Wageningen Academic Publishers

Optimisation of microwave-assisted extraction of triterpenoic acids from olive mill waste using response surface methodology

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Received: 19 October 2015 / Accepted: 27 August 2016

RESEARCH ARTICLE

Abstract

Maslinic acid (MA) and oleanolic acid (OA) are pentacyclic triterpenic phytochemicals found in the olive fruit and leaves as well as wastes. Dried olive pomace is a good source of both triterpenic acids. This study aimed to investigate the potential of microwave-assisted extraction (MAE) to obtain MA and OA from olive pomace and to optimise the MAE process temperature and time by response surface methodology (RSM). The model produced for each compound showed high adequacy to predict the experimental results, hence, the conditions for each triterpenic acid were optimised. The optimal condition for MA extraction was 40 °C for 10 min, whilst OA had optimal extraction at 40 °C for 2.93 min. Compared to conventional Soxhlet extraction, MAE is a promising method to produce MA and OA rich extracts from olive pomace. Also, RSM is a relevant and accurate tool to optimise the MAE parameters.

Keywords: extraction, oleanolic acid, olive pomace, maslinic acid

1. Introduction

Olive oil has been used as a major ingredient of the Mediterranean diet for centuries. Nowadays, it is well-recognised for its nutritional benefits, whilst its derivatives are also used in the cosmetic industry. As the demands for olive oil increase, more factories with increased production capacities are being established. The European Union countries surrounding the Mediterranean Sea, including Turkey, supply more than 30% of the global, annual olive oil production (Azbar *et al.*, 2004). However, an increasing olive oil production results in an increasing amount of waste referred to as olive mill waste. Consequently, environmental pollution is a growing concern for the olive oil industry, particularly in the Mediterranean region. The industry faces a serious environmental challenge and must find an economically viable solution to handle and dispose of the olive mill waste.

Olive mill waste (olive pomace and waste water) includes high amounts of organic compounds. These organic materials are considered the main pollutant and are not readily biodegradable. Despite numerous studies to overcome the environmental effect of olive mill waste (Alba-Mendoza *et al.*, 1996; Kurtuluş and Günerhan, 2003; Öcal, 2005; Parra *et al.*, 2010), many difficulties have been encountered, such as scale-up issues, economic limitations, and long processing time requirements. Therefore, research in this area and in the use of olive mill waste to obtain value-added products is on-going.

Literature shows that the olive mill waste contains high amounts of pentacyclic triterpenic acids, mainly maslinic acid (MA) and oleanolic acid (OA), primarily produced in the lipid layer of olive fruit skin to combat insect attacks (Kombargi *et al.*, 1998) and microbial growth (Bianchi *et al.*, 1994). Plant-derived triterpenic acids are desirable natural compounds for the food, cosmetic and pharmaceutical industries due to their potential health benefits. Recent studies emphasise the anticancer, antitumour, antidiabetic, antihypertensive, antioxidant, antiparasitic, antimicrobial, anti-inflammatory, neuroprotective, and antiproliferative effects of triterpenic acids (Allouche *et al.*, 2011; Braga *et al.*, 2007; De Pablos *et al.*, 2010; Guan *et al.*, 2011; Horiuchi *et al.*, 2007; Juan *et al.*, 2008; Li *et al.*, 2010; Liu *et al.*, 2007; Petronelli *et al.*, 2009; Tang *et al.*, 2008).

The high economic value of these triterpenic acids has contributed to studies focused on identifying new sources of natural plant bioactives. The annual production of olive oil has been estimated at 2.9 million tonnes with 15 million tonnes of olive mill waste (Roig *et al.*, 2006). In Mediterranean countries, olives have been a major part of the agricultural production for many decades (if not centuries). During olive oil production, approximately 35 kg of olive pomace is produced for every 100 kg of olives, thus, the production of olive mill waste and olive pomace are sustainable and readily available as sources of value-added products (Nasopoulou and Zabetakis, 2013). In this context, the present study aimed to improve the economic value of olive mill waste by extracting the major triterpenic acids, MA and OA.

