Food Chemistry 322 (2020) 126676

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Pharmacokinetics of maslinic and oleanolic acids from olive oil – Effects on endothelial function in healthy adults. A randomized, controlled, dose–response study

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ARTICLE INFO

Keywords:
Maslinic
Oleanolic
Bioavailability
Triterpenes
Endothelial function
Pharmacokinetics

ABSTRACT

To date, pharmacokinetics of maslinic (MA) and oleanolic (OA) acids, at normal dietary intakes in humans, have not been evaluated, and data concerning their bioactive effects are scarce. We assessed MA and OA pharmacokinetics after ingestion of olive oils (OOs) with high and low triterpenic acid contents, and specifically the effect of triterpenes on endothelial function. We performed a double-blind, dose–response, randomized, crossover nutritional intervention in healthy adults, and observed that MA and OA increased in biological fluids in a dose-dependent manner. MA bioavailability was greater than that of OA, and consumption of pentacyclic triterpenes was associated with improved endothelial function. To the best of our knowledge, this is the first time MA pharmacokinetics, and effects on endothelial function *in vivo*, have been reported in humans.

1. Introduction

Maslinic (MA) and oleanolic (OA) acids are among the main triterpenes present in olives and olive oil (OO). Their concentrations in the oil depends on the type of OO and the variety of olive tree (Sánchez-Quesada et al., 2013). In experimental studies, MA and OA have been reported to have anti-cancer, anti-inflammatory, and antioxidant activities as well as being cardioprotective (Rodriguez-Rodriguez, 2015; Žiberna et al., 2017). In order to obtain positive opinion regarding a health claim (i.e. EFSA or FDA) for OO triterpenes in foods, the bioavailability of these compounds in humans must be fully characterized. The bioavailability of triterpenes, both *in vitro* (i.e. Caco-2 cells) and *in vivo* (mainly rodents), was reviewed in 2017 (Furtado et al., 2017). Triterpenic acids appeared to have poor gastrointestinal fluid solubility and absorption, but these characteristics differ depending on whether the compounds are administered as isolates or in a complex matrix, such as food (Furtado et al., 2017). Studies on the bioavailability of MA and OA in humans, in amounts typical of dietary consumption, have been hampered by a lack of assays with adequate sensitivity and specificity. Recently, we validated a method for analysis

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https://doi.org/10.1016/j.foodchem.2020.126676
Received 27 September 2018; Received in revised form 20 February 2020; Accepted 21 March 2020
Available online 27 March 2020
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of MA and OA in human plasma and urine (Pozo et al., 2017), and performed population studies in which the mean steady state of OO OA concentrations were determined to be from 0.72 ng/mL in non-consumers OO to 1.32 ng/mL in high-consumers (Buckland et al., 2017).

Currently, the only pharmacokinetic study performed in humans with a triterpenic acid (OA) was conducted in Chinese subjects who received a 40 mg oral dose (Song et al., 2006). Typically, concentrations of triterpenes in OO are approximately 40 mg/kg, Thus, considering typical OO intakes within the framework of the Mediterranean diet (around 30 mL), about 1 mg of these compounds are ingested daily. Given the matrix dependency of triterpene bioavailability and dietary dose, further pharmacokinetic studies under real-life conditions are required.

In experimental studies, triterpenic acids from OO have demonstrated vasoactive properties, improving endothelium-dependent nitric oxide-mediated vasodilatation (Rodriguez-Rodriguez, Perona, Herrera, & Ruiz-Gutierrez, 2006; Simonsen, Rodriguez-Rodriguez, Dalsgaard, Buus, & Stankevicius, 2009). In animal models, a triterpene-enriched pomace oil has been reported to improve endothelium-dependent relaxation in spontaneously hypertensive rats (Rodriguez-Rodriguez, Herrera, de Sotomayor, & Ruiz-Gutierrez, 2007; Valero-Muñoz et al., 2014). In human studies, OOs rich in phenolic compounds have been shown to improve endothelial function (Moreno-Luna et al., 2012; Valls et al., 2015). In this context, we took the advantage of the NUTRAOLEUM study (Biel et al., 2016) to assess MA and OA pharmacokinetics in humans after a single dose of OOs with high and low triterpene acid contents, and the acute effects of OO triterpenic acid on endothelial function. Our hypothesis was that OA and MA would have similar bioavailabilities and their presence in OO would improve endothelial function in healthy adults.

2. Material and methods

2.1. Olive oil characteristics

Characteristics of the OOs used in the NUTRAOLEUM study have been described elsewhere (Biel et al., 2016). Briefly the edible oils used were: 1) a virgin olive oil (VOO) obtained using a traditional procedure; 2) a natural optimized VOO (OVOO) with high phenolic content, but with the same triterpene content as the VOO; and 3) a functional OO (FOO) obtained from OVOO and enriched with triterpenic acids. The phenolic compound contents were 124 mg/kg, 490 mg/kg, and 487 mg/kg, and triterpene acid concentrations were 83.3 mg/kg, 83.6 mg/kg, and 389 mg/kg, for VOO, OVOO, and FVOO, respectively. With the exception of their phenolic/triterpenic acid contents, the OOs had similar fat and micronutrient (e.g. carotenoids, sterols) compositions (Supplementary Table 1). All OOs were stored in dry, dark, cool conditions.

2.2. Participants

Eighteen individuals (9 male) aged between 20 and 50 years (average 29.3 ± 8.6 years) with a body mass index (BMI) of 24.0 kg/m^2 ± 3.6 were included. They were healthy on the basis of physical examination and routine biochemical and hematological laboratory results, and capable of providing written informed consent and adhering to the protocol. Exclusion criteria were: smoking, intake of supplements or medications with antioxidant properties, hyperlipidemia, obesity (BMI > 30 kg/m^2), diabetes, hypertension, celiac or any other intestinal disease, any condition limiting mobility or life-shortening conditions (e.g. cardiovascular disease). Participants were recruited from the general population through newspapers and advertisements in civic centers.

