Application of response surface methodology for the optimization of supercritical CO_2 extraction of oil from olive paste: yield, content of bioactive molecules and biological effects *in vivo*.

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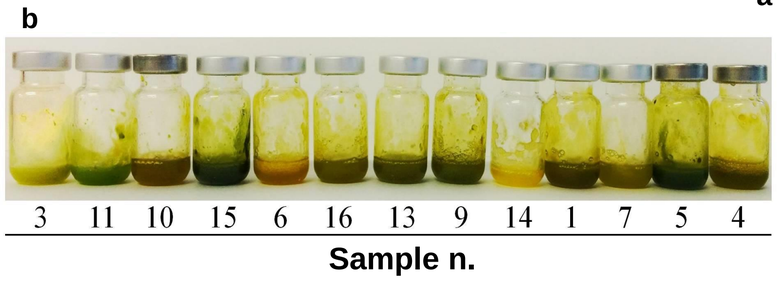
The modern two-phase technology of the **Leopard** series for olive oil industrial extraction developed by the **Pieralisi Group** (Pieralisi Maip SpA., Italy) generates large quantities of a novel by-product (**olive paste**, **OP**) constituted of the partially defatted wet drupe pulp without any traces of the kernel (**Fig. 1**). **OP** is rich in lipophilic and hydrophilic molecules able to exert beneficial effects on human health, including triterpenic acids, phytosterols, tocopherols, carotenoids, polyphenols, minerals and fibers.[1] In the perspective of by-product valorization through a **modern biorefinery approach**, it is worth noting that **freeze-dried OP** contains more than 10% oil characterized by a well-balanced lipid profile, rich in mono and polyunsaturated fatty acids, and a very good oxidative stability, due to the high concentration of fat-soluble antioxidants. This makes **OP oil** particularly suitable as **functional ingredient** for food/feed industry, as well as for the formulation of nutraceutical, cosmeceutical and pharmaceutical products.

*Fig. 1: Schematic representation of **OP** processing and extraction by SC-CO_2.*

Supercritical carbon dioxide (**SC-CO_2**) is a green and environmentally friendly technology for the effective extraction of high-value natural molecules. It is gaining a foothold in industrial production of **solvent-free vegetable oils** and has been also applied to concentrate oil products of lipophilic micronutrients (e.g. vitamin E in soybean and olive oils).[2] Nevertheless, the industrial application of SC-CO_2 technology requires a careful optimization of the operative parameters to make the process efficient from both an economic and a productive point of view**. In this work, a response surface methodology approach, based on the Box-Behnken Design, was used to determine the optimal parameters of pressure, temperature and time to simultaneously maximize oil production from the freeze-dried OP of cultivar Leccino and concentrate the most abundant lipophilic bioactives (Fig. 2).**



a

Sample n.	Extraction n.	Temperature (°C)	Pressure (Bar)	Time (min)	Oil yield (%)
3	1	40	600	60	16.9
11	2	60	300	90	17.0
10	3	60	600	30	17.7
15	4	60	450	60	17.8
6	5	80	450	30	17.1
16	6	60	450	60	17.6
13	7	60	450	60	17.5
9	8	60	300	30	9.1
14	9	60	450	60	18.1
1	10	40	300	60	8.7
7	11	40	450	90	16.9
5	12	40	450	30	16.4
4	13	80	600	60	15.1
17	14	60	450	60	18.3
8	15	80	450	90	18.0
12	16	60	600	90	16.4
2	17	80	300	60	10.3

*Fig. 2: Randomized Box-Behnken design matrix and experimental **OP oil yield** (a). In all extractions the CO_2 flux was maintained constant at approx. 0.218 kg·h^{-1}. Appearance of the **OP oils** extracted in the different operative conditions of temperature, pressure, and time.*

The experimental data were fitted to a **second-order polynomial equation** by multiple regression analysis, and examined using appropriate statistical methods (**Fig. 3**). The 3-D response surface plots derived from the mathematical models (**Fig. 4**) were applied to determine the **optimal extraction parameters**, which resulted: **temperature 70 °C, pressure 35.5 MPa and time 62 min**. Under these conditions, the oil experimental yield was 14.0±0.7%, in close agreement with the predicted value.

$$Y = 17.86 + 0.20 \cdot A + 2.63 \cdot B + 1.00 \cdot C - 0.85 \cdot AB + 0.100 \cdot AC - 2.30 \cdot BC - 1.53 \cdot A^2 - 3.58 \cdot B^2 + 0.77 \cdot C^2$$

Fig. 3: Second order polynomial equation. Y, oil yield; A, temperature; B, pressure, C, extraction time. $R^2 = 0.9541$.

