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Ultrasound increases the aqueous extraction of phenolic compounds with high antioxidant activity from olive pomace.

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Abstract

Olive pomace is a waste produced by the olive oil industry in massive quantities each year. Disposal of olive pomace is difficult due to high concentrations of phenolic compounds, which is an environmental concern. However, phenolic compounds have applications in the health industry. Therefore, extraction of phenolic compounds from olive pomace has the potential to remove an environmentally hazardous portion of pomace while creating an additional source of income for farmers and producers. Using advanced technologies including Ultrasound Assisted Extraction (UAE), combined with water as an extraction solvent, has recently gained popularity. The present study outlines the optimal UAE conditions for the extraction of phenolic compounds with high antioxidant activity from olive pomace. Optimal conditions were developed using RSM for parameters power, time and sample-to-solvent ratio. Total phenolic compounds determined by Folin Ciocalteu method and total major bioactive compounds determined by HPLC as well as antioxidant capacity (DPPH and CUPRAC) were investigated. The optimal conditions for the extraction of phenolic compounds with high antioxidant activity were 2 g of dried pomace/ 100mL of water at 250W power for 75mins. UAE improved the extraction efficiency of water and yielded extracts with high levels of phenolic compounds and strong antioxidant activity.

Keywords

Olive pomace; *Olea Europaea*; HPLC; UAE; Response Surface Methodology.

1. Introduction

Olive pomace is the solid waste product of the olive oil extraction process, which retains high amounts of organic substances (14-15%), including sugars, nitrogenous compounds, volatile fatty acids, polyalcohols, pectins and fats (Lafka, Lazou, Sinanoglou, & Lazos, 2011) as well

as a high concentration of phenolic compounds (Goldsmith, Vuong, Stathopoulos, Roach, & Scarlett, 2014a; Ranalli, Lucera, & Contento, 2003). Thousands of tonnes of olive waste are produced each year; these waste products are often dumped in landfill, which is causing a number of environmental concerns due to the presence of phenolic compounds. Therefore, the disposal of olive waste products has been a major environmental issue in a number of olive growing countries (Capasso, Cristinzio, Evidente, & Scognamiglio, 1992).

Extraction of the phenolic compounds from olive pomace has the potential to somewhat limit the environmental damage that can be caused by this waste fraction and may even provide an additional source of income for olive oil producers (Obied, Allen, Bedgood, Prenzler, & Robards, 2005). For example, the extraction of oleuropein, the most abundant phenolic compound in olive products, would add value to the olive oil production process. This is because a number of the beneficial health effects of virgin olive oil have been attributed to consumption of oleuropein, including anti-atherogenic (Covas, 2007), anti-inflammatory (de la Puerta, Ruiz Gutierrez, & Hoult, 1999), anti-cancer (Ahmad Farooqi et al., 2017; Fayyaz et al., 2016; Hadrich et al., 2016; Liu, Wang, Huang, Chen, & Li, 2016; Maalej, Bouallagui, Hadrich, Isoda, & Sayadi, 2017; Morana et al., 2016; Secme, Eroglu, Dodurga, & Bagci, 2016; Sepporta et al., 2016; Xu & Xiao, 2017) and anti-microbial (Bisignano et al., 1999) properties and therefore oleuropein is a valuable product in itself. A number of advanced techniques to extract phenolic compounds have gained popularity in recent years including Microwave Assisted Extraction (MAE), Pressurised Liquid Extraction (PLE) and Solid Phase Extraction (SPE). However, Ultrasound Assisted Extraction (UAE) is considered one of the simplest and most cost-effective techniques to scale up for industrial production.

The UAE method has been used to improve the extraction efficiency of phenolic compounds from a variety of plant matrices. The method has a number of benefits, including as an add on step to existing processes with minimum alteration, as an application in the aqueous

extraction of phenolic compounds therefore reducing the need for harmful organic solvents, which can be difficult and expensive to dispose of. The UAE method often results in shorter extraction times and high yields; importantly, UAE has been shown to improve extraction yield up to 35% (Vilkhu, Mawson, Simons, & Bates, 2008).