2.3. Study design

This work focused on the NUTRAOLEOUM dose–response study (Biel et al., 2016), which comprised a randomized, double-blind, crossover nutritional intervention (n = 58) performed at the Clinical Research Units of Virgen de las Nieves and San Cecilio Hospitals (Granada, Spain). Subjects were enrolled in the study from February 2014 to July 2014. The study was conducted in accordance with the Declaration of Helsinki and Spanish laws concerning clinical trials, and approved by the local institutional review board (Comité de Ética de Investigación de Centro de Granada, Reg: 13/11 C38). Subjects signed informed consent prior to inclusion and were compensated financially for any inconvenience derived from the protocol. The trial was registered at ClinicalTrials.gov (ID: NCT02520739).

Subjects completed a three-day dietary record at the beginning of the study and after each intervention period. Physical activity was recorded at the beginning and at end of the study, and was assessed based on the Minnesota Leisure Time Physical Activity Questionnaire validated for use in Spanish men and women (Elosua, Marrugat, Molina, Pons, & Pujol, 1994; Elosua et al., 2000). A general physical examination, including routine urine and blood biochemical and hematological analyses, was performed at the beginning and end of the study. Participants were allocated to three sequences of OO administration using a stratified block randomization method. They were blinded to the allocation throughout the study.

The study flow-chart is provided in Supplementary Fig. 1. At the beginning of each intervention period, after 12 h fasting, participants (n = 18) received single doses (30 mL daily) of VOO, OVOO or FOO. A schema of the dose–response study shown in Supplementary Fig. 2.

For the assessment of the MA/OA bioavailability and disposal, a subgroup of 12 subjects, with characteristics similar to the whole group, was selected after VOO and FOO interventions. Triterpenic acid pharmacokinetics were evaluated after a single oral dose in a controlled setting on the first intervention day. In addition, further plasma (prior to VOO/FOO intakes after over-night fasting) and urine (24 h) samples were collected after 3 weeks of following a daily 30 mL dose of OOs. Estimated concentrations of triterpenic acids were used to compare simulations of repeated OO doses versus actual plasma concentrations and obtain a preliminary estimation of steady state concentrations.

Participants were asked to follow an antioxidant-free diet (Supplementary Item1) and avoid moderate/intense physical activity for three days prior to each intervention period. On day 1 of each intervention period, baseline (fasted) bloods and urine samples were collected (0 h). Subsequently, subjects received a single oral administration (30 mL) of OO with 80 g of bread. Blood samples were collected at 30 min, 45 min, 1 h, 2 h, 4 h, 5 h, 6 h, 8 h, 10 h, 12 h and 24 h. Urine samples were collected at 0–2 h, 2–4 h, 4–6 h, 6–8 h, 8–10 h and 10–24 h. At 6 h and 10 h after OO ingestion, participants received a low-phenolic content meal or snack. After 3 weeks of each intervention, plasma samples (prior to OO intake following over-night fasting) and 24 h urines were collected. Bloods were collected in 10 mL tubes containing EDTA and centrifuged (1700× g, 10 min, 4 °C) and plasma collected. Both plasma and urine samples were frozen at −80 °C until analysis.

2.4. Determination of oleanolic and maslinic acids in blood and urine

Instrumental conditions for liquid chromatography tandem mass spectrometric determination (LC/MS/MS) of MA and OA have been described previously (Pozo et al., 2017). The lower limits of quantitation (LLOQ) in plasma for MA and OA were 1 ng/mL and 0.7 ng/mL, respectively. For urine, a LLOQ of 0.16 ng/mL was assigned for both (Pozo et al., 2017). Briefly, for plasma samples,1 mL aliquotswere transferred to a glass tube and spiked with d3-OA (25 µL of 1 µg/mL MeOH solution), as an internal standard. A liquid–liquid extraction was carried out with the addition of, to each 1 mL sample, NaCl 1% and

5 mL of ethyl acetate; samples were stirred for 20 min in a shaker rotator before being centrifuged for 5 min at 1700× g, and the organic phase evaporated to dryness under a nitrogen stream at < 30 °C and < 15 103.425N/m^2 pressure. Analytes were reconstituted in 200 μL of MeOH–ammonium acetate (35 mM) (85:15, v/v). In order to remove impurities, samples were centrifuged at 3500× g for 10 min at 4 °C, and the supernatants analyzed using HPLC-MS/MS. Calibration curves, control samples, and human plasma samples were subjected to the same extraction protocol.

For urine samples, 250 μL aliquots were transferred to 15-mL screw-capped glass tubes, spiked with 1 ng/mL of d3-OA. β-glucuronidase (20 μL) from Escherichia coli, and 200 μL of 0.1 M phosphate buffer pH 6.0 were added. After overnight incubation in a water bath at 37 °C, 50 mg of $NaHCO_3/Na_2CO_3$ (1:2, w/w) was added to each tube and thoroughly mixed before extraction. A liquid–liquid extraction with 2 mL of methyl *tert*-butyl ether was performed. The mixture was homogenized in a shaker rotator for 20 min and centrifuged at 1700× g for 5 min at room temperature. The organic phase was transferred to clean tubes and evaporated (40 °C) under a stream of nitrogen. Extracts were derivatized with the admixture of 50 μL of triphenylphosphine (TPP; 10 mM in acetonitrile, ACN), 50 μL of 2,2′-dithiodipyridine (DPDS 10 mM in CAN,) and 50 μL of 2-picolylamine PA (1 μg/μL in ACN). The reaction mixture was incubated for 10 min at 60 °C on a heating block and dried under nitrogen. Samples were reconstituted in 100 μL of ACN-H_2O MilliQ grade (1:1). LLOQs for plasma MA and OA were1 ng/mL and 0.7 ng/mL, respectively. For urine, aLLOQ of 0.16 ng/mL was assigned for both analytes. Limits of detection (LOD) in plasma (0.4 and 0.3 ng/mL) were established for MA and OA, respectively, whereas LOD in urine was 0.05 ng/mL for both (Pozo et al., 2017).