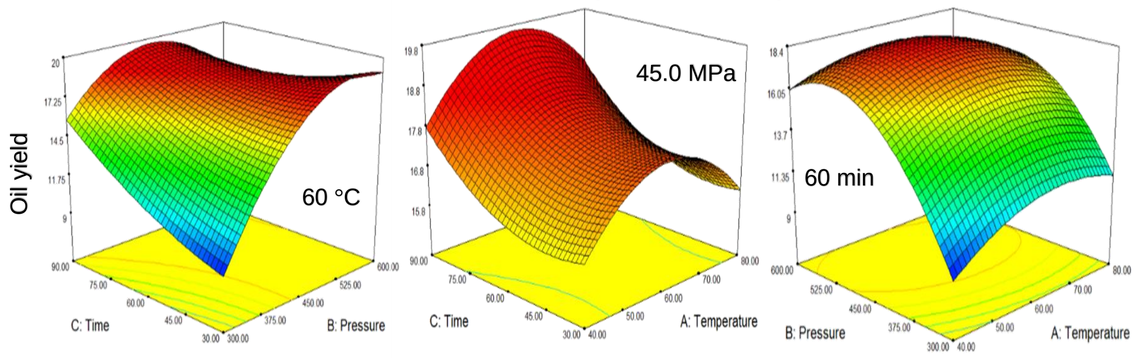


Fig. 4: 3-D response surface plots.

Compared to the **freeze-dried OP**, SC-CO_2 extracted **OP oil** was much concentrated in phytosterols (~13 fold), tocopherols (~6 fold) and squalene (~4 fold); total carotenoid concentration remained almost unchanged, while triterpenic acids, being polar, were substantially reduced (approx. -80 %) (**Fig. 5**). However, their total content was much higher than in extra virgin olive oil.

a

	Freeze-dried OP
Triterpenic acid (mg/g d.w.)	
Maslinic acid	5.62±0.27
Oleanolic acid	1.70±0.26
Total	*7.32±0.53*
Phytosterols (mg/g d.w.)	
Campesterol	0.27±0.02
β-Sitosterol	4.78±0.52
Total	*5.06±0.58*
Squalene (mg/g d.w.)	*3±0.05*
Tococromanols (µg/g d.w.)	
α-Tocopherol	122.92±3.74
ß-Tocopherol	9.20±0.59
Total	*132.12±4.33*
Carotenoids (µg/g d.w.)	
Lutein	11.72±1.65
Zeaxanthin	0.34±0.06
α-Cryptoxanthin	1.32±0.24
β-Carotene	3.90±0.77
13, cis β-Carotene	14.32±0.86
Total	*31.60±3.58*

b

	SC-CO_2 OP Oil
Triterpenic acid (µg/g oil)	
Maslinic acid	225.62±23.71
Oleanolic acid	1144.26±79.99
Erythrodiol	110.82±4.47
Uvaol	5.61±1.04
Total	*1486.31±109.21*
Phytosterols (mg/g oil)	
Campesterol	0.75±0.10
β-Sitosterol	63.93±3.43
Total	*64.68±3.53*
Squalene (mg/g oil)	11.88±1.01
Tococromanols (µg/g oil)	
α-Tocopherol	694.44±2.38
ß-Tocopherol	32.72±1.05
Total	*727.16±3.43*
Squalene (mg/g oil)	*2.12±0.03*
Carotenoids (µg/g oil)	
Lutein	16.32±0.10
Zeaxanthin	0.52±0.01
α-Cryptoxanthin	4.37±0.10
β-Carotene	15.44±0.20
13, cis β-Carotene	4.14±0.16
Total	*40.79±0.57*

*Fig. 5: Profile of the most abundant bioactives in the **freeze-dried OP** obtained from the olive cultivar Leccino (a) and in the **oil** extracted by SC-CO_2 in the optimized operative conditions (b). Values represent the mean ± standard deviation of three independent replicates (n = 3).*

The investigation of the molecular mechanisms of **OP oil** action revealed a significant increase in the activity of carnitine palmitoyl-transferase I (**CPTI**), suggesting a concomitant stimulation of hepatic fatty acid oxidation (**Fig. 6d**). A parallel increase in the activities of mitochondrial respiratory complexes was also observed in freshly isolated mice liver mitochondria. Interesting, the stimulation of respiratory chain activity retained an efficient mitochondrial oxidative phosphorylation, as suggested by the respiratory control ratio values (**Fig. 6e**).

The biological effects produced by OP oil intake were examined on 3 groups of healthy BALB/c mice (n=5/group) orally administered (for 4 weeks) with 20 or 40 mg/die of OP oil. Untreated animals were used as control (**Fig. 6a**). **OP oil** supplementation had no significant effects neither on body weight, nor on the activity of most liver cytoprotective enzymes [glutathione-disulfide reductase (**GDR**), glutathione S-transferase (**GST**) and glucose-6-phosphate dehydrogenase (**G6PD**)] with the exception of NAD(P)H:Quinone Oxidoreductase 1 (**NQO1**), whose activity was significantly increased (P<0.05; 30%) as compared to controls, by the 40 mg treatment (**Fig. 6b**). A significant decrease in the amount of liver triglycerides (**TG**), but not of cholesterol (**Chol**), was also detected in the mice fed with 20 mg **OP oil** (**Fig. 6c**).

