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Application and Optimization of Microwave-Assisted Extraction and Dispersive Liquid–Liquid Microextraction Followed by High-Performance Liquid Chromatography for the Determination of Oleuropein and Hydroxytyrosol in Olive Pomace

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Abstract

In the present study, a new method based on microwave-assisted extraction and dispersive liquid–liquid microextraction (MAE–DLLME) followed by high-performance liquid chromatography (HPLC) was proposed for the separation and determination of oleuropein (Ole) and hydroxytyrosol (HyT) from olive pomace samples. The effective factors in the MAE–DLLME process such as microwave power, extraction time, the type and volume of extraction, and dispersive solvents were studied and optimized with the aid of response surface methodology (RSM) based on a central composite design (CCD) to obtain the best condition for Ole and HyT extraction. At the optimized conditions, parameter values were 220 W microwave power, 12 min extraction time, 60 μL extracting solvent, and 500 μL dispersive solvent. The calibration graphs of the proposed method were linear in the range of 10–500,000 μg L^{-1}, with the coefficient of determination (R^2) higher than 0.99 for Ole and HyT. Repeatability of the method, described as the relative standard deviation (RSD), was 4.12–5.63% ($n = 6$). The limits of detection were 35 and 20 μg L^{-1} for Ole and HyT, respectively. The recoveries of these compounds in the spiked olive pomace sample were from 93 to 98%. The proposed method, MAE–DLLME–HPLC–UV, was an accurate, rapid, and reliable method when compared with previous methods.

Introduction

By-products and wastes from plant food processing, which are principal problems for the food industry, are promising sources of bioactive compounds. A vast amount of different by-products is generated during the olive processing industry, which includes the production of olive oil and table olives, such as olive pomace, wastewater, and leaves (Romero-García et al. 2014). The olive leaves and pomace contain a wide variety of phenolic compounds such as oleuropein (Ole), hydroxytyrosol (HyT), tyrosol, apigenin, and luteolin that are potent antioxidants (Rodrigues et al. 2015). Olive leaf extracts have been shown to possess antioxidant activities upper than those of BHT and vitamin E with regard to the protective effect applied on methyl linoleate (Le Tutour and Guedon 1992). The chemical composition of olive leaves and pomace is changeable due to the origin of the olives, olive variety, culture conditions, and extraction procedure (Dermeche et al. 2013).

Ole is the most abundant phenolic compound in unprocessed olive fruit and leaves and is responsible for the bitter taste of olive fruit (Malik and Bradford 2006). HyT, an Ole derivative, is present in large amounts in the processed olive fruit and olive oil. Health benefits of these compounds have been extensively evaluated. It has been demonstrated that Ole and HyT act as antioxidants and reduce the risk of coronary

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diseases (Manna et al. 2002; Vilaplana-Pérez et al. 2014), they protect enzyme activity and cell death in cancer patients (Tripoli et al. 2005; Vilaplana-Pérez et al. 2014), and they exhibit antimicrobial and antiviral properties (Kubo et al. 1995; Malik and Bradford 2006), and HyT also protects against atherosclerosis (Tripoli et al. 2005) and prevents diabetic neuropathies (Japón-Luján et al. 2006b).

Numerous methods were used for extraction of antioxidant components from natural products such as conventional heat-assisted extraction, soxhlet extraction, maceration, and percolation, among others. These conventional methods require large quantities of solvents and are often time-consuming (Heleno et al. 2016; Yang et al. 2017). Recently, emerging extraction technologies such as ultrasound-assisted extraction, supercritical fluid extraction, pressurized solvent extraction, and microwave-assisted extraction have been applied by the researchers with the aim of more efficient extraction of minor and major antioxidant agents in the natural sources. These emerging methods have several benefits like saving energy and reagents, being fast and efficient, avoiding losses, and optimizing the extraction procedure (Heleno et al. 2016).

Recently, the health benefits of Ole and HyT have stimulated the development of analytical methods for their separation and quantification. Ultrasound-assisted extraction coupled with capillary electrophoresis–diode array detection was used in the extraction and determination of Ole and HyT in alperujo (Priego-Capote et al. 2004). Ultrasound-assisted extraction followed by liquid chromatography–photodiode array detection was applied to determine Ole in the olive leaves (Japón-Luján et al. 2006a). In other studies, the isolation and determination of Ole in the olive leaves were done by microwave-assisted extraction coupled with liquid chromatography–photodiode array detection (Japón-Luján et al. 2006b), and also, microwave-assisted extraction followed by liquid chromatography–tandem mass spectrometry was applied in the extraction and determination of Ole and HyT in the alperujo (Pérez-Serradilla et al. 2007). Godoy-Caballero et al. (2013) employed the reversed-phase dispersive liquid–liquid microextraction for the determination of Ole and HyT in virgin olive oil coupled with rapid resolution liquid chromatography–diode array detection and liquid chromatography–mass spectrometry. In another study, ultrasound-assisted emulsification–microextraction followed by liquid chromatography–photodiode array detection was developed to identify and quantify Ole and HyT from extra-virgin olive oil (Reboredo-Rodríguez et al. 2014). These methods have been evaluated as reliable alternatives to the classic liquid–liquid extraction (LLE) technique.