The extraction of bioactives from natural plant sources has been extensively investigated (Cacace and Mazza, 2002, 2003a,b; Liyana-Pathirana and Shahidi, 2005). Such studies showed that solvent type, temperature, time, solvent/solid ratio, as well as extraction method are important factors of extraction performance, extractive quality and yield, economic cost, and environmental consideration. Consideration of the plant material structure, desired purity and efficiency, have been evaluated and optimised to meet the demands of the target compounds.

Any process and/or system require an evaluation of their performance as a function of its process variables. Optimisation is performed by statistical tools that identify the variable-dependent variations and their interaction effects on the process responses. Thus, in this research, the temperature and time extraction parameters were optimised to determine the associations between process variables and yields of the pentacyclic triterpenic acids (MA and OA).

The current study focused on the recovery of pentacyclic triterpenic acids present in olive pomace by MAE. The temperature and time process parameters were optimised for maximum yield of MA and OA, using response surface methodology (RSM).

2. Material and methods

Material and chemicals

Wet olive pomace was provided by a private olive oil manufacturer located in Aydın, Turkey. Absolute ethanol, high-performance liquid chromatography (HPLC) grade methanol and acetic acid were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Authentic standards for MA and OA were purchased from Sigma-Aldrich (M6699) and Fluka (42515), respectively.

Determination of physicochemical properties of olive pomace

The moisture content of the olive pomace was determined according to the UNE standard Spanish method (Asociacion Espanola de Normalizacion y Certificacion) (Instituto Espanol de Normalizacion, 1973). Approximately 10 g of wet pomace was weighed and then dried for 24 h at 105 °C, cooled for 30 min in a desiccator and reweighed. Moisture and dry matter (DM) content are given as g H_2O/100 g sample, and g DM/100 g sample, respectively. The oil content of the olive pomace was determined by separating the stones from the dried sample by passing it through a sieve (mesh size of 1 mm). Then, 10 g of pomace was extracted with 200 ml of hexane for 4 h in a Soxhlet extractor. After, pomace was dried in an oven at 60 °C to attain a constant weight. The extract was then evaporated under vacuum in a rotary evaporator to remove hexane. The oil content of the pomace sample is given as g oil/100 g dried pomace. All analyses were triplicated.

Drying of olive pomace

Based on preliminary studies, approximately 1 kg of wet pomace was dried on a tray at 1 cm thickness under vacuum (200 mbar) at 60 °C until the moisture content attained approximately 3.87±0.05% and then ground and passed through a sieve with a mesh size of 1 mm to remove olive stones. The dried sample was stored at -18 °C in a sealed plastic bag to avoid water absorption.

Soxhlet extraction of triterpenic acids

A 10 g aliquot of the dried sample was extracted with 200 ml of absolute ethanol (boiling point approximately 80 °C) using a Soxhlet extractor for 4 h (Goulas and Manganaris, 2012) to obtain a final volume of 100 ml and then filtered through a syringe filter (0.45 μm). The filtrate was injected into an HPLC system for analysis of MA and OA.

Microwave-assisted triterpenic acid extraction

A modified, microwave-assisted, Soxhlet extraction system (DryDist, Milestone, Italy) having a temperature controller unit was used. The DryDist model was modified by replacing the glass apparatus for volatile compounds with Soxhlet apparatus. The solvent-based MAE reported by Camel (2000) was modified to extract the triterpenic acids from olive pomace because there is no data on this method for triterpenic acids extraction available in the literature. The extraction used 100 ml ethanol:water mixture (9:1 v/v) and 5 g of ground dried sample without olive stones, which were poured into an extraction cell and then heated using microwave energy, according to the experimental design. The solid phase was removed from the extract by filtration

through filter paper and the extract was made up to a final volume of 100 ml with the extraction solvent.