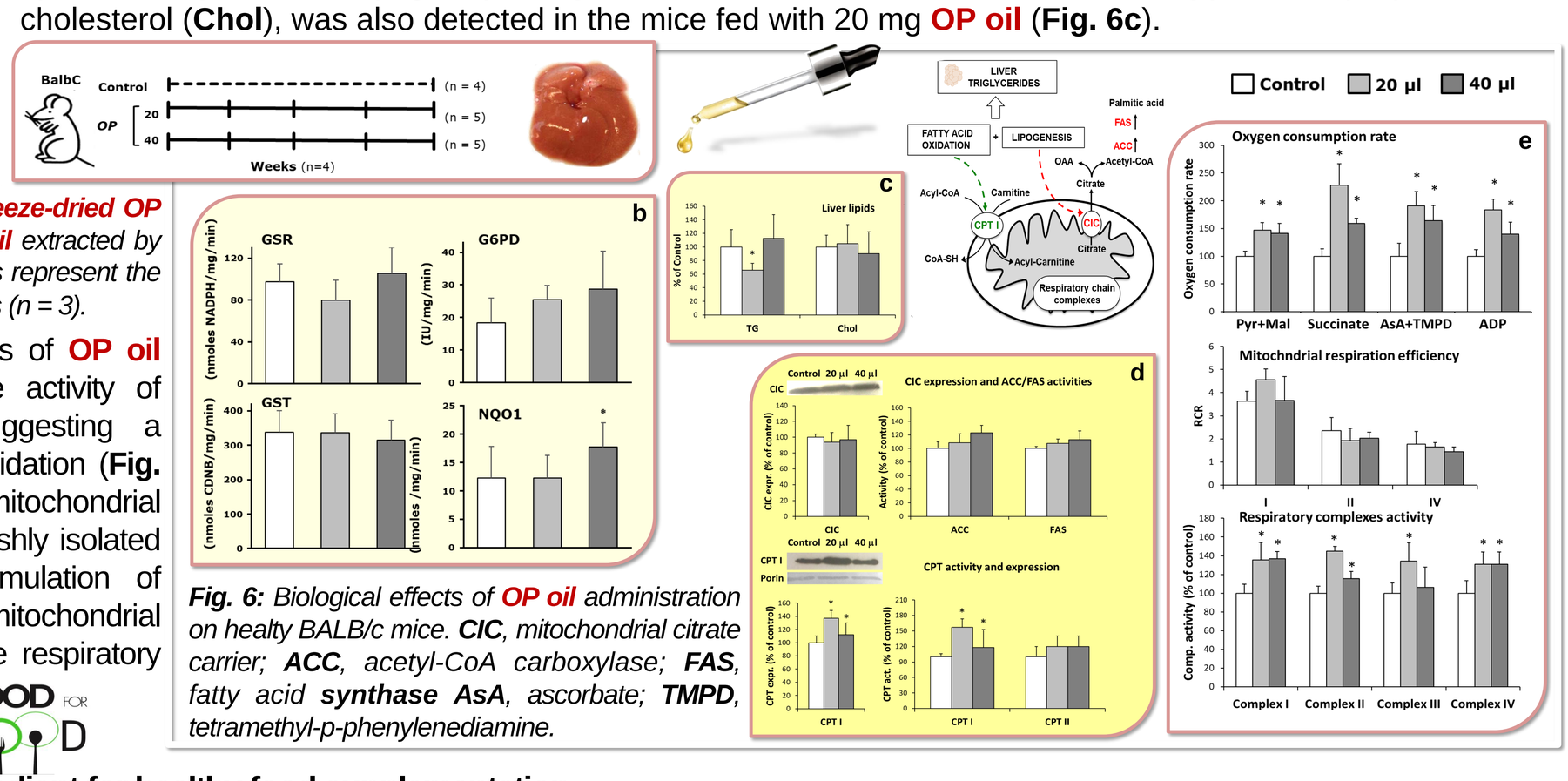


*Fig. 6: Biological effects of **OP oil** administration on healty BALB/c mice. **CIC**, mitochondrial citrate carrier; **ACC**, acetyl-CoA carboxylase; **FAS**, fatty acid **synthase AsA**, ascorbate; **TMPD**, tetramethyl-p-phenylenediamine.*

Conclusion

SC-CO_2 extracted OP oil is a promising ingredient for healthy food supplementation.

References: [1] Padalino et al., 2018. Antioxidants, 7(4), 59. [2] Fine et al., 2016. Eur. J. Lipid Sci. Technol., 118, 680-697.