Despite the clear benefits of UAE, the use of high power levels with the method can lead to the degradation of phenolic compounds. For example, in one of our previous studies we observed a 25% decrease in the extraction of Euphol from Euphorbia Tirucalli when the power was increased from 150-250W (2015). Therefore, it is important to optimise the UAE extraction parameters to ensure the maximum retention of valuable compounds.

Water is classified as a safe and "green" solvent, which is inexpensive, accessible and considered an environmentally friendly alternative to harmful organic solvents (Hartonen & Riekkola, 2017). Therefore, water was the solvent of choice for the recovery of bioactive compounds from olive pomace in the present study. This study, for the first time, optimised the Ultrasound Assisted Extraction (UAE) conditions for maximum recovery of phenolic compounds with high antioxidant activity from olive pomace using water. Our study is the first to investigate water as an extraction solvent and determine the optimal conditions for the extraction of bioactive compounds from olive pomace.

2. Materials and Methods

2.1. Materials and Reagents

Folin–Ciocalteu's reagent, sodium carbonate, gallic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,4,6-Tris(2-

pyridyl)-*s*-triazine (TPTZ), ferric chloride, sodium acetate, acetic acid, copper (II) chloride, ammonium acetate (NH_4Ac), neocuproine methanol and ethanol were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Ultra-pure (type 1) de-ionized (DI) water was prepared by reverse osmosis and filtration using a Milli-Q direct 16 system (Millipore Australia Pty Ltd., North Ryde, NSW, Australia).

2.2. Sample Collection and preparation

Green olives of the Manzanilla cultivar were harvested at Houndsfield Estate (Hunter Valley, NSW, Australia) in July 2015 and processed on-site the next day using a semi-continuous Enorossi 150 traditional olive oil pressing system (Enoagricola Rossi, Calzolaro di Umbertide, Perugia, Italy) standardised to press a maximum of 150kg of olives at a time. Olive pomace was collected and stored at -20ºC until further analysis. Olive pomace was freeze dried until constant weight was achieved before blending in a blender and being passed through a 0.1mm sieve and stored at −20°C until further analysis. Dried pomace was then de-fatted 3 times by adding 100mL of hexane to 10g of pomace and filtering with a Buchner funnel apparatus. For extraction yields, the water was removed from a certain quantity of extract in a vacuum drier (Memmert, Schwabach, Germany) at 50 °C and vacuum pressure of 65 mb until constant weight was achieved (total aqueous extract yield = 208.35 ± 35 mg/g dried sample).

2.3. Response Surface Methodology (RSM)

The RSM with the Box–Behnken design was used to investigate the influence of three independent parameters; power, time and sample to solvent ratio, on the extraction of total phenolic compounds (TPC) and the antioxidant activity of the extracts. An ultrasonic bath was used (Soniclean, 220V, 50Hz and 250W model 250HD, Soniclean, Pty Ltd, Thebarton, SA, Australia). The optimal ranges of power (150-250W), time (45-75 min) and sample-to-

111 solvent ratio (1-3 g/100 mL) were determined based on preliminary experiments (data not
112 shown). A control extraction was conducted at the same optimal time and sample to solvent
113 ratio without ultrasound. Temperature was maintained at 40°C by the ultrasound baths
114 temperature regulator. The independent variables and their code variable levels are shown in
115 Table 1.

116 To express the TPC or antioxidant capacity as a function of the independent variables, a
117 second-order polynomial equation was used as follows and as previously described by Vuong
118 *et al.* (2011):

119 $$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k=1} \sum_{j}^{k} \beta_{ij} X_i X_j + \sum_{i=1}^{k} \beta_{ii} X_i^2,$$

120 Where various X_i values are independent variables affecting the response Y; β_0, β_i, β_{ii}, and β_{ij}
121 are the regression coefficients for the intercept and the linear, quadratic and interaction terms,
122 respectively, and k is the number of variables.

123 *2.4. Total Phenolic Compounds*

124 The TPC were determined according to Thaipong *et al.* (Thaipong, Boonprakob, Crosby,
125 Cisneros-Zevallos, & Hawkins Byrne, 2006). Briefly, samples were added to Folin–
126 Ciocalteu's reagent before adding 5% sodium carbonate solution and incubating in the dark
127 for 1h. Absorbance was then read at 760nm using a UV spectrophotometer (Varian, Melbourne,
128 VIC, Australia). Results were expressed as mg of gallic acid equivalents per gram of dried
129 olive pomace (mg GAE/g).

