a well ventilated and shady place. The leaves were grinded and separated by a 3 mm sieve. The supercritical fluid extraction (SFE) apparatus (Separex 1-2) is a semi – pilot scale device and it is designed to allow the study of a wide range of conditions. Also, an accelerated solvent extraction apparatus (Dionex ASE 300) was used for the pressurized liquid extraction (PLE).

Experimental design

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. 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 indicated the main factors influencing the measured responses. These factors have been 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.

SFE of olive leaves

Supercritical CO_2 extraction was implemented in order to obtain an extract rich in oleuropein that possess high antioxidant activity. The following conditions were applied:

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

The procedure was designed in two stages: In the first step, up to Ratio 120*, the flow rate of EtOH was 5% (w/w) to CO_2. Afterwards, the flow rate of EtOH raised to 20% (w/w) to CO_2 until Ratio 290. The purpose was to remove, in a first step, the lipophilic constituents of the leaves as well as a significant part of the chlorophylls. This was based on previous trials that had been conducted, which showed that addition of up to 5% of EtOH (mass) in the supercritical CO_2 does not lead to the extraction of oleuropein. The results for this experiment are summarized in the following table:

SAMPLE	DETAILS	% Yield	% Oleuropein	Recovery**	DPPH IC_{50} (μg/ml)
SFE 1	CO2+5%EtOH	14,70	0,00	0,00	752,1
SFE 2	CO2+20%EtOH	17,00	30,00	5,04	113,9

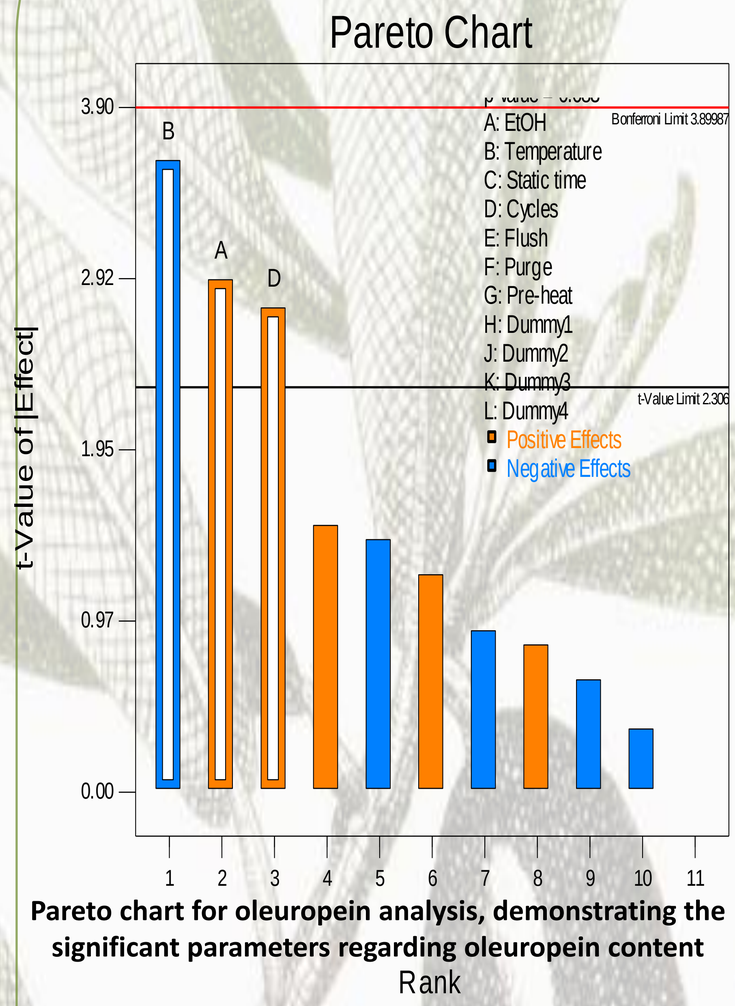
$$*Ratio = \frac{Mass\ of\ CO2(kg)}{Mass\ of\ olive\ leaves\ (kg)}$$