In recent years, much attention has been considered on the application of microwave-assisted extraction (MAE) in analytical and biological chemistry (Bener et al. 2016). MAE is the process by which microwave energy is applied to heat extraction solvents in contact with solid samples. This phenomenon leads to migration of target compounds from the sample into the extraction solvent while the extraction solvent diffuses into the samples and extracts the target components. It is noticeable that MAE has several advantages when compared with traditional methods. It can be done in a short time and at a high rate with good product quality and the solvent consumption can be reduced; polar or non-polar extraction solvents can be used for extraction of target compounds; all parameters in the MAE method can be controlled by a software-based system (Bener et al. 2016; Khajeh and Ghanbari 2011); better reproducibility, higher recoveries, and extraction efficiency can be achieved; and the thermal degradation effects can be avoided (Bener et al. 2016). Besides, in terms of used energy, sample preparation, and extraction solvents, MAE is a green extraction and green chemistry (Rombaut et al. 2014).

Dispersive liquid–liquid microextraction (DLLME) is an alternative sample preparation method which was introduced in 2006 (Rezaee et al. 2006) and is based on a ternary component solvent. The emulsion phenomena are performed by injecting a mixture of the extraction and disperser solvents in the aqueous sample containing the target analyte and a cloudy emulsion form. After a very short time, equilibrium is achieved and the separation process is done by centrifugation (Habibi et al. 2017; Sereshti et al. 2014). This technique is simple, rapid, and inexpensive and provides high recovery and enrichment factors (Farajzadeh et al. 2016). However, the chlorinated solvents as extraction solvents such as chlorobenzene, chloroform, or carbon tetrachloride that were used in most applications of DLLME procedure were typically highly toxic (Habibi et al. 2017; Madani-Tonekaboni et al. 2015).

MAE–DLLME has been used for extraction of PAHs from grilled meat (Kamankesh et al. 2015), polyamines from turkey breast meat (Bashiry et al. 2016), and nitrosamines from sausage and salami (Ramezani et al. 2015). Nevertheless, MAE–DLLME–HPLC has not been used to facilitate the extraction and determination of Ole and HyT in olive pomace or the other food samples.

Response surface methodology (RSM) is an empirical model method useful to improve, develop, and optimize processes. RSM is a multivariate statistic technique for designing the experiments, describing the interactive effects and the relationship between the factors and the response, making models, and looking for the optimal condition (Habibi et al. 2013; Sereshti et al. 2014). RSM minimizes the number of experimental runs and is capable of finding the interactive effect of the independent variables on the response and leads to estimate the best response and optimal conditions (Habibi et al. 2017).

Therefore, in this study, we propose for the first time to the best of our knowledge a new and reliable MAE–DLLME–HPLC-UV method to extract and determine Ole and HyT from olive pomace samples. Important parameters that may

affect extraction efficiency in the MAE-DLLME procedure, such as microwave power, extraction time, type and volume of extraction, and dispersive solvents, were optimized with the aid of RSM based on a CCD to introduce the best conditions for the separation and determination of Ole and HyT in olive pomace samples.

Materials and Methods

Reagent and Chemicals

Ole and HyT with purity higher than 98% were purchased from Sigma-Aldrich (Hohenbrunn, Germany). 1-Octanol, ethanol, methanol, glacial acetic acid, and acetonitrile were prepared from Merck (Darmstadt, Germany). The single stock solution of Ole and HyT was prepared at a concentration of $1000 \ \mu g \ mL^{-1}$ in methanol. For preparation of standard solution with a concentration of $500 \ \mu g \ mL^{-1}$, the stock standard solution was diluted with methanol. Working standard solutions from 10 to $500,000 \ \mu g \ L^{-1}$ were prepared by diluting stock standard solution with methanol. All solutions were refrigerated at 4 °C.

Sample and Sample Preparation

Olive pomace samples, as olive oil industry by-products, were prepared from the olive oil industry in November 2016. Olive pomace samples were dried and milled in the laboratory. 0.5 g of dried olive pomace samples was accurately weighed and transferred to a quartz reaction vessel, and 5 mL of water:ethanol solution (40:60) was added to extract Ole and HyT from olive pomace. The vessel was closed and placed in a Multiwave 3000 microwave system (Anton Paar, Austria) for microwave-assisted extraction. The extraction was carried out at a power of 220 W for 12 min. After the extraction, the solution was transferred into a 10-mL conical flask and centrifuged for 5 min at 4000 rpm. The supernatant was separated and filtered with a syringe filter (0.45 μm) and transferred to another conical flask containing 5 mL deionized water where the DLLME procedure was performed. All experiments were done in triplicate.

DLLME Technique

A solution consisting of 60 μL 1-octanol (extraction solvent) and 500 μL ethanol (disperser solvent) was rapidly injected into the conical flask containing sample solution, and then the mixture was thoroughly agitated using a shaker for 2 min. In this step, Ole and HyT were inserted into fine droplets of 1-octanol. Thereafter, the cloudy solution was centrifuged for 10 min at 4000 rpm to separate the organic phase. After this process, a tiny droplet of 1-octanol was floated on the aqueous

sample and 20 μL of the floated phase was injected directly into the HPLC using a Hamilton microsyringe.