130 *2.5. Total Major Bioactive Compounds*

131 For determination of total major bioactive compounds, HPLC was performed according to
132 Goldsmith et al., (2014a) with minor modifications. The extracts were analysed using a

Shimadzu HPLC system (Shimadzu Australia, Rydalmere, NSW Australia) and a 250 ± 4.6mm Synergi 4 µm Fusion-RP 80A reversed-phase column (Phenomenex Australia Pty. Ltd., Lane Cove, NSW Australia) with detection at 254nm. The column was maintained at 30°C, the flow rate was 1 ml/min and three solvents were used for the mobile phase Solvent **A**: 0.1% orthophosphoric acid, Solvent **B**: 100% Methanol, Solvent **C**; 100% Ethanol. A gradient elution schedule was used according to the following: 0-40 mins A 96%, B 2%, C 2%; 40-60 mins A 40%, B 30%, C 30%; 60-62 mins A 96%, B 2%, C 2%. Syringic acid was used as internal standard. Values for total major bioactive compounds were determined using a tyrosol standard curve; they were expressed as µg Tyrosol equivalents (TYE) per gram of dried olive pomace.

2.6. Antioxidant Activity Assays

Two assays were employed to assess the antioxidant activity of the pomace extracts:

The cupric reducing antioxidant capacity (CUPRAC) assay was conducted as previously described by Apak *et al.* (2004). Results were expressed as mg of trolox equivalents per gram of dried olive pomace (mg TRE/g).

The DPPH free radical scavenging capacity of the extracts were analysed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, as described by Goldsmith et al, (2014b). The results were expressed as mg of trolox equivalents per gram of dried olive pomace (mg TRE/g).

2.7. Statistical analysis

The RSM was designed and analysed using JMP Version 11 (SAS Cary, NC, USA). JMP was also used to develop the model equation, graph the 2D and 3D prediction profiler plots to predict the optimum values of the response variables in order to maximise the TPC and

antioxidant capacity of the extracts. The original values and ranges of the parameters under investigation as well as their parameter symbols and codes are presented in *Table 1*.

3. Results and discussion

3.1. Fitting the models for the prediction of TPC and antioxidant capacity

Based on preliminary experiments (not shown), time, power and sample-to-solvent ratio were identified as important parameters which could impact upon the extraction of phenolic compounds from olive pomace, the ranges for each variable were determined and are listed in *Table 1*.

Table 2 shows the reliability of the mathematical model in predicting variances between actual and predicted values. The analysis of variance for the experimental results for the Box Behenkin design showed the coefficient of determination (R^2) for the fit of the model of TPC was 0.8, CUPRAC was 0.81 and DPPH was 0.69; suggesting that 80%, 81% and 69% of the actual TPC, CUPRAC and DPPH values could be predicted by the model, respectively. This relationship is further supported by the values for Predicted Residual Sum of Squares (PRESS is a measure of how well each point fits the experimental design) and the F-ratio of the model: 3128 and 15.1 for TPC, 3001 and 6.56 for CUPRAC and 1566 and 6.15 for DPPH (respectively). In summary, analysis of variance showed that the models are reliable for prediction of TPC and antioxidant capacity.

3.2. The effect of the test parameters on the extraction of TPC

The effect of the test parameters (coded variables in Table 1) on the response variable (Y) TPC is shown in the following equation:

$$Y = 8.3 + 2.4\,X_1 + 0.1\,X_2 - 0.7\,X_3 + 1.6\,X_1\,X_2 - 1.0\,X_1\,X_3 - 1.1\,X_2\,X_3 + (6.2\,X_1)^2 + (3.5\,X_2)^2 - (2.6\,X_3)^2$$

Table 3 presents the linear regression coefficients for each variable and indicates their statistical significance. Power and time both had positive relationships with the extraction of TPC, while the sample-to-solvent ratio had a negative effect; that is, as we increased the amount of sample while keeping the amount of solvent that same, we saw a decrease in TPC. Therefore, as power and time were increased and as the amount of solvent /g of sample were increased, the extraction of TPC also increased. However, the only individual variable that had a significant influence on the extraction of TPC within the ranges tested was power ($p = 0.0001$). Power has previously been shown to increase the extraction of phenolic compounds from a variety of sources (Altemimi, Watson, Choudhary, Dasari, & Lightfoot, 2016). Moreover, the combination of power and time also had a significant influence on the extraction of TPC ($p = 0.03$); this is also in accordance with the literature (Falleh, Ksouri, Lucchessi, Abdelly, & Magné, 2012). In addition, the interaction between power and time within the ranges tested had a significant impact on extraction of TPC whereas, there was no interactive relationship between power and ratio or time and ratio (Table 3); indicating that increasing both power and time can result in a higher TPC being extracted from the olive pomace.

