INVESTIGATION OF COMBINED ULTRASOUND AND MICROWAVE PRETREATMENTS AND ENZYME ADDITION ON THE MAIN PHENOLICS AND SOME QUALITY PARAMETERS OF OLIVE OIL

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Olive oil was obtained by applying laboratory scale microwave (MW) and ultrasound (US) technologies and enzymes (E) after olive crushing and malaxation. The effects of these technologies on some physical and chemical properties of olive oil have been examined with focus on phenolics in olive oil. The aim was to evaluate the combined effect of MW and US applications and E to olive paste by comparing with conventional production.

The secoiridoids were present with lower values in US applied samples; as an example, the amount of 3-4-DHPEA-EDA in US treated sample was 59.36 mg kg^{-1} oil, whereas its amount was 92.36 mg kg^{-1} in the sample of conventional extraction. US resulted in the highest decrease (62%), and MW+US condition resulted in the lowest decrease (25.7%) in terms of total phenolics content. The US and MW pretreatments resulted in more advantageous properties in terms of free acidity, peroxides, and phenolics when applied together than their single applications.

Keywords: olive oil, microwave, ultrasound, enzymes, phenolics, secoiridoids

A current trend in virgin olive oil extraction is to enhance malaxation procedure applying some new emerging technologies, such as pretreatment of olive paste by high power US, MW, pulsed electric field, flash thermal preheating methods, etc., which could improve the oil yield by reducing the time of treatment (Leone et al., 2014).

In conventional malaxer, the olive paste gets heated via a thermal gradient, resulting in excess expenditures of time and thermal energy. However, MW heating is characterised as volumetric heating, reducing the thermal gradient and saving thermal energy. Thus, the application of MW energy was recommended as a cost effective option in the olive oil processing industry.

Recently, high power US has been proposed for olive paste preparation (Bejaoui et al., 2016a). The main physical effect of US application is the mechanical movement generated by high and low pressure cycles. The resulting mechanical and shear forces help to increase mass transfer and can also break cell walls.

It has been shown that the addition of exogenous enzymes to the olive paste increases oil yield. The enzymes break up the emulsions mainly developed by crushing and centrifuging the paste. This results in the release and merging of oil droplets into larger ones, which is more easily extracted mechanically (Iconomou et al., 2010).

In the literature, the combined application of MW and US technologies including commercial enzyme preparates has not yet been published. Therefore, the purpose of this paper was to study the commercial enzyme addition, US and MW applications alone and

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their combinations for olive paste pretreatment after malaxation at laboratory scale, and their effect on oil extraction process yield, olive oil composition, and quality parameters with special emphasize on phenolic compounds.

1. Materials and methods

1.1. Olive fruit samples

Olive fruit, *Olea europaea* L. (Gemlik, from the city of Bursa, Turkiye), were picked by hand in 2015/2016 crop season. The ripening degree was close to black stage (MI=6). The sampling was done from all sides of the tree, about 20 kg, with three replicates.

1.2. Oil extraction system and pretreatments

After leaf-removal and washing, olives were divided in sixteen homogeneous batches of 2.5 kg. For laboratory scale simulation of olive oil extraction, a hammer crusher (stainless steel, 3000 r.p.m., 5 mm sieve), a malaxer (Hobart Corporation, USA), and a centrifuge (Awel Industries, France) were used. Enzyme was added to the paste with a ratio of 0.09% (Olivex, Novo Nordisk Ltd., Switzerland).

The US pretreatment was applied for 10 min on olive paste after crushing, using an ultrasonic bath (Elmasonic S60H, Germany), then centrifuged at a speed of 5000 r.p.m. for 5 min. Olive oil was filled into dark colored glass bottles under nitrogen flux and kept at +4 °C (CLODOVEO et al., 2013a).

The MW pretreatment of olive paste was performed in a microwave oven (LG, Korea, 23 L; absorbed power: 1200; 800 W (IEC-705); 2450 MHz). The temperature of olive paste was measured employing a mercury-in-glass thermometer submerged in the olive paste for 1 min. The temperature of olive paste did not exceed 25 °C for US and 40 °C for MW pretreatments, where the initial temperature of the paste was 18 °C.

1.3. Extraction of phenolic fraction from olive oil and analysis of individual phenolics

The methanolic extraction of phenolics and HPLC analysis was performed as previously reported by VINHA and co-workers (2005). Phenols were quantified by a four-point regression curve on the basis of standards obtained from commercial suppliers with the exception of secoiridoid aglycones, which were identified based on earlier reports and reference chromatograms (MATEOS et al., 2001), and were quantified using gallic acid as internal standard.