**g of oleuropein obtained from 100 g of olive leaves

PLE of olive leaves

For the PLE, an optimization process was designed. It comprised of 2 stages:

I. Screening procedure: Plackett Burman design



Pareto chart for oleuropein analysis, demonstrating the significant parameters regarding oleuropein content

Eleven parameters have been considered in the screening design (low and high value for each). Thus, a series of 24 experiments were performed in order to decide on the significant parameters that affect the extraction.

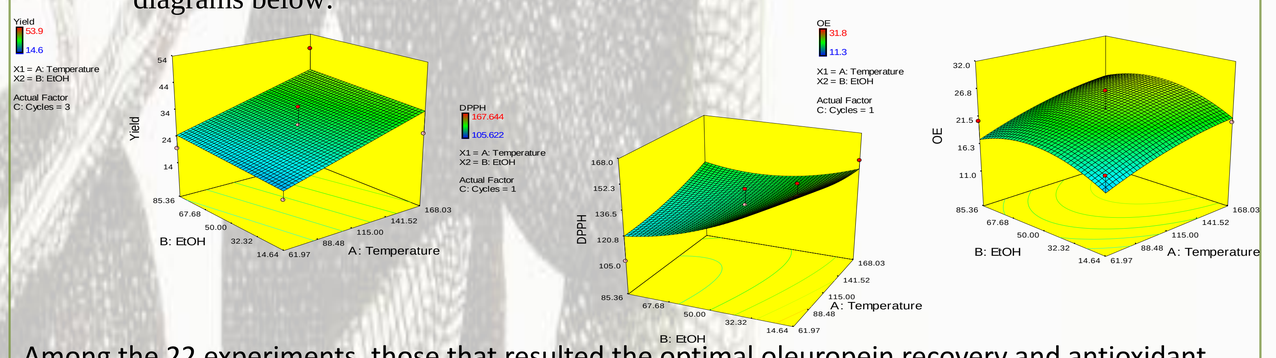
Three responses have been taken under consideration, namely yield (%), oleuropein content (%) and radical scavenging activity (IC_{50} at DPPH test). In order to identify the main effects the Pareto chart for the influence on the studied parameters has been used. The effects of each parameter is proportional to the height of the bar. Below are the parameters that are statistically significant at a level of p=0.05:

- 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 (%), is the only main influential parameter on the DPPH activity of the extracts.

As a consequence, temperature, EtOH (%) and extraction cycles have been used to construct the model.

II. Optimization: Central composite design (CCD)

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. The response surface that resulted from the 22-experiments (Run 1-22) CCD for the three measured responses are shown in the 3D diagrams below:



Among the 22 experiments, those that resulted the optimal oleuropein recovery and antioxidant activity respectively are presented in the following table :

SAMPLE	DETAILS	% YIELD	%OLEUROPEIN	RECOVERY	DPPH IC_{50} (μg/ml)
RUN 15	168°C, 85% EtOH,3 Cycles	48,30	19,01	9,18	127,40
RUN 7	62°C, 85% EtOH,1 Cycle	16,70	20,78	3,47	105,62

Sequential 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 the undesirable compounds, such as chlorophylls, lipids, 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/Sample	Temperature (°C)	EtOH (%)	Cycles
1/SFE-ASE 1	190.00	56.96	1
2/SFE-ASE2	81.76	56.29	3

The results of the two extractions that were depicted by the statistical model 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

Conclusions

Three different basic extraction schemes have been investigated: i) PLE, ii) SFE and iii) SFE-PLE sequentially. 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}, all of the obtained extracts exhibited good antioxidant activity, and the ratio of max. to min. values among them did not vary significantly, as it can be seen in the previous tables. 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 PLE solely (9.18%).

References

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