3.3. The effect of the test parameters on antioxidant activity

The effect of the test parameters (coded variables in Table 1) on the response variable DPPH scavenging capacity (Y) is shown in the following equation:

$$Y = 22.4 + 2.5\,X_1 + 0.3\,X_2 + 5.7\,X_3 + 3.6\,X_1X_2 - 0.9\,X_1X_3 - 3.0\,X_2X_3 + (1.3\,X_1)^2 - (3.8\,X_2)^2 - (0.2\,X_3)^2$$

Similarly, the effect of the test parameters (coded variables in Table 1) on the response variable cupric reducing antioxidant capacity (Y), is shown in the following equation:

$$Y = 37 + 6.8\,X_1 + 1.6\,X_2 - 0.9\,X_3 + 4.8\,X_1X_2 - 1.6\,X_1X_3 - 6.3\,X_2X_3 + (19.9\,X_1)^2 + (10.4\,X_2)^2 - (5.1\,X_3)^2$$

The results showed that the individual variables of power and time had a positive influence on both the DPPH scavenging capacity and the cupric reducing antioxidant capacity of the extracts. Sample-to solvent ratio on the other hand, had a positive influence on the DPPH scavenging capacity but had a negative influence on the cupric reducing antioxidant capacity. In addition, power and time as well as time and sample to solvent ratio, in the tested ranges, had a significant interactive effect on DPPH scavenging capacity and the cupric reducing antioxidant capacity of the extracts. Of interest, power and ratio in the tested ranges did not show a significant interactive effect on DPPH scavenging capacity and cupric reducing antioxidant capacity of the extracts.

3.4.Optimisation of the extraction conditions for maximum extraction of TPC with high antioxidant activity from olive pomace

Based on the predictive models (Figures 1 and 2), the optimal conditions for the extraction of phenolic compounds from olive pomace were 2g of dried pomace/ 100mL of water at 250W power for 75mins. These conditions were the same for the optimisation of antioxidant activity via DPPH and CUPRAC; therefore, these conditions were used for further validation (Table 4). The resulting values fell inside the proposed ranges for TPC and antioxidant activity. As

such, these conditions were proposed as optimal for the extraction of phenolic compounds with high antioxidant activity from olive pomace waste.

3.5.Optimal UAE conditions compared to control conditions

The principle of UAE extraction is to disrupt plant cell walls and increase mass transfer of intracellular components into the extraction solvent (Yingngam, Monschein, & Brantner, 2014). To assess the efficacy of ultrasound in extracting phenolic compounds with high antioxidant activity from olive pomace, validation was also conducted comparing the optimal conditions with and without ultrasound. The optimised UAE conditions increased the extraction of TPC by 24% (Table 4). This was also reflected in the HPLC results where by the UAE improved total peak area by 20.4% (Table 5). Typical chromatograms produced from optimised UAE extracts as well as control extracts are pictured in Figure 3. The UAE conditions yielded a higher level of TPC as well as antioxidant activity compared to the control. Figure 3 shows that the optimised UAE extracts had a higher area for most of the peaks compared to the control extracts; however, the UAE extracts did not have any additional peaks. This suggests that UAE enhanced the ability of water to extract compounds from the pomace without extracting any additional compounds. This increase can be attributed to the ability of Ultrasound to impact the microstructure of plant materials; since ultrasonic cavitation creates shear forces that disrupt cell walls, which enabled the extraction solvent to penetrate the pomace tissue and extract the phenolic compounds. Similar results have been reported previously (Chen et al., 2018; Feng, Luo, Tao, & Chen, 2015; Tian, Xu, Zheng, & Martin Lo, 2013).