Instrumentation

An Agilent 1260 infinity liquid chromatographer consisting of a vacuum degasser, an autosampler, and a quaternary pump equipped with a C18 ODS (250 × 4.6 mm, 5 μm) column from Agilent Technologies (Santa Clara, CA, USA) was applied for quantitative determination of Ole and HyT. The mobile phases used were water with glacial acetic acid (0.5%) (eluent A) and pure acetonitrile (eluent B), and the elution gradient program for the HPLC was as follows: 0–30 min, 5–20% B; 30–40 min, 20–30% B; and 40–45 min, 30–35% B. The flow rate was set at $1 \ mL \ min^{-1}$ throughout the gradient. The column temperature of 25 °C was used and the injection volume was 20 μL. The effluent was monitored at 280 nm for Ole and HyT.

Experimental Design and Statistical Analysis

In the present study, RSM based on a CCD was performed to determine the most effective independent factors in the MAE-DLLME method such as microwave power, extraction time, extraction solvent volume, and dispersive solvent volume. A four-factorial CCD was used to reach the best value of these factors on the response variable, namely, the sum of the peak area of Ole and HyT. The experimental levels and codes of variables in this study are shown in Table 1. The total experimental points (N) needed for the development of CCD are determined as $N = 2^f + 2f + n$, where f is the number of factors that should be optimized and n is the number of central points (Habibi et al. 2013). Therefore, the employed design included 30 randomly experimental runs. The design was complete with six replicates of the central point to estimate the experimental error. Three replicate extractions and quantitative determinations were performed for each experiment. Three-dimensional surface plots were employed to demonstrate the effects of the independent variables on the response variable. The adequacy of the MAE-DLLME method was evaluated by testing for lack of fit. Significant lack of fit ($p < 0.05$) displays that the proposed model is inadequate and requires improving. The experimental data were analyzed with Design-Expert version 8.0.5 (Minneapolis, USA).

Analytical Figures of Merit

The calibration curves of Ole and HyT were investigated by the peak area of Ole and HyT versus its concentrations for ten points, and a coefficient of determination (R^2) was represented. The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated based on a signal-to-noise ratio of 3 and 10, respectively. Precision, which is a measure of

Table 1 The experimental range and levels of the variables in the CCD

Variables	Effect symbol	Levels in CCD design		
		$-\alpha$	0	$+\alpha$
Microwave power (W)	A	50	200	350
Extraction time (min)	B	4	12	20
Volume of extraction solvent (μL)	C	40	80	120
Volume of dispersive solvent (μL)	D	300	600	900

repeatability, is expressed as percent relative standard deviation (RSD %) which was calculated from six analyses of the olive pomace sample. The recovery for Ole and HyT was also studied for the proposed method by comparing the amount of Ole and HyT added to the olive pomace sample with the concentration found after the procedure.

Results and Discussion

Selection of the Best Ratio of Water:Ethanol

Water:methanol and water:ethanol mixtures as extraction solvents with different ratios depending on the target analytes and sample matrix were usually applied for extraction of phenolic compounds (Pérez-Serradilla et al. 2007). The use of organic solvents such as methanol, ethanol, and acetone strongly affects the extraction efficiency and improves the recovery of target analytes from the solid or semi-solid samples, and it is noticeable that the MAE technique reduces extraction time and helps rapidly transfer the target analytes into the liquid phase when compared with routine methods (Aeenehvand et al. 2016). As shown in Fig. 1, water:ethanol (W:E) mixtures as cheap and non-toxic extraction solvents were used for extraction of Ole and HyT at different ratios. A mixture of water

and ethanol as an extraction solvent has a higher extraction efficiency than water and ethanol alone in the extraction of Ole and HyT that it is in agreement with other studies (Aeenehvand et al. 2016; Pérez-Serradilla et al. 2007). It is clear that the mixture of water and ethanol had better solubility features and enhanced the release of Ole and HyT from the olive pomace sample. The highest extraction efficiency for HyT was observed at a ratio of 50W:50E, and the highest extraction efficiency for Ole was found at a ratio of 20W:80E; this is related to the difference in the chemical structure of Ole and HyT, and also, it may be because HyT is more soluble in water and polar organic solvents in comparison with Ole (Vilaplana-Pérez et al. 2014). No significant difference was observed between 50W:50E and 40W:60E for HyT and between 20W:80E and 40W:60E for Ole (Fig. 1). Based on this result, the ratio of 40W:60E was selected as the best ratio for simultaneous extraction of Ole and HyT.

Selection of Extraction and Dispersive Solvents

The selection of a suitable organic solvent is very important for efficient DLLME. In DLLME technique, a high partitioning coefficient of the target analytes in the solvent, high efficiency, low density of water, good chromatographic behavior, immiscibility with water, and high solubility in