1.4. Quality indices, carotenoids, chlorophylls

Determination of oil quality indices was performed according to Regulation EEC/2568/91 (EU, 1991). Carotenoids (lutein) and chlorophylls (pheophytin a) (mg kg^{-1} oil) were determined at 470 and 670 nm, respectively, in cyclohexane using the specific extinction values. The concentration of total phenolic compounds was determined with Folin-Ciocalteu assay (CLODOVEO et al., 2013a). DPPH radical scavenging activity was evaluated as reported by SINGH and co-workers (2002).

1.5. Determination of the olive oil yields

The oil extraction yield (% w/w) was calculated as follows:

$$\text{Olive oil extraction yield, } \% = \frac{\text{Mass of extracted oil (g)}}{\text{Mass of olive paste (g)}} \times 100$$

1.6. Microscope images of olive paste

An inverted light microscope (Motik AE31, Wetzlar, Germany), coupled with a digital colour camera (Moticam® 5-mP), was used to determine surface morphology. Image acquisition was performed using the Motic Images Plus 2.0.23 capture software, and objective 10×0.25 (CCIS® Plan Achromatic) as hardware.

1.7. Statistical analysis

The data was subjected to an ANOVA using SPSS 10.0 for Windows. Significant differences between mean values were evaluated using the Duncan's multiple range test ($P<0.05$).

2. Results and discussion

The olive paste samples exhibited more homogeneous structure with single applications and E+MW application in the microscope image (Fig. 1). However, in applications such as E+US, MW+US, E+MW+US, in which the US pretreatment was coupled with exogenous enzymes and MW, the olive pastes showed more heterogeneous structure. Microscope images of E and US treated olive pastes were more blurred and dispersed than the others, and those were the applications that provided the highest oil yield. These two applications formed a more juicy olive paste. This may be correlated with the preventive effect of exogenous enzymes and US on occurrence of stabil emulsion between oil droplets and water droplets. A similar trend was also reported by LEONE and co-workers (2014), as they did not observe any significant effect on oil drop aggregation, when the olive paste malaxed for 15 min after the MW treatment.

E, US, and MW led to significant rise in olive oil yield compared to traditional extraction (Table 1). Oil yield ranged between 14.25–28.29%, where the lowest yield percent among samples was obtained from the E+MW pretreatment, and E alone gave the highest oil yield. US resulted in more increase than that of MW in terms of oil yield. Previously it was reported that MW application did not end with significant increase in oil yield (LEONE et al., 2014; TAMBORRINO et al., 2014). AYDAR and co-workers (2017) also reported that the highest oil yield values were observed when olive pastes were subjected to 10 min of US at 35 °C with 50 min of malaxation and 2 min of US at 35 °C with 50 min of malaxation, respectively. These pretreatments have been reported to exert a mechanical effect on olive paste causing the rupture of cell walls and recovering the oil trapped in the uncrushed olive tissue (CLODOVEO & HACHICHA, 2013). There are of course many works proving the higher oil extraction yield by using US during the olive paste kneading (CLODOVEO et al., 2013a; BEJAOUI et al., 2016a).

Free acidity of oil samples varied between 1.39–1.55%. E did not exhibit significant change in free acidity, while the free acidity values of US applied samples reached around 1.55%. MW was also the most effective treatment on free acidity (1.49%), though not to such big extent as US. Nevertheless, this increase was not observed when US and MW were applied together. TAMBORRINO and co-workers (2014) and BEJAOUI and co-workers (2016b) reported that MW and US did not significantly affect the free acidity. CLODOVEO and co-workers (2013a) reported also no significant effect of US and MW treatments on free acidity