The antioxidant activity of the UAE extracts (Table 4) was also higher than the controls (an increase of 11% and 12% for the DPPH and CUPRAC assays respectively). The application of UAE has been shown to increase the antioxidant activity of extracts from a variety of plant

materials, including olive leaves (Sahin & Samli, 2013), peach, pumpkin (Altemimi et al., 2016) and green tea (Nkhili et al., 2009). This is likely due to the improvement in the extraction of total phenolic compounds. In the present study, no new peaks were identified in the chromatograms from the UAE extracts (Figure 3) when compared to the controls; therefore, the increase in antioxidant activity is likely due a larger quantity of each compound being extracted. However, since the peak area (mg TYE equivalents) increased by 26% with the application of UAE (Table 4) the peaks that were significantly increased must correspond to compounds with high antioxidant activity. Therefore, UAE can be considered as an effective technique to increase the levels of the extracted compounds with high antioxidant activity in olive pomace extracts.

4. Conclusions

UAE increased the quantity of phenolic compounds extracted from olive pomace. The proposed optimal conditions for the extraction of phenolic compounds with high antioxidant activity from olive pomace were 2 g of dried pomace/ 100mL of water at 100% power (250W) for 75mins maintained at 30°C. This simple and inexpensive method could be readily up-scaled to add a source of income to olive farmers and olive oil processors, a viable use for this agricultural waste product.

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Table 1. Values of the independent parameters and their coded forms with their symbols employed in RSM for optimization of UAE conditions for phenolic compounds from olive pomace.

Independent Parameters	Symbols of the Parameters	Original Values of the Parameters	Parameter Coded Forms*
Power (W)	X_1	100	-
		150	0
		250	+
Time (min)	X_2	45	-
		60	0
		75	+
Ratio (g/100mL)	X_3	1	-
		2	0
		3	+

*Parameter coded forms -, 0 and + are the minimum point, centre point and maximum point (respectively) for the independent parameters temperature, time and ratio.

Table 2

Table 2. Analysis of variance for determination of the model fit. Total Phenolic Compounds (TPC) and antioxidant capacity (CUPRAC and DPPH).

Sources of Variation	**TPC**	**Antioxidant Capacity**	
		CUPRAC	**DPPH**
Lack of fit (*p*-value)	>0.0001*	>0.0001*	0.0076*
R^2	0.8	0.81	0.69
PRESS	3128	3001	1566
F-ratio of model	15.1	6.56	6.15
p of model > F	>0.0001*	>0.0001*	>0.0001*

* Denotes significant result ($p < 0.05$)

Table 3

Table 3. The analysis of variance for the experimental results.

Parameter	DF	TPC		Antioxidant Capacity			
				DPPH		CUPRAC	
		Estimate	Prob>|F|	Estimate	Prob>|F|	Estimate	Prob>|F|
β_0	1	8.26	<0.0001*	22.4	<0.0001*	37.14	<0.0001*
$\beta_{1\ power}$	1	2.4	<0.0001*	2.53	0.0288*	6.79	<0.0001*
β_{2time}	1	0.068	0.89	0.29	0.7921	1.61	0.2950
β_{3ratio}	1	-0.70	0.11	5.68	<0.09	-0.91	0.4832
$\beta_{12power.time}$	1	1.59	0.025*	3.64	0.0209*	4.81	0.0250*
$\beta_{13power.ratio}$	1	-0.97	0.10	-0.92	0.4758	-1.60	0.3650
$\beta_{23time.ratio}$	1	-1.12	0.065	-2.96	0.0272*	-6.33	0.0009*
$\beta_{11power2}$	1	6.24	<0.0001*	1.26	0.4260	19.93	<.0001*
$\beta_{22time2}$	1	3.53	<0.0001*	-3.79	0.0210*	10.37	<.0001*
$\beta_{33ratio2}$	1	-2.62	0.0026*	-0.24	0.8914	-5.06	0.0445*

* Significantly different at $p < 0.05$; β_0: intercept; β_1, β_2 and β_3: linear regression coefficients for power, time and ratio; β_{12}, β_{13} and β_{23}: regression coefficients for interaction between power × time, power × ratio and time × ratio; β_{11}, β_{22} and β_{33}: quadratic regression coefficients for power × power, time × time and ratio × ratio.