Fig. 1 Extraction recovery results for selecting the best ratio of water (W):ethanol (E) for MAE
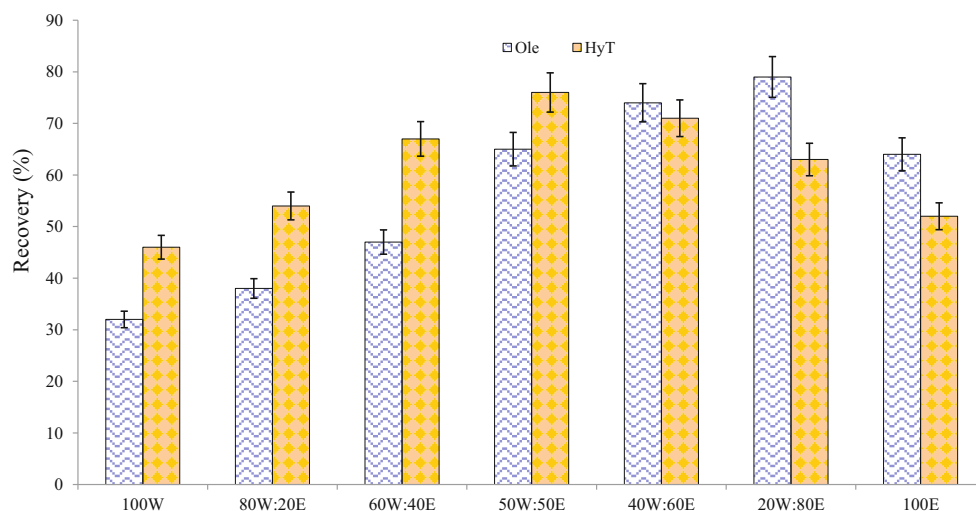
dispersive solvent are important properties in selecting an appropriate extracting solvent (Habibi et al. 2017; Kamankesh et al. 2015). Moreover, solvents with a higher density than water, the chlorinated solvents, that are used for extraction of target analytes require additional steps such as evaporation and dissolution of the residue analyte into a polar solvent while they have a non-polar nature and therefore cannot be injected into the reverse HPLC which increases the time of extraction and the risk of analyte losses (Madani-Tonekaboni et al. 2015). Based on the above considerations, 2-ethyl-1-hexanol and 1-octanol were tested as extraction solvents (Fig. 2). After the evaluation, 1-octanol was selected as the extracting solvent because it has lower density in comparison with water, high extraction capability of Ole and HyT, and good HPLC behavior. The miscibility of the dispersive solvent, both in the aqueous phase (water) and organic phase (extracting solvent), is the important factor in the selection of dispersive solvent (Madani-Tonekaboni et al. 2015; Rezaee et al. 2006). Therefore, in this study, four organic solvents including methanol, ethanol, acetonitrile, and acetone were tested to select the best dispersive solvent for the DLLME in the model sample under the same conditions. The results indicated that ethanol gave the maximum recovery (Fig. 2). Therefore, ethanol was chosen as a proper dispersive solvent. Finally, 1-octanol-ethanol as the most suitable solvent pair was selected for extraction of Ole and HyT.

RSM for the Selection of Optimum Conditions

Optimal separation and detection of Ole and HyT was obtained by optimizing four important variables such as microwave power, extraction time, volume of extraction solvent, and volume of disperser solvent in the DLLME method. RSM based on CCD was applied to optimize the DLLME process and increase the extraction efficiency. It also provides acceptable response at low analyte concentration. The results obtained from CCD were fitted to a second-order polynomial model. Equation 1 shows the RSM model in terms of coded factors:

$$\begin{aligned} R = {} & +7388.71 + 575.50A \\ & + 316.50B{-}337.44C{-}141.17D{-}701.19AB \\ & + 293.06\,AC{-}7.81\,AD + 309.06\,BC{-}203.06BD \\ & + 49.94CD{-}1119.75A^2{-}361.75B^2{-}205.25C^2{-}397.75D^2 \end{aligned}$$
$$(1)$$

where R is the sum of the peak areas of Ole and HyT (response) as a function of A (microwave power), B (extraction time), C (volume of extraction solvent), and D (volume of dispersive solvent).

There was a good correlation between predicted and actual responses under the studied condition which suggests good fitness of the second-order polynomial model. To evaluate the significance of each factor and interaction terms, analysis of variance was used (Table 2). Regarding the estimated p value from the ANOVA results, the effects of extraction conditions on the response were considered to be significant ($p < 0.05$). According to the ANOVA summary, all of the variables on the DLLME process had a significant effect on the response except D (p value < 0.05). Significant interactions have been viewed between microwave power and extraction time, microwave power and volume of extraction solvent, extraction time and volume of extraction solvent, and extraction time and volume of dispersive solvent. Other interactions were not significant (p value > 0.05).

The "Model F-value" of 23.97 implied that the model was significant with a p value less than 0.0001. The "Lack of Fit P-value" of 0.2857 implies the LOF is not significantly

Fig. 2 Extraction recovery results for selecting a solvent pair for MAE–DLLME. 2-Ethyl-1-hexanol (2-E-1-H) and 1-octanol (1-OC) as extraction solvents; methanol, ethanol, acetonitrile, and acetone as dispersive solvents
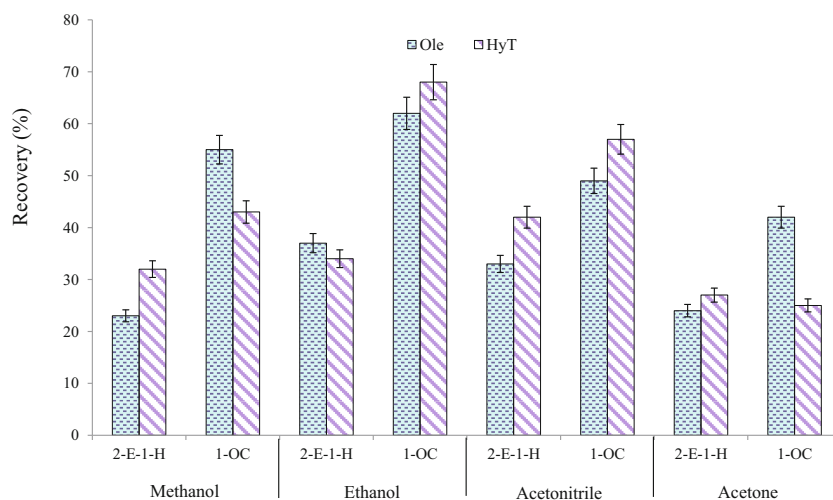
Table 2 Analysis of variance (ANOVA) for the response surface quadratic model