values. In this study, the lowest free acidity value was determined in the oils of enzyme incorporated extraction. Similarly, Iconomou and co-workers (2010) reported that the addition of a mixture of exogenous enzymes improved the quality characteristics of the obtained olive oil, such as acidity, peroxide value, and chlorophyll.
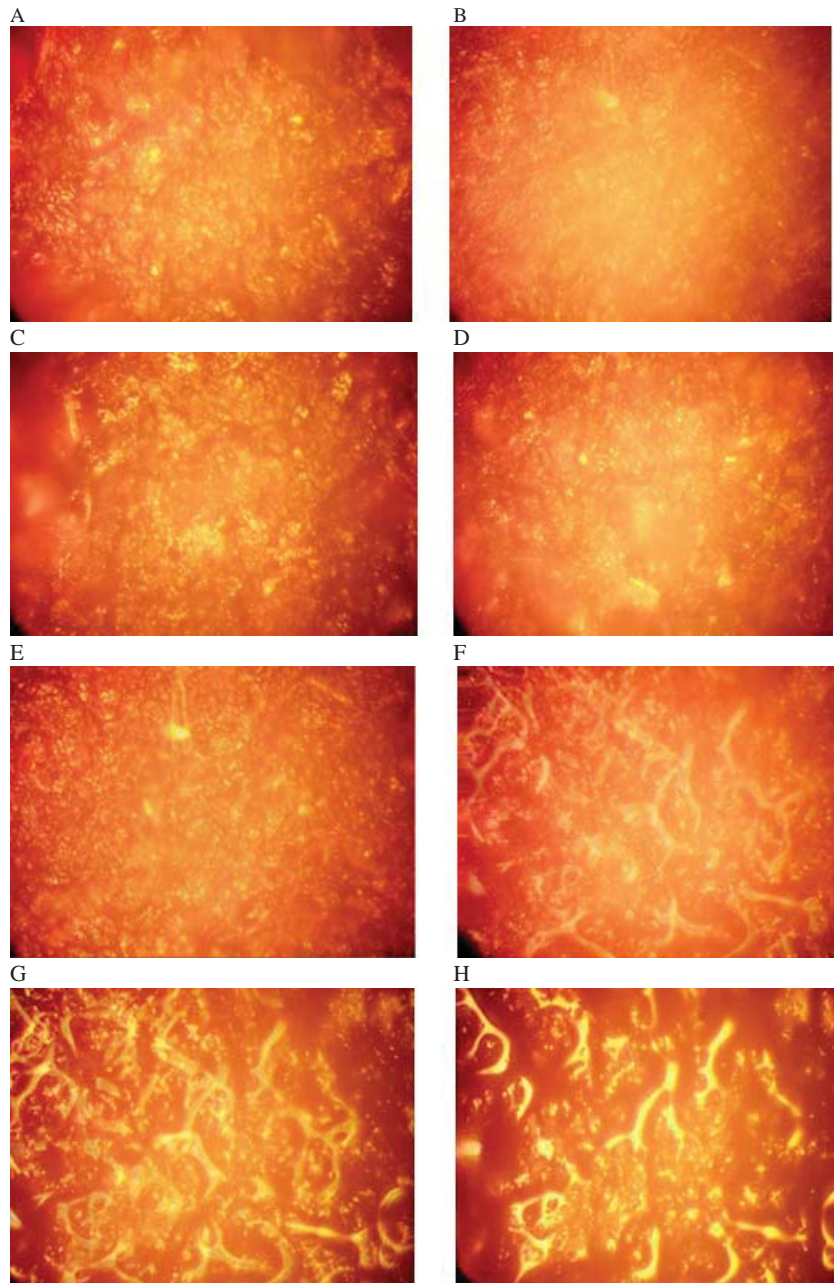
Fig. 1. Microscope images of olive pastes after malaxation and pretreatments
A: Control; B: Enzyme; C: Microwave; D: Ultrason; E: Enzyme+microwave; F: Enzyme+ultrason; G: Microwave+ultrason; H: Enzyme+microwave+ultrason

Table 1. Effects of different pretreatments on oil yield and some quality parameters of olive oil

Treatments	Oil yield (%)	Free fatty acids (%, oleic acid)	Peroxide value (meq O$_2$/kg oil)
Control	14.3±0.2h	1.40±0.09c	8.0±0.5ab
Enzyme	28.3±0.2a	1.39±0.01c	9.0±0.3a
MW	24.9±0.1f	1.49±0.04ab	8.7±0.50a
US	27.4±0.2b	1.55±0.02a	8.3±0.9ab
E+MW	22.8±0.1g	1.45±0.01bc	5.7±0.7d
E+US	27.1±0.1c	1.48±0.01ab	8.8±0.3a
MW+US	26.6±0.1d	1.40±0.01c	7.3±0.3bc
E+MW+US	26.4±0.1e	1.43±0.05bc	6.5±0.7cd

Mean values±standard deviations; significant differences in the same column are shown by different lowercase letters (P≤0.05)

There was no difference between the control group and MW and US application in terms of peroxide value, whereas there was an increase with E application. E+MW and E+MW+US led the lowest peroxide values, which were lower than that of control. No significant change was reported in peroxide value of MW pretreated samples, and additionally it was claimed that the reduction in kneading resulted in a decrease in the peroxide number (TAMBORRINO et al., 2014). BEJAOUI and co-workers (2016b) reported a small increase in peroxide value when US was applied to olive paste.