2.5. Pharmacokinetic assessment

MA and OA pharmacokinetic analyses were performed after intake of VOO and FOO, when plasma and urine concentrations were above the LLOQs for the analytical method.

2.5.1. Non-compartmental pharmacokinetic analyses for maslinic and oleanolic acids

Plasma pharmacokinetic parameters for MA and OA, after FOO intakes, were extrapolated from plasma concentrations over time curves, i.e. maximum peak concentrations (C_{max}), time to reach peak concentrations (T_{max}), and areas under the concentrations-time curves, between times 1 and 2 (AUC t_1-t_2). AUCs were calculated using the linear trapezoidal rule. In the case of OA, due to its poor bioavailability, determinations at the terminal phases of plasma concentrations over time curves were below LLOQs for the method in half the subjects (6/12). Therefore, pharmacokinetics parameters for MA are reported for 12 subjects and, for OA, only for 6 subjects.

2.5.2. Compartmental model for maslinic acid kinetics

Only MA plasma concentrations, after FOO intakes, were modeled compartmentally. Pharmacokinetic data analysis was achieved with compartmental modeling SAAM II software System (The Epsilon Group, Charlottesville, VA, https://tegvirginia.com/software/saam-ii-popkinetics/) (Barrett et al., 1998). Best fit lines from actual plasma concentrations, and amounts excreted in urine, were selected after visual inspection, analysis of the objective function, Akaike information criteria, correlation matrix, and weighted residual plots. Plasma concentrations and urine excretion versus time, after a single oral administration, were characterized simultaneously using a two-compartment open model with first order oral absorption and linear elimination described by the following equation:

$$C_t = \frac{k_a \bullet F \bullet D \bullet (k_{21} - \beta)}{V_c \bullet (k_a - \beta) \bullet (\alpha - \beta)} \bullet e^{(-\beta \bullet t)}$$

Where Ct (ng/ml) and t (h) are plasma concentrations of MA and time, respectively, ka (h^{-1}) the absorption rate constant, F (%) the oral bioavailability, D the dose, k_{21} (h^{-1}) the constant rate from peripheral to central compartment, and α and β the pharmacokinetic constants (h^{-1}) corresponding to distribution and post-distribution phases, respectively, in a bi-compartmental model. Vc (liters) is the volume of distribution of the drug in the central compartment. The multi-compartmental model, which included a 5-compartment system, used to describe MA kinetic behavior is shown in Supplementary Fig. 3.

Dietary MA, albeit in limited quantities, might have been present in measurable quantities in the plasma of subjects prior to the intervention, so an adjustment was performed introducing pre-dose plasma concentrations to the pharmacokinetic fitting of observed values. This baseline was a forced function in the central compartment, and the adjustment was specific for each subject. Elimination constant ke was obtained from the constants describing the bi-compartmental model. The elimination half-life $t_{1/2}$ (h) obtained was 0.693/ke. Drug plasma clearance (Clp/F) was calculated from central compartment volume and the elimination constant as:

$$Clp/F = Vc * ke$$

AUC_0-last is the area under the plasma concentration versus time curve from time 0 to the last time post-dose and was calculated using the trapezoidal rule; AUC_0-∞ is the area under the plasma concentration versus time curve from time 0 to infinity. AUC_0-∞ was obtained as the sum of AUC_0-last and the extrapolated AUC from last observed concentration time point to infinity. Absorption half-life $ka_{1/2}$ (h) and mean absorption time MAT (h) were calculated as 0.693/ka and 1/ka, respectively. The fraction of administered dose excreted unchanged or conjugated in urine (Fe) was calculated as:

$$Fe = Ae/D$$

Where Ae is the cumulative amount of unchanged drug recovered in urine and D the dose. Renal clearance (Clr) between two time points was calculated from cumulative amounts of unchanged MA in urine between time t_1 and t2 (Ae t_1-t_2), and AUC for the same time interval ($AUCt_1$-t_2) as follows:

$$Clr = Aet1 - t_2/AUCt_1 - t_2$$

Pharmacokinetic parameters obtained after single dose administrations were used for multiple dose simulation. Pharmacokinetic simulation profiles in plasma and urine after dietary intakes of 6.0 mg MA (1.5 mg four times per day) was performed. Simulated plasma concentrations and urine excretion profiles were generated by introducing associated errors of 10% and 20%, respectively. Simulated plasma at T_{max} time and predicted cumulative urine data were compared with those observed on day 7 under the same multiple dose schedules.

2.6. Endothelial function and blood pressure assessment

Endothelial function was assessed at baseline and 4 h and 6 h after consumption, by monitoring endothelium-mediated changes (ischemic reactive hyperemia, IRH) in the digital pulse waveform, known as the peripheral arterial tone (PAT) signal (EndoPAT 2000; Itamar Medical Inc., Caesarea, Israel). Specially designed finger probes were placed on the middle finger of subjects' hands. These probes comprised a system of inflatable latex air cuffs connected by pneumatic tubes to an inflating device controlled via a computer algorithm. A constant counter pressure (pre-determined by baseline diastolic blood pressure [DBP]) was applied through the air cushions. Pulsatile volume changes of the distal digit induced pressure alterations in the finger cuff, which were perceived by pressure transducers and transmitted to and recorded by the EndoPAT 2000 device. Hyperemic reactivity measured by Endopat 2000 has been shown to predict cardiovascular disease (Rubinshtein et al., 2010). Systolic blood pressure (SBP) and DBP were measured with a mercury sphygmomanometer after a minimum of 10 min rest in

the seated position; the average of two measurements was recorded.