Source	Sum of squares	df[a]	Mean square	F value[b]	p value [c]prob > F	
Model	47,407,998.13	14	3,386,285.58	23.97	< 0.0001	Significant
A—microwave power	5,961,604.50	1	5,961,604.50	42.20	< 0.0001	
B—extraction time	1,803,100.50	1	1,803,100.50	12.76	0.0028	
C—volume of extraction solvent	2,049,637.56	1	2,049,637.56	14.51	0.0017	
D—volume of dispersive solvent	358,704.50	1	358,704.50	2.54	0.1319	
AB	7,866,622.56	1	7,866,622.56	55.69	< 0.0001	
AC	1,374,170.06	1	1,374,170.06	9.73	0.0070	
AD	976.56	1	976.56	0.01	0.9348	
BC	1,528,314.06	1	1,528,314.06	10.82	0.0050	
BD	659,750.06	1	659,750.06	4.67	0.0473	
CD	39,900.06	1	39,900.06	0.28	0.6029	
A^2	3,248,611.07	1	3,248,611.07	23.00	0.0002	
B^2	339,062.52	1	339,062.52	2.40	0.1421	
C^2	109,153.35	1	109,153.35	0.77	0.3932	
D^2	409,903.97	1	409,903.97	2.90	0.1091	
Residual	2,118,805.87	15	141,253.72			
Lack of fit[d]	1,641,602.53	10	164,160.25	1.72	0.2857	Not significant
Pure error	477,203.33	5	95,440.67			
Cor total	49,526,804.00	29				

[a] Degrees of freedom

[b] Test for comparing model variance with residual (error) variance

[c] Probability of seeing the observed F value if the null hypothesis is true

[d] The variation of the data around the fitted model

associated with the pure error. The regression equation obtained from the ANOVA showed that the coefficient of determination (R^2) was 0.9572. This is an estimate of the fraction of the overall variation in the data accounted by the model, and thus, the model is capable of explaining 95.72% of the variation in response. The "adjusted-R^2" was found to be 0.9173 that showed a good relationship between the experimental data and the fitted model, and the "predicted-R^2" of 0.8149 indicated that the model had a high potential to predict the response.

Three-dimensional model graphs were applied to evaluate the interactive effect of two variables on the extraction response. Investigation of the interactive effect of microwave power vs. extraction time, volume of extraction, and dispersive solvents (Fig. 3a–c) showed that an increase of microwave power resulted in a higher response in the middle range of extraction time, volume of extraction, and dispersive solvents while the other variables were fixed in their center points. It can be explained that microwave energy leads to the breakdown of the plant cell walls and consequently results in a higher release of phenolic compounds such Ole and HyT from the plant matrices (Gögüs and Maskan 2001). The combination effect of microwave power and extraction time has shown that shorter extraction time at high microwave power

seems to release and maintain Ole and HyT better than long extraction time in the middle range of microwave power (Ahmad-Qasem et al. 2013). In addition, the short time required for extraction by the microwave device can keep the phenolic content such as Ole and HyT against oxidation. High microwave power may inactivate and reduce the enzymatic oxidation caused by polyphenol oxidases, which are widely distributed among the different plant tissues (Ortega-García et al. 2008). A slight decrease in total response can be observed in the higher range of microwave power and long extraction time (Fig. 3a, d), and it may be due to the increase in the thermal degradation of phenolic compounds like Ole and HyT during the extraction procedure (Gögüs and Maskan 2001). According to Fig. 3d–f, the extraction efficiency decreased when the volumes of extraction solvent and dispersive solvent were increased to higher values, and it could be due to