Pentacyclic triterpenic acid determination using LC-DAD analysis

Before HPLC analysis, the extract was passed through a 0.45 µm PVDF membrane disc held in a 13-mm diameter syringe filter holder (Chromatographic Specialties, Brockville, Ontario, Canada) and then stored at 4 °C until analysis. The method of Fu *et al.* (2014) was modified for triterpenic acid determination in extracts as follows: the liquid chromatography system (Agilent 1200 infinity series; Agilent Technologies, Palo Alto, CA, USA) was equipped with a photodiode array detector, an autosampler, and a control module. Aliquots of 5 µl were injected into a reversed-phase C18 column (Zorbax SB, 5 µm, 250×4.6 mm ID; Agilent Technologies) preceded by a guard column (Inertsil 5 ODS, 5 µm, 30×4.6 mm ID; Phenomenex, Torrance, CA, USA). An isocratic solvent system consisting of 30:70 solvent A (acetic acid/ultra-pure water, 0.05%, v/v): solvent B (pure methanol) was used. The solvent flow rate was 1.0 ml/min. The MA and OA (detection at 205 nm) were analysed qualitatively by comparing their retention times and UV spectra with authentic standards and their concentrations calculated using their peak areas and standard curves.

Optimisation of process parameters

Optimisation of the extraction parameters (temperature, X1 and time, X2) was performed using RSM with Minitab software. A central composite experimental design was used. The process responses were yields of MA (Z1) and OA (Z2) in the extract. Coded and uncoded values of each independent variable are given in Table 1. The experimental design, showing the extraction conditions and corresponding MA and OA contents of the extracts, are given in Table 2. The experimental data were fitted to a second-order polynomial regression model containing the coefficient of linear, quadratic, and two factors interaction effects:

$$Z = \beta_0 + \sum_{i=1}^{2}\beta_i X_i + \sum_{i=1}^{2}\beta_{ii} X_i^2 + \sum_{i=1}^{1}\sum_{j=1+1}^{2}\beta_{ij} X_i X_j \quad (1)$$

Where, Z is the dependent variable, β_0 is the constant coefficient, β_i is the linear coefficient (main effect), β_{ii} is the quadratic coefficient, and β_{ij} is the two factors interaction coefficient. The model performance, for each compound, was determined by evaluation of R^2, adj-R^2 and lack-of-fit test (Myers *et al.*, 2009).

Table 1. Coded and uncoded values of independent variables.

Independent variable	Factor level				
	(-1.41)	(-1)	(0)	(1)	(1.41)
Temperature (°C, X1)	25.86	30	40	50	54.14
Time (min, X2)	2.93	5	10	15	17.07

Table 2. Experimental design and corresponding responses.

	Independent variables				Dependent variables	
	Coded values		Uncoded values		Maslinic acid[2]	Oleanolic acid[3]
Run order[1]	X_1	X_2	Temperature	Time		
1	0	0	40.0	10.0	17.30	4.54
2	0	0	40.0	10.0	17.33	4.38
3	1.41	0	54.14	10.0	15.02	4.49
4	0	0	40.0	10.0	16.81	4.22
5	1	-1	50.0	5.0	14.87	4.55
6	-1	-1	30.0	5.0	14.46	4.46
7	-1	1	30.0	15.0	15.09	4.41
8	0	0	40.0	10.0	16.78	4.25
9	-1.41	0	25.86	10.0	13.84	4.03
10	0	-1.41	40.0	2.93	15.89	4.96
11	0	1.41	40.0	17.07	14.95	4.66
12	0	0	40.0	10.0	16.81	4.23
13	1	1	50.0	15.0	15.46	4.76

[1] Randomised run order.
[2] mg maslinic acid/kg pomace.
[3] mg oleanolic acid/kg pomace.

3. Results and discussion

Physicochemical properties of olive pomace

The physicochemical properties of the olive mill pomace are presented in Table 3. Before extraction of the triterpenic acids, the sample was dehydrated to a certain moisture level under vacuum. Olive pomace has a high moisture content and is dried before storage to avoid any chemical and microbiological activities. The oil content of the pomace was too low to have an economical value. Thus, olive pomace may serve to produce value-added bioactives instead of oil (Parra *et al.*, 2010).