Table 4

Table 4. Validation of the RSM models; the predicted values and the actual values obtained at the maximum desirability for the UAE conditions of 2 g of dried pomace/ 100mL of water at 100% power for 75 min maintained at 30°C.

	Phenolic compounds	**Antioxidant activity**	
	TPC	**DPPH**	**CUPRAC**
	(mg GAE g^{-1})	**(mg TRE g^{-1})**	**(mg TRE g^{-1})**
Predicted	22.02 ± 2.66^{a}	26.37 ± 5.85^{a}	80.57 ± 7.99^{a}
Actual (UAE)	19.71 ± 1.41^{a}	31.23 ± 1.42^{a}	73.54 ± 2.54^{a}
Control (no UAE)	13.76 ± 0.91^{b}	28.07 ± 3.24^{a}	65.36 ± 1.77^{b}

[a, b] Values in the same column with a different superscript are significantly different from one another ($p<0.05$)

Total yield of extracts (UAE = 222.2 ± 48.1, Control = 194 ± 39.6)

Table 5

Table 5. Quantification of selected HPLC peaks expressed as µM Tyrosol Equivalents (TYE)/g of dried pomace. Peak numbers correspond to the peaks in Figure 3.

Peak number	*Retention time (mins)*	*UAE (µM TYE/g)*	*Control (µM TYE/g)*
1	*7.20*	*0.95 ± 0.1* [a]	*0.46 ± 0.07* [b]
2	*8.46*	*13.65 ± 0.84* [a]	*10.01 ± 0.12* [b]
3	*10.14*	*1.38 ± 0.02* [a]	*0.64 ± 0.06* [b]
4	*12.27*	*0.08 ± 0.03* [a]	*0.00* [b]
5	*16.26*	*6.24 ± 1.01* [a]	*4.99 ± 0.03* [b]
6	*16.89*	*1.29 ± 0.01* [a]	*0.61 ± 0.04* [b]
7	*17.41*	*0.00* [a]	*0.69 ± 0.07* [b]
8	*19.47*	*20.01 ± 0.04* [a]	*15.87 ±0.09* [b]
9	*19.98*	*5.68 ± 0.07* [a]	*4.22 ± 0.03* [b]
10	*22.74*	*2.24 ± 0.12* [a]	*1.58 ± 0.15* [b]
11	*23.86*	*3.95 ± 0.01* [a]	*2.86 ± 0.49* [b]
12	*24.69*	*0.80 ± 0.01* [a]	*0.76 ± 0.31* [a]
IS	*26.11*	*na*	*na*
13	*31.46*	*3.76 ± 0.25* [a]	*0.72 ± 0.24* [b]
14	*42.91*	*5.78 ± 0.05* [a]	*5.21 ±0.73* [a]
Total		***62.05 ± 1.87*** [a]	***49.98 ± 2.27*** [b]

[a, b] Values are means ± SD in the same row with a different superscript are significantly different from one another ($p<0.05$).

Figure 1

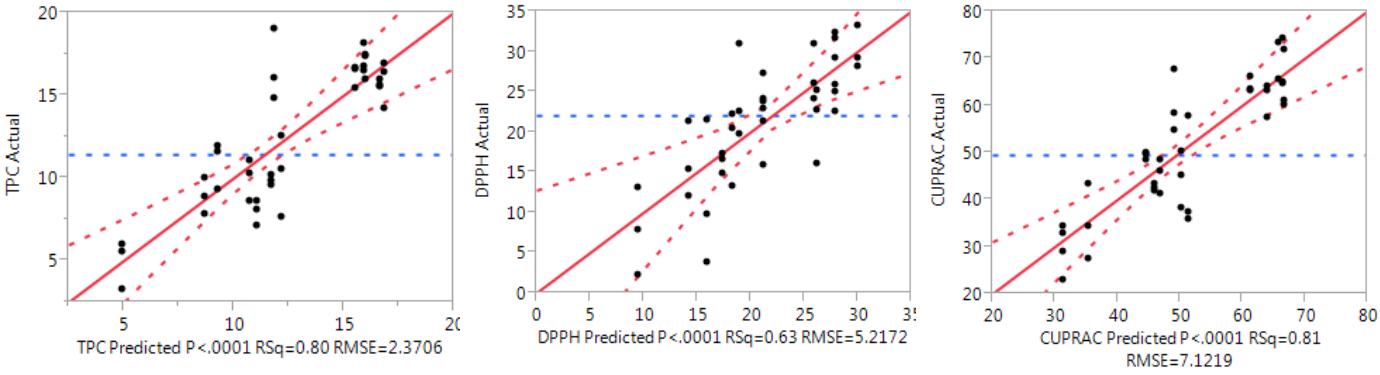

Figure 1. Correlation between the actual and predicted values for TPC, DPPH and CUPRAC of the aqueous olive pomace extract.