Fig. 3 Response surfaces using the central composite design obtained by plotting: **a** microwave power vs. extraction time; **b** microwave power vs. volume of extraction solvent; **c** microwave power vs. volume of dispersive solvent; **d** extraction time vs. volume of extraction solvent; **e** extraction time vs. volume of dispersive solvent; **f** volume of extraction solvent vs. volume of dispersive solvent (In all response surfaces, other variables are in their central points)
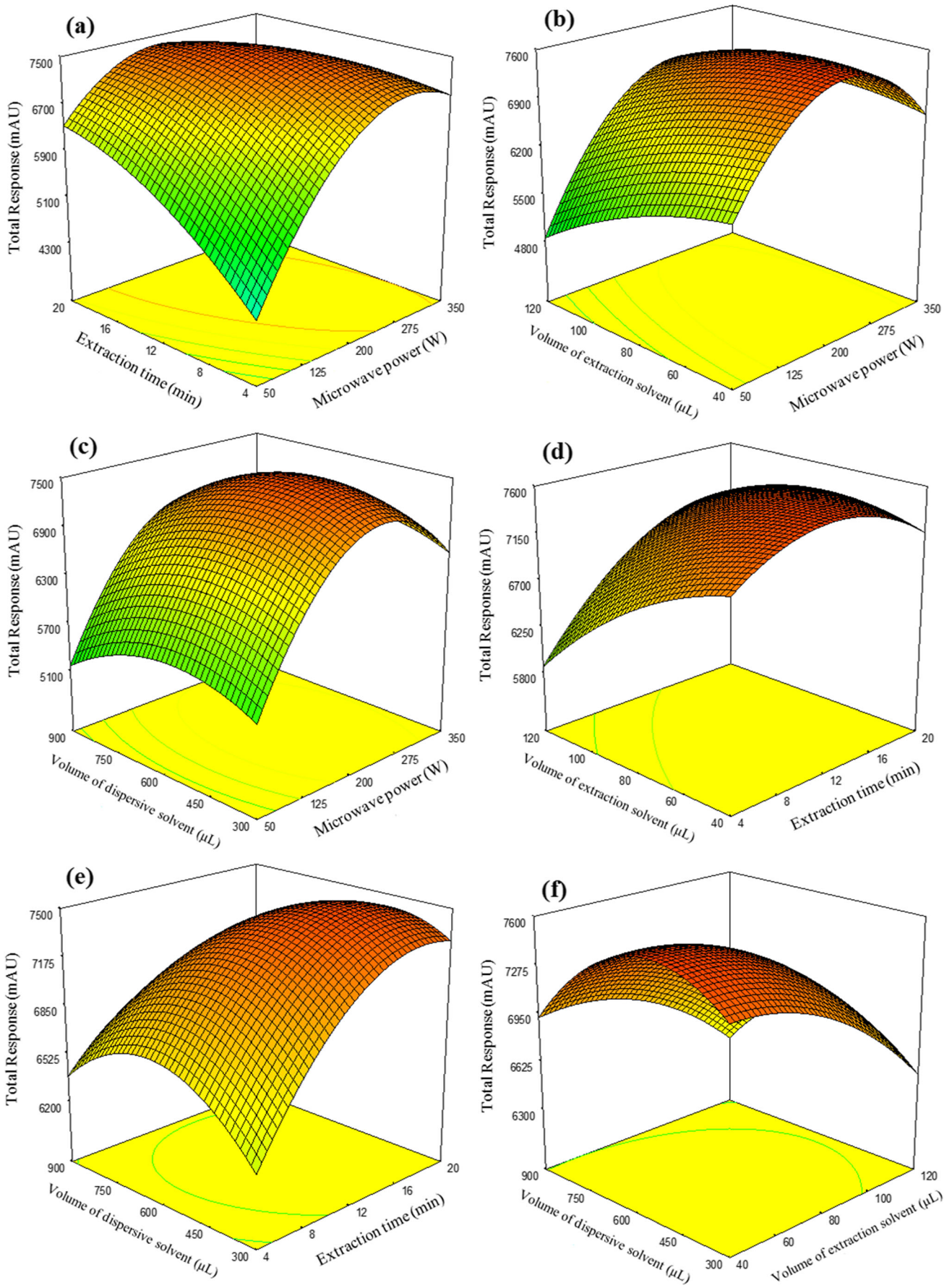
Table 3 Dynamic linear range (DLR), repeatability (RSD %), recovery, limit of detections (LOD), and limit of quantification (LOQ) for the analysis of HMF in beverage samples with other methods and comparison with the proposed method

Method	Matrix	Analyte	DLR (µg L⁻¹)	RSD (%)	Recovery (%)	LOD (µg L⁻¹)	LOQ (µg L⁻¹)	Reference
UAE–CE–DAD	Alperujo	Ole	1,000,000	–	–	2600	8710	Priego-Capote et al. 2004
		HyT	500,000			6560	21,650	
MAE–HPLC–MS–MS	Alperujo	Ole	–	3.35–4.64	–	5.15–11.01	16.99–33.03	Pérez-Serradilla et al. 2007
		HyT						
UAE–HPLC–DAD	Olive leaves	Ole	30,000,000	5.49	–	11,460	30,670	Japón-Luján et al. 2006a
MAE–HPLC–DAD	Olive leaves	Ole	30,000,000	–	–	11,040	29,590	Japón-Luján et al. 2006b
RP–DLLME–RRLC–DAD	Virgin olive oil	Ole	400–100,000	–	–	100	300	Godoy-Caballero et al. 2013
		HyT	520–51,500			400	1000	
RP–DLLME–LC–MS	Virgin olive oil	Ole	100–20,000	–	–	20	70	Godoy-Caballero et al. 2013
		HyT	52–51,500			20	70	
SPE–HPLC–DAD	Virgin olive oil	Ole	–	4.9	40.8	–	–	Pizarro et al. 2013
		HyT		7.7	73.0			
LLME–HPLC–DAD	Virgin olive oil	Ole	–	3.2	85.0	–	–	Pizarro et al. 2013
		HyT		4.7	83.6			
USLLE–HPLC–DAD	Virgin olive oil	Ole	–	7.2	89.7	–	–	Pizarro et al. 2013
		HyT		2.7	93.0			
USAEME–HPLC–DAD	Extra-virgin olive oils	Ole	670–3330	7.9	–	140	470	Reboredo-Rodríguez et al. 2014
MAE–DLLME–HPLC–UV	Olive pomace	Ole	10–500,000	5.63	93.4	35	100	This study
		HyT		4.12	98.7	20	60	