Table 3. Physico-chemical properties of olive pomace.[1]

Physico-chemical properties	Wet pomace sample	Dried pomace sample[2]
Moisture content (g H_2O/100 g sample)	65.11±0.61[a]	3.87±0.034[b]
Dry matter (g DM/100 g sample)	34.89±0.61[a]	96.13±0.034[b]
g oil/100 g dried pomace	8.64±0.28[a]	14.41±0.060[b]

[1] Values are given as a mean of parallels (n=3) with standard deviation. Different letters in the same row means significant difference between measured properties for two different samples.
[2] Sample was dried in a vacuum oven at 60 °C and 200 mBar.

Table 4. Model coefficients and performance parameters for both triterpenic acid.[1]

Model terms related to independent variable	Maslinic acid	Oleanolic acid
β0	-7.82605*	4.87486**
β1	1.05401***	0.02423^{ns}
β2	0.63067*	-0.25501**
β11	-0.01277***	-0.00029^{ns}
β22	-0.03127**	0.00982**
β12	-0.00020^{ns}	0.00130^{ns}
Model	**	*
R^2	93	83
adj-R^2	87	72
Lack-of-fit	ns	ns

[1] ns = not significant (P>0.05); *significant at P<0.05; **significant at P<0.01; ***significant at P<0.001.

Modelling of maslinic and oleanolic acid extraction from olive pomace

Triterpenic acids obtained from natural sources are used in the food, cosmetic and pharmaceutical industries due to their health benefits. Olive pomace is a good source of the triterpenic acids, MA and OA (Parra *et al.*, 2010). However, it is considered an environmental threat due to its high organic content and a large financial loss occurs if it is disposed of as waste, as it is not used as an industrial feedstock. Thus, developing a suitable technique and/or process to use olive pomace, such as extraction of its triterpenic acids, may increase its economic value and decrease its adverse environmental effect. In this study, MAE, which is considered a green technology, was used to leach MA and OA from olive pomace. The temperature and time process parameters were examined to determine their effects on the MA and OA extraction yields.

The MA and OA contents of the extracts obtained according to the experimental design are given in Table 2. The corresponding coefficients of the model terms for each response and the goodness-of-fit values are given in Table 4. In the model developed by RSM for the yields of both target compounds, the effect of temperature as a first and second order term significantly influenced the MA content of the extract (P<0.001), whereas no change in OA content, was observed (P>0.05) (Table 4). Remarkable changes in both target compounds were observed with process time (P<0.05). The time associated second order term affected the extraction of both compounds to the same extent but in different directions (P<0.01). No significant interaction between temperature and time occurred during the extraction of both triterpenoids. The adequacy of the model developed for each compound was interpreted by considering the goodness-of-fit values (determination of coefficient (R^2), adjusted determination of coefficient (adj-R^2) and lack-of-fit test) (Table 4). More than 90 and 80%, of the variations in the MA and OA contents in the extracts, as affected by the extraction conditions, were explained by their respective models.

The influences of MAE temperature and time parameters on the MA and OA extraction yields from olive mill pomace are shown in Figure 1 and 2, respectively. As shown in Figure 1, maximum MA extraction occurred around 40 °C, with lower or higher temperatures causing a decrease in the yield of this triterpenic acid. The process time showed a similar trend, and optimal MA extraction was attained after 10 min (Figure 1).

By considering the maxima process time, it could be concluded that a shorter process provided insufficient contact time for an MA rich extract. However, extending the process beyond 10 min adversely affected the content of this compound in the extraction solution. Mandal and Mandal (2010) reported a similar trend in extraction time on the yield of triterpenoids using MAE. The authors found that time favoured the transition of target compounds from the solid matrix up to a certain process time (around 8 min) but reported adverse effects with a further increase in time. Figure 2 shows the change in OA content of the extraction solution with temperature and time. Although there was a weak increase in OA content with temperature, it was insignificant (Table 4). The influence of time on OA content of the extract showed a decrease in the target compound yield with a prolonged process but beyond a certain time, the yield of OA started to increase (Figure 2).