2.7. Nitric oxide determinations

Nitrites and nitrates were determined in plasma at baseline and at 2, 4, 6, and 8 h after consumption. Concentrations were determined using a colorimetric kit (Cayman Chemical, Michigan, USA). Briefly, a simple two-step process was developed: first, nitrate (NO_3–) was converted to nitrite (NO_2–) with nitrate reductase and, second, Griess reagent was added, which converts nitrite into a deep purple azo compound that can be quantified by spectrophotometry.

2.8. Sample size

A total of 14 participants would provide at least 80% power to determine a statistically significant difference among OO groups of 0.25 units in IRH, assuming a dropout rate of 10% and type I error of 0.005 (2-sided). Standard deviation of the measurement was 0.5 (Rubinshtein et al., 2010). We retained an additional four participants, who met the inclusion criteria after screening, to ensure statistical power, if differences among the treatment groups were lower than expected.

2.9. Statistical analyses

Group characteristics were compared by analysis of log-transformed data. In order to assess interactions for MA and OA pharmacokinetic parameters, volunteers were assigned to one of two groups, based on sex, age (below 26 years, n = 7; > 29 years, n = 5) and BMI, corresponding to normal weight (between 18.5 and 25 kg/m^2) and overweight (> 25 to 30 kg/m^2). General linear modeling was used to assess the main and interactive effects of interventions. Changes in IRH and nitrites/nitrates were assessed using an ANCOVA model with age and sex as covariables. Normality of continuous variables was evaluated using probability plots; non-normally distributed variables were log transformed. Significance was defined at 5% using a two-tailed test. All statistical analyses were performed with SPSS 17.0 (SPSS Software, Chicago, IL).

3. Results

3.1. Participant characteristics and compliance

No significant differences in participants' baseline characteristics were observed among OOs intervention sequence groups (Supplementary Table 2). No changes in daily energy expenditure in leisure-time physical activity were reported during the study. No changes in energy and selected nutrients, after the three interventions, were observed (Supplementary Table 3). We could not identify any adverse effects related to OO intake.

3.2. Noncompartmental pharmacokinetics

3.2.1. Single dose

Intakes of a single dose (30 mL) of FOO containing 6.0 mg MA and 4.7 mg of OA were associated with a rise in their plasma concentrations. The observed plasma concentrations versus time profiles are shown in Fig. 1. In OA plasma samples where concentrations were below the LLOQ, LOD was used instead. Baseline plasma MA concentrations (mean ± SD) were 1.9 ± 1.0 ng/ml. Plasma concentrations of OA from 0 to 10 h equal to or greater than the LLOQ (n = 6) were used for calculation of pharmacokinetic parameters and compared to MA values obtained at the same time interval (n = 12). Pharmacokinetic experimental parameters are shown in Table 1. No sex differences were observed. Although the administered dose of MA was only 1.28-fold higher than that of OA, C_{max} and $AUC_{0\text{-}10}$ values were 6.4- and 7.4-times higher for MA than OA. Renal fraction elimination ($fe_{0\text{-}10}$) of MA, from time 0 to 10 h, was twice that of OA (0.38 ± 0.17 vs. 0.15 ± 0.08 p < 0.001), which was corroborated urinary recoveries over time (Fig. 2).

3.2.2. Repeated doses

MA and OA plasma concentrations showed progressive accumulation over the one-week intervention periods. MA plasma concentrations ranged from 1.8 ng/mL at baseline to 6.7 ng/mL at 24 h after intakes of a single dose (30 mL), and up to 21.5 ng/mL three weeks later. OA plasma concentrations ranged from 0.31 ng/mL at baseline to 0.49 ng/mL at 24 h after intakes of FOO (30 mL), and up to 2.5 ng/mL three weeks later (Fig. 3A). 24-h urinary recoveries, over three weeks for both triterpenic acids, also revealed differences (MA: P = 0.006; OA: P = 0.003) (Fig. 3B). There were no differences in plasma concentrations of either triterpenic acid according to sex. However, when 24-h urinary recoveries were adjusted for body weight, male recoveries on day 1 were greater (MA, P = 0.048; OA, P = 0.012) than those of females. After three weeks of repeated interventions, only a very small trend for OA (p = 0.059) was observed (Supplementary Fig. 4).

3.3. Compartmental analyses for maslinic acid kinetics

Shape of the observed plasma MA kinetic profile (Fig. 1) indicated a substantial two compartment open model with first order oral absorption and linear elimination. The model, applied to observed plasma concentrations and urine excretion versus time, describing the MA pharmacokinetic profile with mean observed and fitted values, is shown in Supplementary Fig.5. The model showed a good individual visual inspection of the fitting, specifically distribution of residual plots, and low values for objective function and Akaike Information Criteria (AIC) values (mean ± SD: 3.8 ± 1.7 for plasma and 3.5 ± 1.0 for urine), indicating a good fit for experimental values. Table 2 shows the MA pharmacokinetic parameters obtained. After 6.0 mg MA intakes, maximum plasma concentrations C_{max} (32.8 ± 10.4 ng/ml) in the central compartment were achieved at 3 h. This relatively fast absorptive phase was concomitant with an apparent rapid absorption half-life and mean absorption time (0.7 ± 0.5 h and 1.1 ± 0.7 h, respectively). The calculated elimination constant (0.06 ± 0.03 h^{-1}) corresponded to an elimination half-life $t_{1/2}$ of 16.3 ± 9.7 h, which explains the relatively slow terminal slope in the fitted kinetic profile. Despite few experimental data defining the terminal slope of the model, extrapolated AUC values explained < 30% (mean 28.5 & IC95%:19.3–37.8) of the total MA disposition from 0 to infinity (AUC $_0$-last versus $AUC_{0\text{-}\infty}$; 265.2 ± 106.0 vs 387.3 ± 157.9 ng*h/mL, respectively).