UAE–CE–DAD ultrasound-assisted extraction–capillary electrophoresis–diode array detection, *MAE–HPLC–MS–MS* microwave-assisted extraction–liquid chromatography–tandem mass spectrometry, *UAE–HPLC–DAD* ultrasound-assisted extraction–liquid chromatography–photodiode array detection, *MAE–HPLC–DAD* microwave-assisted extraction–liquid chromatography–photodiode array detection, *RP–DLLME–RRLC–DAD* reversed-phase dispersive liquid–liquid microextraction–rapid resolution liquid chromatography–diode array detection, *RP–DLLME–LC–MS* reversed-phase dispersive liquid–liquid microextraction–liquid chromatography–mass spectrometry, *SPE–HPLC–DAD* solid-phase extraction–liquid chromatography–photodiode array detection, *LLME–HPLC–DAD* liquid–liquid micro extraction–liquid chromatography–photodiode array detection, *USLLE–HPLC–DAD* ultrasound liquid–liquid extraction–liquid chromatography–photodiode array detection, *USAEME–HPLC–DAD* ultrasound-assisted emulsification–microextraction liquid chromatography–photodiode array detection

Table 4 Analytical results of Ole and HyT in real samples by the MAE–DLLME–HPLC–UV method

Samples	Ole (mg Kg⁻¹)ᵃ	HyT (mg Kg⁻¹)ᵃ
Olive pomace sample 1	2183.51 ± 5.24	1572.89 ± 2.87
Olive pomace sample 2	3539.66 ± 4.50	859.52 ± 3.53
Olive pomace sample 3	1763.67 ± 5.03	1239.98 ± 2.84

[a] ± Standard deviation ($n = 3$)

the decrease in concentration of Ole and HyT in 1-octanol. On the other hand, the solubility of Ole and HyT in aqueous phase may be increased by adding more than 500 μL of ethanol that eventually causes a decrease in response. Also, as shown in Fig. 3e, f, the volume of ethanol lower and higher than 500 μL reduces total response because the droplet of 1-octanol was not shaped well. It is remarkable that the volume of extraction solvent, lower than 60 μL, was difficult to handle for collecting the extraction phase. To compare the effect of variables on extraction efficiency, there is no data about the determination of Ole and HyT in olive pomace based on the MAE–DLLME–HPLC/UV method, but it was found in a

study that 14 min of microwave irradiation at 200 W was sufficient for extraction of Ole and HyT from alperujo by microwave-assisted extraction coupled with liquid chromatography–tandem mass spectrometry method (Pérez-Serradilla et al. 2007).

According to the results of the present study, the optimal experimental conditions for extraction of Ole and HyT from olive pomace samples were microwave power, 220 W; extraction time, 12 min; volume of extraction solvent, 60 μL; and volume of dispersive solvent, 500 μL.

Figures of Merit of the Proposed MAE–DLLME–HPLC/UV Methodology

To evaluate the proposed method, linearity, recovery, repeatability, limit of detection (LOD), and limit of quantification (LOQ) were studied under optimized conditions. The figures of merit for this method are comparable to other methods (Table 3). The calibration curves of Ole and HyT (standard solution) were linear over the range of 10–500,000 μg L⁻¹ for ten points with a coefficient of determination (R^2) more than 0.99 for Ole and HyT. The peak area, calculated from six

Fig. 4 The chromatogram obtained by MAE–DLLME–HPLC–UV under optimum conditions. **a** Non-spiked and **b** spiked with 100 μg kg⁻¹ of HyT (1) and Ole (2)
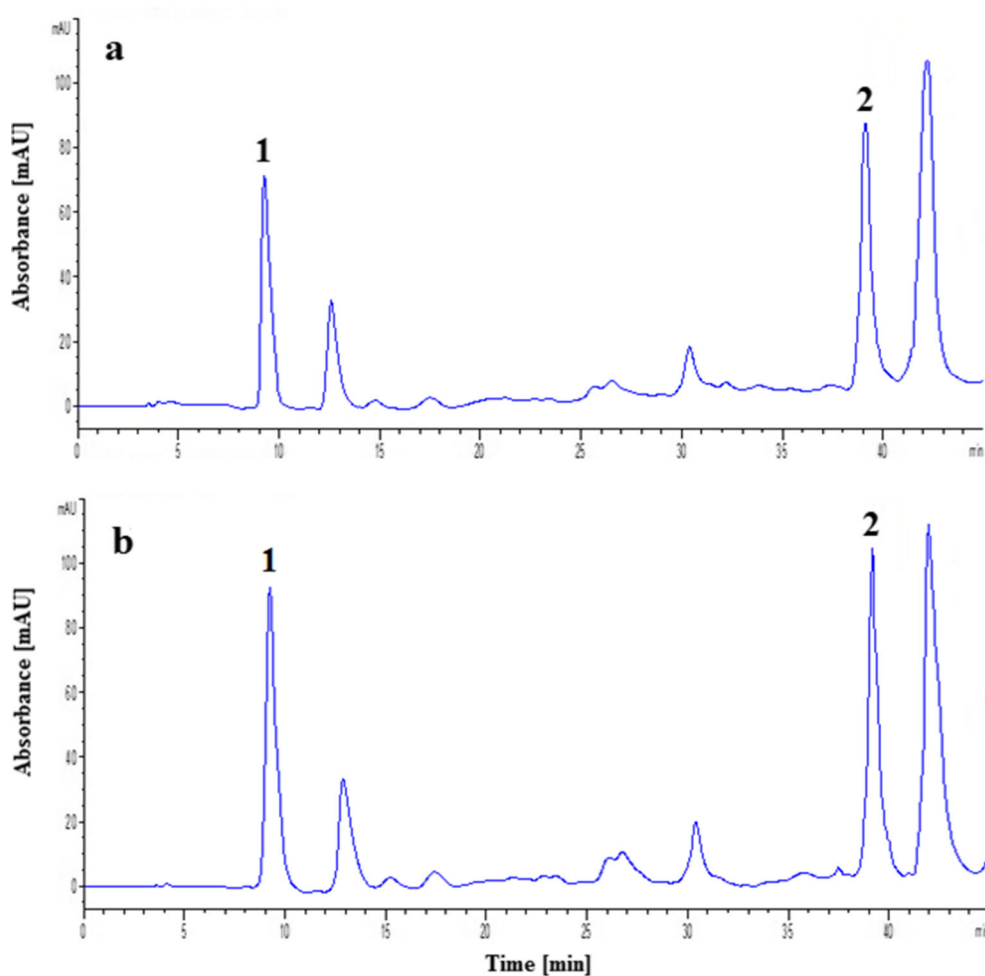
replicate extractions from the olive pomace sample, was used to estimate the repeatability and was represented as relative standard deviation percentage (RSD%). As Table 3 shows, the RSD% was 5.63 for Ole and 4.12 for HyT that were more than the results reported by Pérez-Serradilla et al. (2007) with the aid of the MAE–HPLC–MS–MS method in the alperujo sample. The RSD% of HyT in virgin olive oil evaluated by USLLE–HPLC–DAD was the lowest value reported in Table 3 (Pizarro et al. 2013). The values of recovery using the proposed method were 93.4 and 98.7 for Ole and HyT, respectively, which were better than the values evaluated by SPE, LLME, and USLLE-HPLC-DAD method in the virgin olive oil (Pizarro et al. 2013). The LOD and LOQ for Ole and HyT at the optimized conditions were 35 and 100 $\mu g\ L^{-1}$ for Ole and 20 and 60 $\mu g\ L^{-1}$ for HyT, respectively, and these values were lower than those of the other methods (reported in Table 3), except the amounts of LOD and LOQ reported by the MAE–HPLC–MS–MS method in the alperujo sample (Pérez-Serradilla et al. 2007). These differences can be due to the type of samples, the extraction methods, and the type and conditions of the apparatus used for quantitative determination.