Additionally, the performance of Soxhlet extraction and MAE of MA and OA from olive pomace were compared. The MA and OA contents of the extract obtained by

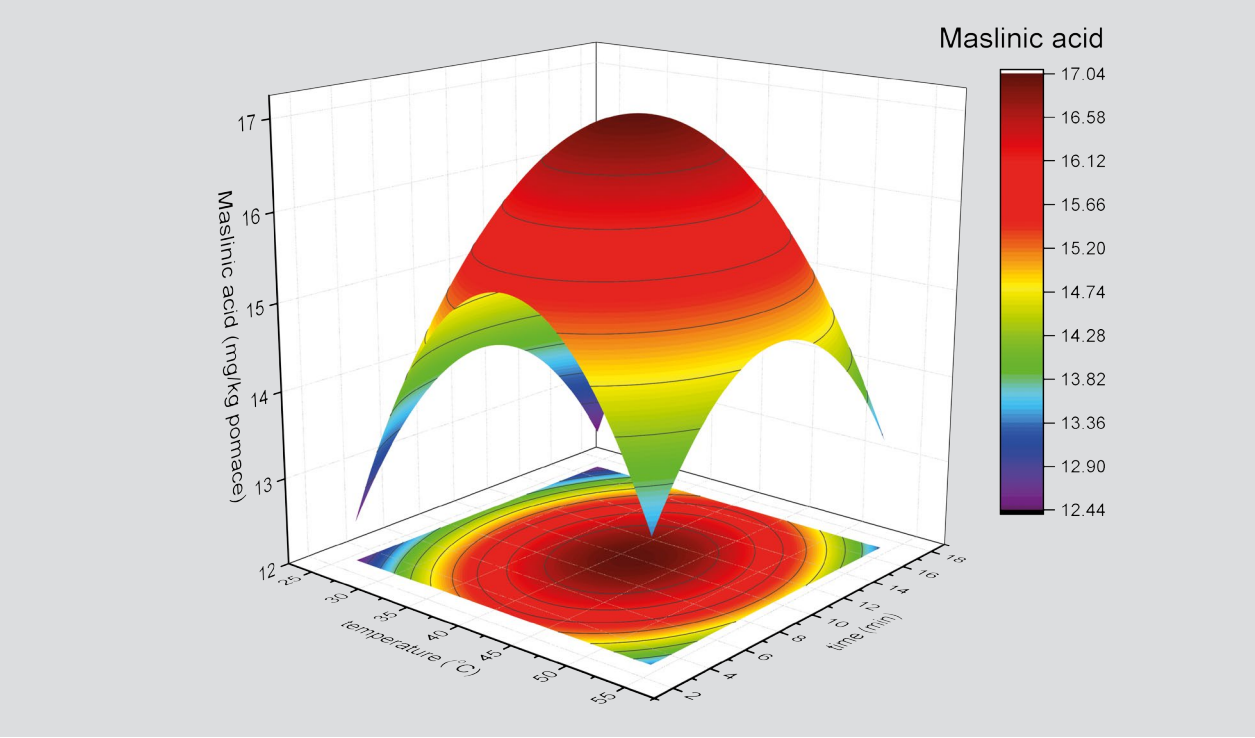

Figure 1. Response surface effects of extraction parameters (temperature and time) on maslinic acid content (mg/kg olive pomace) of extract.