We did not observe unaltered MA or phase I metabolites in urine. Thus, cumulative urine excretion (Ae $_0$-last) and the fractions of doses excreted (fe 0-last) corresponded to MA conjugates with glucuronic acid. In order to assess model compliance in a multiple dose regimen, simulated data obtained after 21 days of a daily MA intake of 6 mg were compared with experimental plasma concentrations and cumulative amounts excreted. No differences were observed in plasma concentrations after 21 days (6.0 mg/day intakes) or C_{max} values predicted by the model (P = 0.812). In addition, there were no differences in cumulative amounts between observed and predicted values for MA in urine under multiple dose regimen (P = 0.291). These results indicate that the model is a suitable tool to simulate the kinetic profile of MA.

3.4. Endothelial function biomarkers and blood pressure

IRH increased after OVOO and FOO ingestion at 4 h and 6 h; changes at 4 h after FOO ingestion being greater than those after VOO (Fig. 4A). SBP decreased (P < 0.05) after 4 h and 6 h regardless of the oil type. DBP decreased at 4 h after OVOO (P = 0.011) and FOO (P = 0.003); decreases were greater than those observed after VOO (P < 0.03). At 6 h, decreases in DPB after OVOO and FOO were only marginally significant (P = 0.075 and P = 0.057 for VOO and FOO,

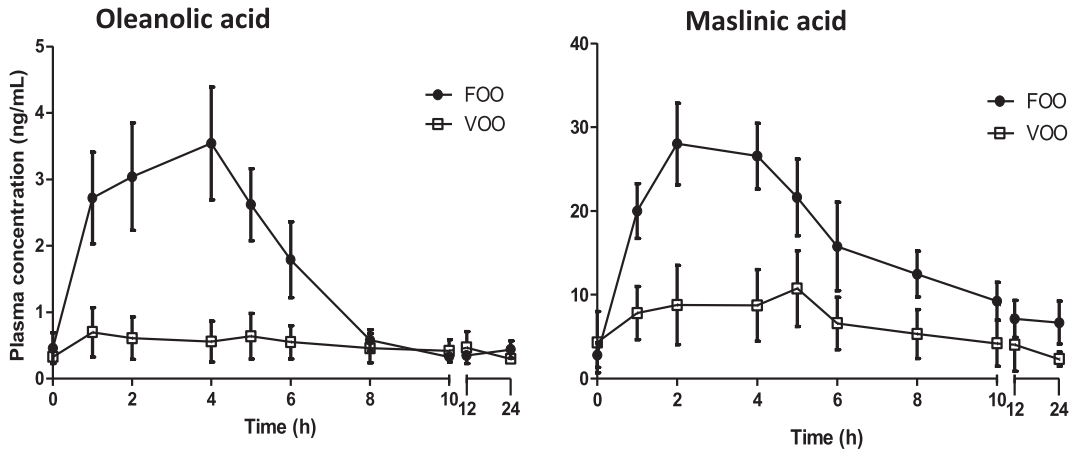

Fig. 1. Plasma concentrations of oleanolic and maslinic acids after ingestion of 30 mL of traditional virgin olive oil (VOO) and functional olive oil (FOO) (n = 12).

respectively). No differences, either intra- or inter-interventions, were observed for nitrites and nitrates. However, and only in the case of FOO, nitrites values were related directly to IRH at 4 h after OO ingestion (Fig. 4B).

4. Discussion

We assessed the bioavailabilities, and the non-compartmental kinetics, of MA and OA from an enriched FOO, and their effects on endothelial function in healthy volunteers. A bi-compartmental model (including a 5-compartment system) was fitted for MA. Both triterpenic acids increased in a dose-dependent manner with their content in the OO administered, but the bioavailability of MA was greater than that for OA. Triterpenic acid ingestion was also associated with an increase in IRH at 4 h after FOO ingestion that was related directly to concentrations of nitrites in urine

Pentacyclic triterpenes are components of medicinal plants, fruit, vegetable oils, and cereals while MA is the main triterpene found in the leaves and fruits of *Olea europaea L* (Pérez-Camino & Cert, 1999; Sánchez-Avila, Priego-Capote, Ruiz-Jiménez, & de Castro, 2009; Furtado et al., 2017). Pentacyclic triterpenes and their derivatives have gain attention as dietary supplements (Sheng & Sun, 2011) but, their efficacy and effectivity as part of the diet, in a functional food or nutraceutical, cannot be established without human bioavailability studies.

Due to the structural similarities of the two triterpenic acids, we hypothesized a similar bioavailability. Contrary to our hypothesis, however, MA bioavailability, on the basis of the C_{max} and $AUC_{0\text{-}10}$, was 7-fold higher than that of OA, despite only a 1.3-fold difference in doses administered. This finding cannot be attributed to differences in lipophilicity, given that octanol/water coefficient, a predictor of absorption by passive diffusion (Artursson, Palm, & Luthman, 2001), is lower in MA (5.52) than in OA (6.47) (Furtado et al., 2017). Although both triterpenic acids are present in typical European diets, basal concentrations of OA were lower thanthe LLOQ in half the subjects. Our data agree with previous studies in rats, suggesting a low OA oral bioavailability, due to poor gastrointestinal absorption and subsequent hepatic microsomal metabolism (Jeong et al., 2007). Oral bioavailability of MA has been reported previously to be about 6.25%, but only 0.7% has for OA in animal models at the same doses (50 mg/kg) (Jeong et al., 2007; Sánchez-González, Colom, Lozano-Mena, Juan, & Planas, 2014). Differences reported in the bioavailabilities of both compounds previously, correspond to those reported in the present study.

MA volume of distribution and plasma clearance were calculated considering the lack of information concerning absolute bioavailability (F). Additionally, in the case of poor permeability and/or bioavailability, the terminal slope in the elimination phase might represent the absorption phase, as a result of flip-flop kinetics. In the case of MA, cumulative urine excretion and the fraction of dose excreted corresponded to MA conjugates with glucuronic acid, in agreement with our preliminary results (Pozo et al., 2017). Calculated MA renal clearance can be taken as a useful, roughly estimated parameter for comparative purposes between the triterpenic acids, since values for oral bioavailabilities and fractional conversions of the parent to metabolites are unknown. Recent studies following MA oral administration to Sprague Dawley rats have shown the prevalence of unaltered compound in plasma and urine (Sánchez-González et al., 2014). Despite potential species differences, these results concur with the absence of phase I

Table 1
Non-compartmental single-dose kinetics after oral ingestion of oleanolic (OA) and maslinic (MA) acid after functional olive oil (FOO).