Application of the MAE–DLLME–HPLC/UV Methodology to Olive Pomace Samples

To evaluate the applicability of the proposed method for quantitative determination of Ole and HyT in olive pomace samples, three samples were evaluated under optimized conditions and the concentrations of the Ole and HyT in the olive pomace samples were determined by the standard addition method (Table 4). As seen in Table 4, the amount of Ole and HyT in this study is more than the Ole and HyT levels found in the previous studies which were a value of 21.30 ± 2.95 to 644 ± 35 mg kg^{-1} for Ole and a value of 149 ± 5 to 890.93 ± 1.75 mg kg^{-1} for HyT in alperujo samples (Pérez-Serradilla et al. 2007; Priego-Capote et al. 2004), but the HyT content of olive pomace sample 2 (859.52 ± 3.53 mg kg^{-1}) is in agreement with the results reported by Pérez-Serradilla et al. (2007), who achieved a value 890.93 ± 1.75 mg kg^{-1} for HyT in the alperujo sample. The diversity in the amount of Ole and HyT among the olive pomace samples of this study and the other studies can be related to differences in the olive variety, origin of the olives, culture conditions, and olive oil extraction method. The average value of Ole in the olive pomace samples (from 1763.67 ± 5.03 to 3539.66 ± 4.50 mg kg^{-1}) was lower than the levels reported in the olive leaves (23,200 ± 0.85 mg kg^{-1}) which confirms the olive leaves as a better source of Ole in comparison with olive pomace, and conversely, the HyT content of olive pomace is higher than that of the olive leaves (Japón-Luján et al. 2006b). The mean concentrations of Ole and HyT in the olive pomace samples are higher than those reported in the olive oil by some researchers (Godoy-Caballero et al. 2013; Reboredo-Rodríguez et al.

2014), who obtained values of non-detectable to < 0.14 mg kg^{-1} for Ole and values of 0.31 to 29.7 mg kg^{-1} for HyT. These findings confirm that olive pomace (as a by-product of the olive oil industry) is a good and inexpensive source of both Ole and HyT. Figure 4 depicts the chromatograms obtained by MAE–DLLME–HPLC/UV for non-spiked (a) and spiked (b) olive pomace sample (sample 1). The concentration of Ole and HyT in the spiked sample was 100 ng g^{-1}. The analytical results showed that MAE–DLLME–HPLC/UV is a powerful method for monitoring Ole and HyT in olive pomace samples.

Conclusion

In the present study, a new, simple, efficient, and reliable microextraction method was applied and validated to extract Ole and HyT in olive pomace samples. RSM based on CCD design was used to optimize and determine the interaction and quadratic effects of important parameters on the performance of the microextraction process. In comparison to other methods, the proposed method has significant advantages, including low consumption of solvent, good precision, low cost, repeatability, high recovery, short extraction time, and good merit figures. Finally, the proposed method is capable of extracting and determining low levels of Ole and HyT from olive pomace samples.

Compliance with Ethical Standards

Conflict of Interest Hossein Habibi declares that he has no conflict of interest. Abdorreza Mohammadi declares that he has no conflict of interest. Mehdi Farhoodi declares that he has no conflict of interest. Sahar Jazaeri declares that she has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animal performed by any of the authors.

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