The total amount of chlorophylls was determined to be 3.99±0.03 and 7.58±0.04 in the minimum US and maximum E+MW+US samples, respectively. The single use of E, MW, and US resulted in oils with lower values of chlorophylls. MW treatment also reduced the chlorophylls in the oil when combined with E and US. This trend was the opposite in terms of carotenoids. E, when used together with US and US+MW, allowed for the production of virgin olive oils with a higher content of chlorophylls, however not exceeding the values of control. Higher amounts of carotenoids were obtained with E alone (slightly higher than that of control sample) and combined applications of MW. BEJAOUI and co-workers (2016b) found that carotenoids and chlorophylls increased significantly with US practices. In the present study, chlorophyll and carotenoid amounts generally declined. When E+MW+US were applied together, chlorophyll value showed the same value as control. It was observed that the use of enzymes in olive oil extraction increased the color transition of oil (NAJAFIAN et al., 2009). In our work, this was true only for carotenoids. Unlikely to our results, CLODOVEO and HACHICHA (2013) reported that the oils obtained by US and MW pretreatments were more pigmented than the conventional ones. Their explanation was the mechanical effects of these treatments, which were able to break the cell walls of vegetal tissue, spreading pigments. The reduction of these minor compounds in olive oil was explained by the duration of sonication and increased temperature (AYDAR et al., 2017).

Total phenolics content of the olive oil samples ranged between 212–410 mg kg^{-1} (Table 2). MW and US pretreatments both led to decrease in total phenolics. MW+US and E pretreatments ended up with higher total phenolics content in the olive oil compared to other pretreatments. The lowest total phenolic values were determined for US and E+US applied samples. MW application leads to the reduction of phenolic compounds due to the shortening of the time required for the effect of the depolymerizing enzymes (TAMBORRINO, 2014). Similarly, US was also reported to exhibit a lowering effect on the total phenolics in olive oil (BEJAOUI et al., 2016b).

All of the emerging technologies produced a significant increase in DPPH radical scavenging activity (RSA), as the control sample showed 52% inhibiton, while the treated samples showed inhbition values ranging between 84.67–93.66% (Table 2). The E, E+MW, MW+US, and E+MW+US treatments resulted in the highest RSA%, which showed activities higher than 90%. MW together with other applications except US had positive results in terms of RSA.

Table 2. Effects of the pretreatments on pigments (mg kg^{-1} oil) and radical scavenging activity of olive oil

Treatments	Total chlorophylls	Total carotenoids	DPPH-RSA (% inhibition)
Control	7.2± 0.06a	5.1± 0.20bc	52.4± 0.9e
Enzyme	4.8± 0.03c	5.5±0.05a	90.2± 0.9ab
MW	4.3± 0.01d	4.7±0.04d	88.4± 3.6bc
US	3.9± 0.03d	4.8±0.04d	78.0± 4.7d
E+MW	4.1± 0.05d	5.2±0.08b	91.2±0.3ab
E+US	6.2± 0.01b	4.5±0.01e	84.7± 3.2c
MW+US	3.9± 0.12d	5.0±0.09c	91.0± 1.9ab
E+MW+US	7.6± 0.04a	5.1±0.08c	93.7± 0.2a

Mean values±standard deviations; significant differences in the same column are shown by different lowercase letters (P≤0.05); DPPH-RSA: DPPH radical scavenging activity

The secoiridoids as the main phenolic compounds in olive oil were significantly influenced by the pretreatments (Table 3). Compared with the control, decreases in the amounts of *p*-HPEA-EA and 3,4-DHPEA-EDA were observed in all treatments, and increases in the amounts of *p*-HPEA-EDA and 3,4-DHPEA-EA were determined. This was associated with the E, as the samples showed higher concentrations of these compounds extracted from all the tests where enzyme was involved. Therefore, we can say that commercial enzyme preparates increase the transition of *p*-HPEA-EDA and 3,4-DHPEA-EA compounds from the paste to the oil.