Parameter	Oleanolic acid				Maslinic acid				
	Male (n = 6)	Female (n = 6)	p^a	Total (n = 12)	Male (n = 6)	Female (n = 6)	p^a	Total (n = 12)	*p* OA vs MA
Dose *(mg)*	4.7	4.7	–	4.7	6.0	6.0	–	6.0	
C_{max} *(ng/mL)*	5.1 (± 1.3)	5.2 (± 2.7)	0.930	5.1 (± 2.1)	33.6 (± 9.1)	31.9 (± 12.4)	0.79	32.8 (± 10.4)	< 0.001
T_{max} *(h)*	3 (2–6)	4 (1–6)	0.88	4 (1–6)	3 (2–5)	3 (1–6)	1.00	3 (1–6)	0.54
Ke (h^{-1})	0.52 (± 0.12)	0.48 (± 0.03)	0.53	0.50 (± 0.09) [b]	0.39 (± 0.13)	0.34 (± 0.19)	0.69	0.37 (± 0.15) [c]	0.03
$t_{½}$ *(h)*	1.38 (± 0.28)	1.45 (± 0.10)	0.65	1.41 (± 0.21) [b]	2.08 (± 1.03)	2.27 (± 1.10)	0.56	2.26 (± 1.03) [c]	0.027
$fe_{0\text{-}10}$ *(‰)*	0.18 (± 0.10)	0.12 (± 0.05)	0.17	0.15 (± 0.08)	0.41 (± 0.05)	0.34 (± 0.10)	0.56	0.38 (± 0.17)	< 0.001
$AUC_{0\text{-}10}$ *(ng.h^{-1}/mL)*	26.6 (± 7.3)	29.7 (± 12.7)	0.73	28.2 (± 9.4)[d]	178.7 (± 43.6)	192.8 (± 92.4)	0.75	185.1 (± 66.5) [c]	< 0.001

Data expressed as mean (± standard deviation) except T_{max} which is expressed as median (min-max). [a]P values for gender comparisons. C_{max}, plasma maximal concentration; T_{max}, time to maximal concentration; Ke, elimination rate constant; $t_{½}$, elimination half-life; fe, cumulative fraction of the dose excreted in urine; $AUC_{0\text{-}10}$, area under the curve from 0 to 10 hours. Calculations made in [b] 11 subjects (5 men and 4 women), [c] 11 subjects (4 men and 5 women), and [d] 6 subjects (3 by gender), given that terminal plasma concentrations were below the limit of quantification.

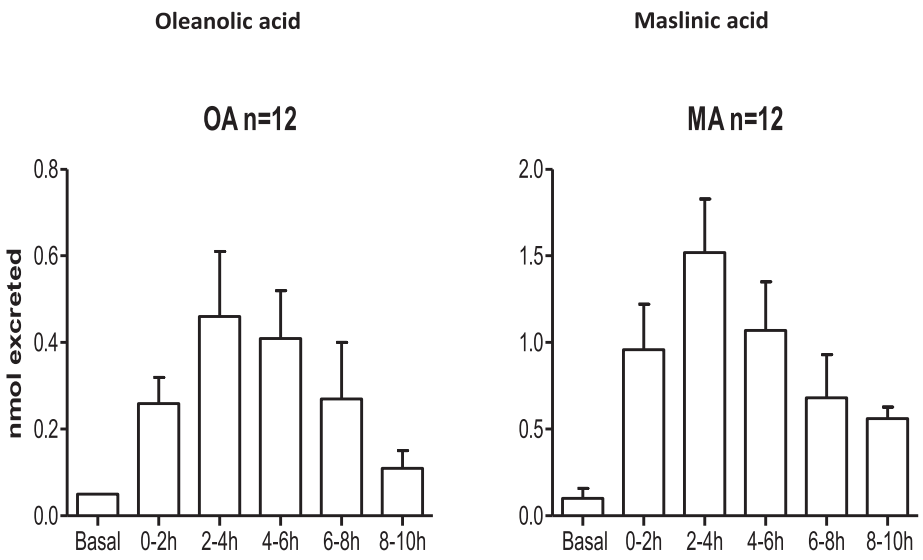

Fig. 2. Urinary recoveries (from 0 h to 10 h) of oleanolic and maslinic acids (n = 12).