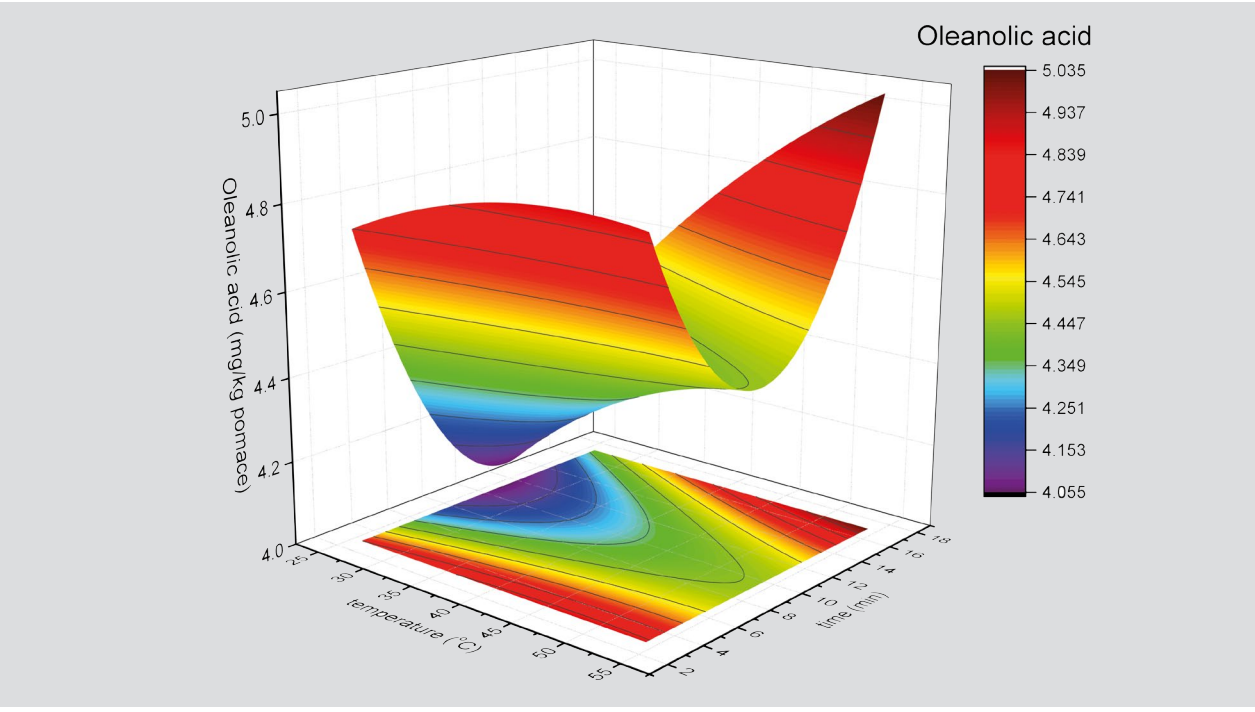

Figure 2. Response surface effects of extraction parameters (temperature and time) on oleanolic acid content (mg/kg olive pomace) of extract.

Soxhlet extraction were 10.80±0.93 and 3.90±0.50 mg/kg pomace, respectively. The relatively high temperature and extensive process time (80 °C for 4 h) required for Soxhlet extraction, were substantially lowered by using MAE (Table 2). Moreover, MAE resulted in extracts with higher MA and OA content than those by Soxhlet extraction, despite

the drastically decreased extraction conditions. Mandal and Mandal (2010) also reported the favourable effect of MAE of triterpenic acids.

The MA (mg/kg pomace) and OA (mg/kg pomace) contents of the extracts obtained by MAE varied from 13.84-17.33 and 4.03-4.96, respectively. Allouche *et al.* (2010) reported 5.87-16.05 and 3.83-10.66 MA (mg/kg olive paste) in olive pastes belonging to Arbequina and Picual species, respectively, whilst the corresponding OA (mg/kg olive paste) contents varied from 3.46-14.20 and 2.80-7.41, respectively. Thus, our results concurred with literature values. Small differences may be attributed to differences in the studied cultivars and extraction processes. The extraction temperature and time of the triterpenic acids were optimised separately. The highest MA yield occurred at 40 °C after 10 min whilst the optimal conditions for OA were 40 °C and 2.93 min.

Model validation for maslinic and oleanolic acid extraction from olive pomace

The MA and OA contents of the extract were maximised by optimising the extraction conditions because these compounds have high economic potential. Optimum conditions for extraction of MA and OA from olive pomace were determined as 42 °C and 3 min. The respective models estimated the yields of MA and OA contents in the extract obtained under the optimal extraction conditions as 14.20-16.75 and 4.47-5.27 mg/kg pomace, respectively. For model validation, the extraction was performed under the optimal conditions. The MA and OA contents of extract were 16.47 and 4.85 mg/kg pomace, respectively. Thus, the experimental results for both triterpenoids were within the corresponding prediction range specified for each triterpenoid. Therefore, the developed models were accurate and appropriate.

Acknowledgements

This study was financially supported by The Scientific and Technological Research Council of Turkey (grant number: 2241-A).

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