Table 3. Main phenolic compounds in olive oil with regard to different pretreatments (mg kg^{-1} oil)

Treatments	3,4-DHPEA	*p*-HPEA	*p*-HPEA-EA	3,4-DH-PEA-EDA	*p*-HPEA-EDA	3,4-DH-PEA-EA	Total phenolics
Control	21.5±7.6a	37.1±7.3a	55.0±7.3a	92.4±5.6a	34.3±4.5d	0.1±0.02d	552±28a
Enzyme	8.4±0.5b	7.6±0.5b	9.3±1.6d	35.4±5.5d	65.6±6.2ab	0.3±0.05a	393±30a
MW	2.7±0.2cd	3.8±0.4b	8.5±1.1d	33.5±5.0d	64.3±12.8ab	0.2±0.04bc	257±19bc
US	7.4±1.1b	2.3±0.3b	28.0±0.0c	59.4±9.0c	48.1±4.8c	0.2±0.01bc	212±28c
E+MW	6.6±0.8bc	2.6±0.4b	27.8±4.3c	36.8±0.0d	70.1±16.1a	0.3±0.15a	306±31b
E+US	2.5±0.7cd	3.1±0.5b	24.4±7.0c	32.3±10.4d	63.7±4.8b	0.3±0.03ab	217±18c
MW+US	0.9±0.1d	4.6±0.3b	37.0±2.1b	69.8±3.9b	45.2±2.6c	0.2±0.06c	410±55a
E+MW+US	1.9±0.6cd	6.1±0.8b	11.5±2.8d	33.5±10.0d	62.0±10.8ab	0.3±0.06a	258±41bc

Mean value±standard deviation; Significant differences in the same row are shown by different lowercase letters (P≤0.05); 3,4-DHPEA: hydroxytyrosol, *p*-HPEA: tyrosol, 3,4-DHPEA-EDA: dialdehydic form of decarboxymethyl oleuropein aglycone; *p*-HPEA-EDA: dialdehydic form of decarboxymethylligstrosideaglycone; *p*-HPEA-EA: aldehydic form of ligstrosideaglycone; 3,4-DHPEA-EA: aldehydic form of oleuropein aglycone

The *p*-HPEA-EA content showed the lowest value by MW application (8.53 mg kg^{-1}), with a reduction of about 7-fold compared to the control. E+MW showed the highest *p*-HPEA-EDA values (70.13 mg kg^{-1}). Iconomou and co-workers (2010) reported that the addition of enzymes increased the amount of total phenols, as well as 3,4-DHPEA, *p*-HPEA, and the secoiridoid derivatives (3,4-DHPEA-EDA and 3,4-DHPEA-EA) in olive oil. Clodoveo and co-workers (2013b) attributed this reduction in olive oil phenolics to the temperature increase, increase in the effect of oxygen for the non-enzymatic oxidation, the effect of sonication on endogenous enzymes, and the orientation of hydrophilic phenolics to the air-oil interface. Regarding MW, reduction in phenolics was due to decreased time employed, which prevented the enzyme from hydrolysing the phenolic glycosides, resulting in decreased amounts of secoiridoid derivatives (Tamborrino et al., 2014).

The concentrations of 3,4-DHPEA and *p*-HPEA in olive oils obtained from pretreated olive paste were found to be lower compared to control. Pretreatments showed the most spectacular effect on the phenolic alcohols, as their concentrations diminished by up to 90%. On the contrary, Iconomou and co-workers (2010) reported that enzyme depletion enhances simple phenolic compounds (3,4-DHPEA, *p*-HPEA) in olive oil. When US treatment was applied on olive paste, the phenols became more susceptible to oxidation due to the increased release of enzymes, phenols, and atmospheric oxygen (Clodoveo et al., 2013b), which gives a start for non-enzymatic oxidation.

3. Conclusions

Microscope images of the olive pastes displayed the heterogeneous structure of the US pretreatment coupled with exogenous enzymes and MW, revealing the coalescence of minute oil droplets into large droplets. US and US+MW were the pretreatments that increase the transition of 3,4-DHPEA-EDA to the oil. These pretreatments exhibited the least protective effect on *p*-HPEA-EDA in the olive oil. E increased the transition of *p*-HPEA-EDA and 3,4-DHPEA-EA compounds from the paste to the oil. However, when the secoiridoid derivative 3,4-DHPEA-EDA considered, enzyme preparates along with its combined applications showed the most disruptive effect. Commercial enzyme addition during extraction resulted in the highest amounts phenolic alcohols in the oil. MW pretreatment was less harmful than US in terms of total phenolics. On the contrary, regarding the amount of 3,4-DHPEA, *p*-HPEA-EA, and 3,4-DHPEA-EDA compounds, MW was more defective than US.

US and E gave higher oil yield, but at the same time peroxide number also increased in these samples. The lowest yield percent among samples of emerging technologies was obtained from the "E+MW" pretreatment. US resulted in more increase than that of MW treatment in terms of oil yield. MW when applied together with US and E had positive results in terms of radical scavenging activity. Considering some advantages mentioned above, the benefits of combining MW application with enzyme and US applications were demonstrated.

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