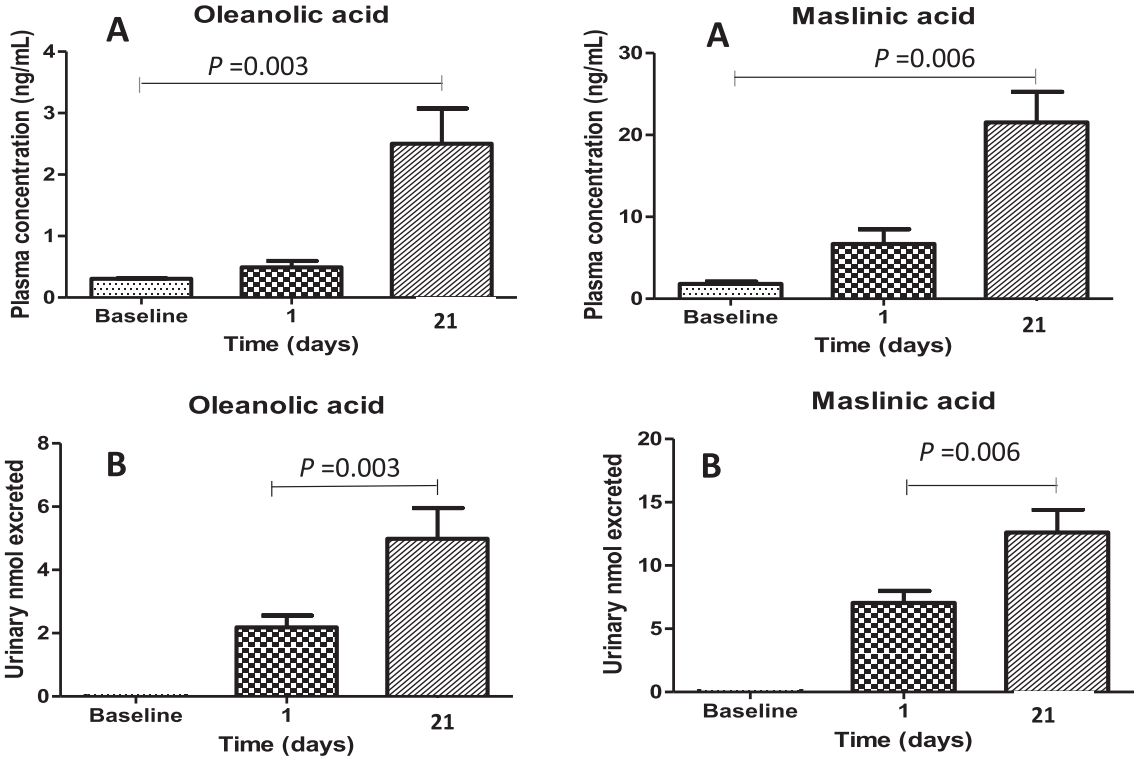

Fig. 3. Panel A. Oleanolic and maslinic acid plasma concentrations at baseline and after 24 h and 1 week of consumption (30 mL/day) of functional olive oil (FOO). Panel B. Oleanolic and maslinic acid urinary recoveries at 24 h, and at three weeks after (30 mL/day) after consumption of FOO. Data expressed as mean and standard error.

metabolites observed in our study. Moreover, the lack of differences between plasma concentrations and cumulative amounts of MA excreted, and those predicted by the model, indicated its suitability to simulate MA kinetic profiles.

Slight differences were observed in the bioavailability of both triterpenic acids, which were greater in males. Our sample size was however, small and further studies are warranted in larger populations. We also observed that, after considering the elimination half-life of both triterpenic acids, steady state concentrations were reached after repeated doses. These concentrations were 3- to 4-times higher than those obtained after a single administration. This concurs with observations in animal models after sustained regimens of administration (Yin, Lin, Mong, & Lin, 2012).

Endothelial dysfunction is considered to be an early sign of atherosclerosis and has been attributed to unfavorable changes in nitric oxide (NO) metabolism (Ignarro, Cirino, Casini, & Napoli, 1999), related to oxidation and inflammation (De Haro Miralles et al., 2009). Moreover, impairment of endothelial-dependent vasodilatation occurs in the postprandial state (Ghiadoni, Taddei, & Virdis, 2012). Improvements in endothelial function, associated with olive oil phenolic compounds (Moreno-Luna et al., 2012; Ruano et al., 2005; Valls et al., 2015) and other polyphenols (Balzer et al., 2008), have been reported previously. Results from the NUTRAOLEUM sustained-consumption study showed that decreases in plasma endothelin *in vivo* occurred were

Table 2
Compartmental pharmacokinetic parameters for the maslinic acid model after oral intake of 30 mL of FOO containing 6 mg of MA (n = 12).

	Mean ± SD	IC95%
Baseline, *ng/ml*	1.9 ± 1.0	(1.4–2.5)
Plasma maximal concentration (C_{max}), *ng/ml*	32.8 ± 10.4	(26.9–38.6)
Time to C_{max} (T_{max}), *h* [a]	4.0 (2–10)	(2.0–3.1)
Absorption rate constant (Ka), h^{-1}	1.5 ± 1.0	(0.9–2.1)
Absorption half-life ($t_{1/2\ abs}$), *h*	0.7 ± 0.5	(0.5–1.0)
Mean absorption time (MAT), *h*	1.1 ± 0.70	(0.7–1.5)
$AUC_{0\text{-last}}$, $ng.h^{-1}/ml$	265 ± 106	(205–325)
$AUC_{0\text{-}\infty}$, $ng.h^{-1}/ml$	387 ± 158	(298–477)
AUC_{extr}, %	28.5 ± 16.4	(19.3–37.8)
Elimination rate constant (Ke), h^{-1}	0.06 ± 0.03	(0.04–0.07)
Elimination half-line ($t_{1/2\ elim}$), *h*	16.3 ± 9.7	(10.8–21.8)
Vc/F, *L*	97.8 ± 48.9	(70.2–125.4)
Clp/F, *L/h*	18.6 ± 9.6	(13.2–24.1)
$Clr_{0\text{-last}}$, *L/h*	0.01 ± 0.06	(0.01–0.012)
Last plasma concentration (Clast), *ng/ml*	4.9 ± 2.4	(3.6–6.3)
$Ae_{0\text{-last}}$, *ng*	3322 ± 1562	(2438–4205)
$fe_{0\text{-last}}$, %	0.06 ± 0.03	(0.04–0.07)

FOO, functional olive oil. Data expressed as mean ± standard deviation [a] Median (min-max).IC, 95% interval of confidence;$AUC_{0\text{-last}}$, area under the curve from 0 to the time of the last quantifiable concentration; $AUC_{0\text{-}\infty}$, area under the curve from 0 to infinity; AUC_{extr}, extrapolated area under the curve from t_{last} to infinity; Vc/F, apparent central volume of distribution following extravascular administration; Clp/F, apparent total body clearance following extravascular administration; $Clr_{0\text{-last}}$, renal clearance calculated from 0 to the time of the last quantifiable concentrations; Ae, cumulative amount of drug excreted in urine from 0 h to last time; fe, cumulative fraction of the dose excreted in urine.

similar, regardless of the intervention, although *ex vivo* decreases in blood cell cultures were greater after consumption of triterpene-rich FOO (Sanchez-Rodriguez et al., 2018). No data, however, exist regarding the effects of triterpenic acids from OO on direct measurement of endothelial function in humans.