Figure 2

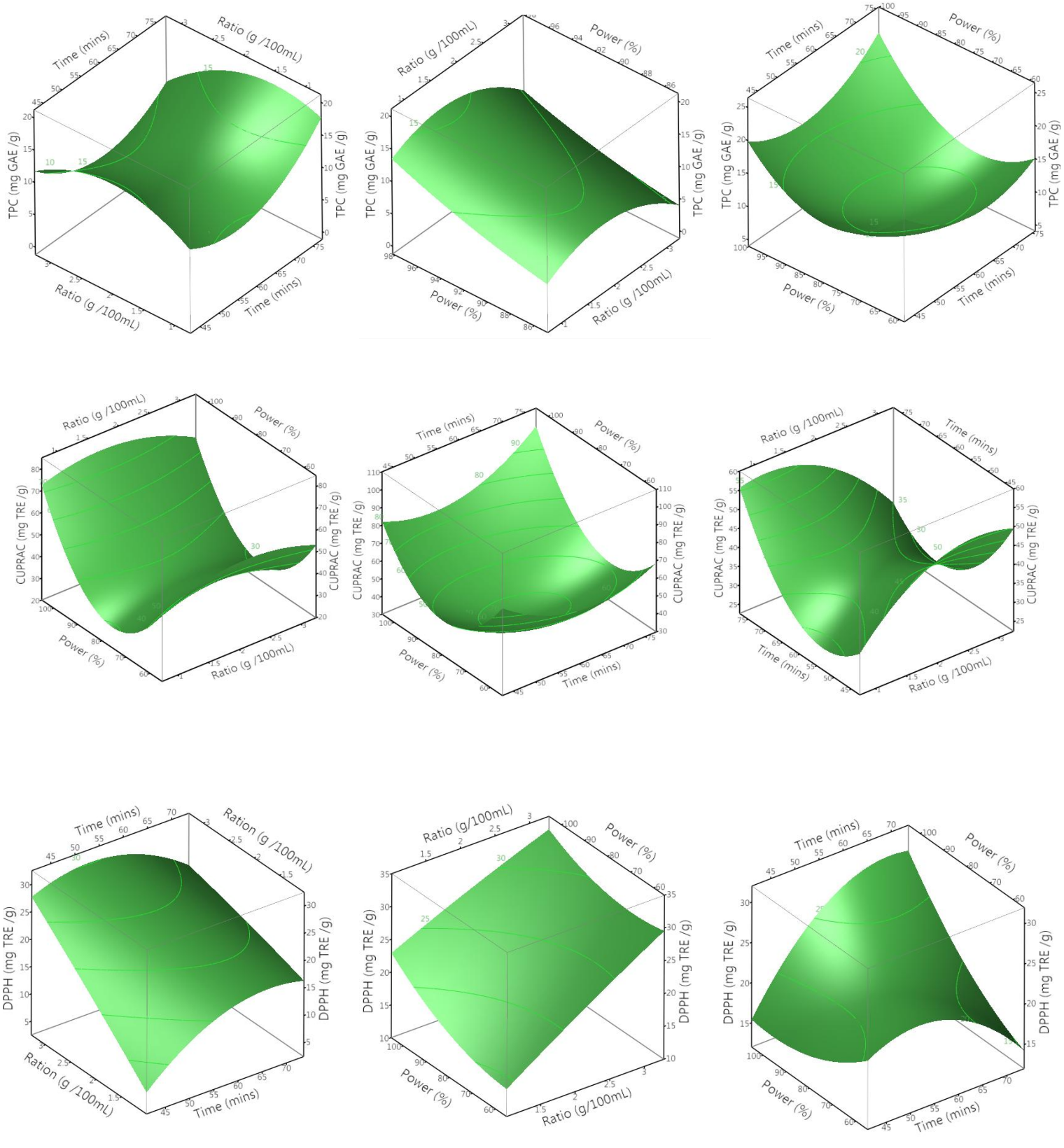
TPC (mg GAE /g)
Time (mins)
Ratio (g /100mL)
Power (%)
CUPRAC (mg TRE /g)
DPPH (mg TRE /g)
Ration (g /100mL)
Ratio (g/100mL)