Our aim was, therefore, to assess whether enrichment of OO with triterpenic acids could provide additional benefits inhuman endothelial function, based on IRH values, beyond those provided by OO phenolic contents. In our study, improvement in endothelial function at 4 h-postprandial reached significance only when the triterpenic acids were added to a phenol-rich OO. In agreement with this finding, at this time point, and only in the case of FOO, was a direct relationship observed between increased IRH and nitrite concentrations, a surrogate marker for NO bioactivity, given around 80% of nitrites in plasma stem from endothelial nitric oxide synthase (eNOS) activity (Kleinbogard et al., 2003). Based on these results, further studies are warranted to elaborate the mechanisms of action and implications for human health.

Our study has strengths and limitations. The model baseline was established as a single fixed value prior to administration of the OOs, and not multiple experimental time points. Nevertheless, introduced as a forced function in the model, the background facilitated slope estimation to characterize bi-compartmental behavior of MA. Although compartmental MA was suitable for simulating the MA kinetic profile, assessment over a wider range of doses, including a parenteral administration (e.g. intravenous), is needed for both dose non-linearity detection and calculation of absolute oral bioavailability (F). We were unable to assess potential interactions between FOO and other dietary components with respect to endothelial function. Furthermore, and given that no differences were observed between IRH changes after OVOO and FOO, synergisms between phenolic compounds and triterpenic acids cannot be discounted. Indeed, synergistic associations between plant triterpenes and phenolic substances have been described previously (Macedo dos Santos, Pereira dos Santos, Castro-Gamboa, BoldrinZanoni, & Furlan, 2010). The randomized crossover intervention, however, minimized the effects of possible confounders, with each individual acting as their control. To the best of our knowledge, this is the first-time pharmacokinetics for MA, and effect of triterpenic acids on endothelial function *in vivo*, have been reported in humans.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank ACER CAMPESTRES, San Francisco de Asis Coop., and AGROINSUR SL, for providing the materials and processes for obtaining the olive oils used in the project. These sources do not play any role or authority in the study design, collection, management, analyses, and interpretation of data, writing or submission of the manuscript. This work was supported by grants from DIUE de la Generalitat de Catalunya (2017 SGR 138). CIBEROBN is an initiative of Health Institute Carlos

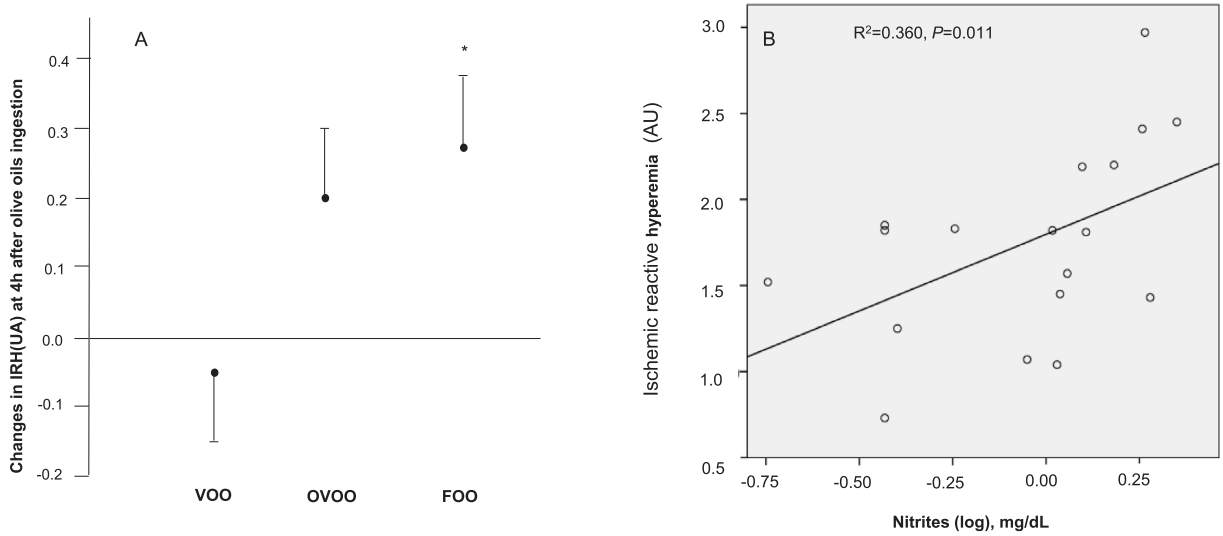

Fig. 4. Panel A. Changes in ischemic reactive hyperemia (IRH) after 4 h of olive oil ingestion (n = 18). VOO, traditional virgin olive oil (control); OVOO, optimized VOO rich in polyphenols; FOO, functional VOO rich in polyphenols and triterpenes. *P = 0.032 versus changes after VOO. Panel B. Relationship between plasma nitrites and ischemic reactive hyperemia at 4 h after ingestion of FOO rich in polyphenols and triterpenes. AU, arbitrary units.

(ISCIII). The NUTRAOLEOUM Study has been supported by the grant 20131031 from the FEDER-INTERCONNECTA (CDTI) and Junta de Andalucía, Spain.

Funding sources

The NUTRAOLEOUM Study has been supported by the grant ITC-20131031 from the FEDER-INTERCONNECTA (CDTI) and Junta de Andalucía, Spain.

Conflict of interest

José Jimenez-Valladares is an employee of the Cooperativa San Francisco de Asís, Andalucía Spain where the olive oils used were prepared. The other authors have no conflict of interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.126676.

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