Figure 2. 3D response surface and 2D contour plots for the effect of the test parameters on the total phenolic compounds (TPC) and antioxidant activity (DPPH and CUPRAC) of the aqueous olive pomace extracts.

Figure 3

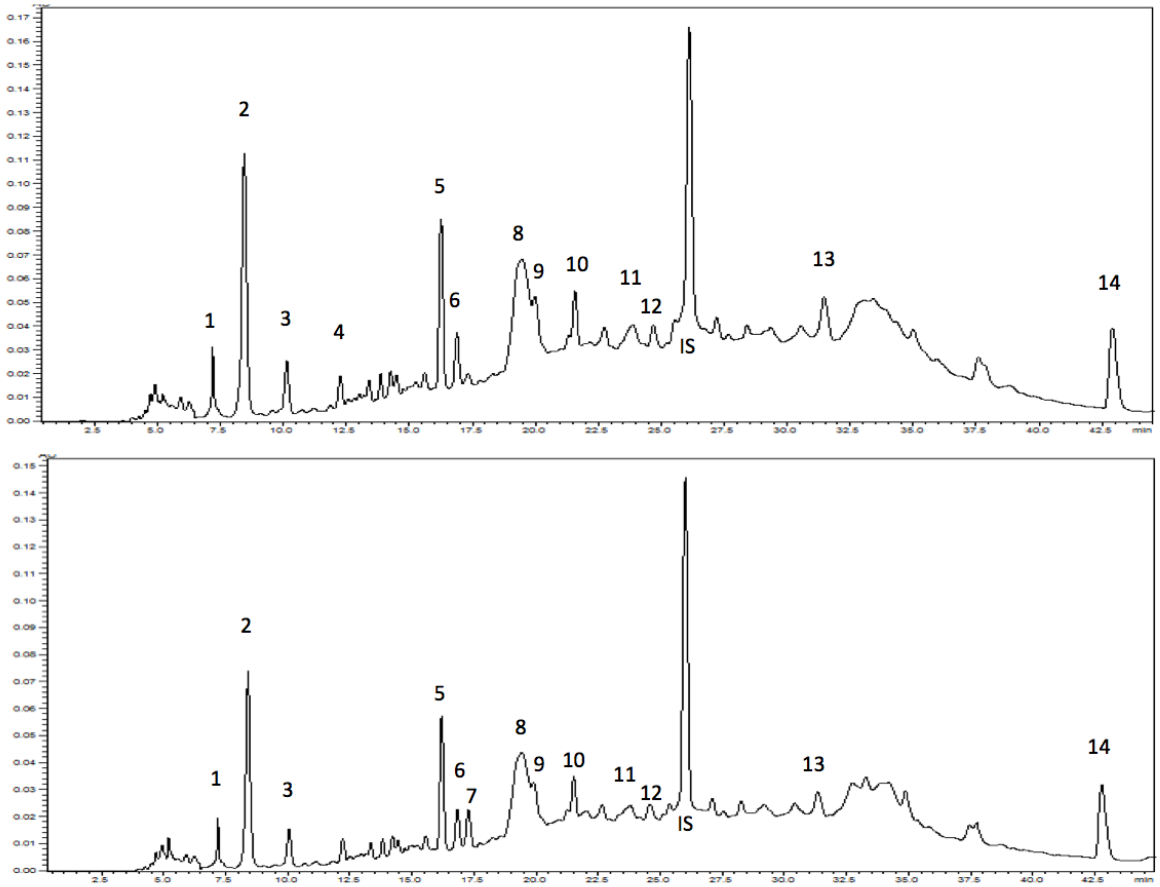

Figure 3. Typical HPLC chromatogram at 254nm of; (Top) optimal UAE extract (Bottom) control extract. The internal standard (IS) was syringic acid.
*Axes on chromatograms are not the same.