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Microwave-assisted extraction of hydroxytyrosol from alperujo and its impact on the stability of mayonnaise

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Extraction is one of the crucial steps for research and development of plant secondary metabolites. Microwave power and extraction time were selected as extraction parameters of hydroxytyrosol from alperujo. To achieve this goal, the optimization of physical parameters was carried out by analyzing the obtained extracts using GC-MS apparatus. Optimum conditions of microwave-assisted extraction based on maximum levels of responses were medium microwave power with 15 min of alperujo treatment. Maximum levels of hydroxytyrosol were obtained under these conditions (~1.9 g of hydroxytyrosol/kg of alperujo). Furthermore, we have studied the stability of mayonnaise after addition of hydroxytyrosol extract ranging between 65 and195 micrograms by following the changes on free acidity, peroxide value, conjugated dienes as well as content of polyphenols and squalene. This study was conducted for 4 weeks and the mayonnaise samples were stored protected from light at room temperature. These results showed that 195 µg of hydroxytyrosol rich extract may be of great benefit to improve the stability of the mayonnaise against oxidation.

Keywords: Alperujo, microwave treatment, GC-MS, hydroxytyrosol.

Introduction

The alperujo is a by-product of a two-phase centrifugation olive oil milling process. It is all the olive waste that is left over solid and liquid after all the olive oil has been extracted[1]. Thus, olive cake "alperujo" is an inexpensive biomass that is available in large quantities in the Mediterranean countries. However, it represents a serious environmental problem for olive-oil-producing countries. Therefore, many studies have aimed to either reduce the environmental impact of olive cake and/or harness its potential economic value[2]. Phenolic compounds have been identified in olive fruits and processing by-products. Hydroxytyrosol stands out as a compound of high added value, due to its high antioxidant and beneficial properties (regarding both nutrition and oil stability), that could be recovered from the solid by-product. Therefore, it is not surprising that many chemical efforts have been made to collect pure hydroxytyrosol. For example, Cappasso's research team suggested a method to prepare hydroxytyrosol[3]. This molecular was obtained by reducing 3,4-dihydroxyphenylacetic with $LiAlH_4$ (hydrure mixture of lithium and aluminium) in tetrahydrofuran under refluxing for 2 h and the reaction yield's was 82.8%[3]. More recently, Gambacorta and collaborators[4] proposed a new method in order to recover high yield hydroxytyrosol. This method was based on the reduction of oleuropein. On the other hand, three teams of researchers from Tunisia have proposed proceeds in order to recover high quantity of hydroxytyrosol from olive waste, for example Allouche *et al.*[5] from the waste water (1.225 g/L of waste water), Bouaziz *et al.*[6] from olive leaves (2.3 g/100 g of fresh olive leaves), while Khoufi *et al.*[7] recovered 0.8 g of hydroxytyrosol/L of waste water after an enzymatic hydrolysis of olive waste water. More recently, Rigane *et al.*[2] mentioned that by using autoclave apparatus, it was possible to recover 1.9 g of hydroxytyrosol per kg from two-phase *Chemlali* olive pomace. Until now, only few papers in the literature have focused on the evaluation of the phenolic con-

tent of solid olive residues from different milling processes. Thus, one of the most promising techniques is microwave-assisted extraction[8]. The later used microwave energy to heat solvents that are in contact with solid samples. In contrast with classical heating, the uniform heating by microwave energy allows for the sample to be heated. This allows the solvent to heat rapidly, resulting in short extraction times. Furthermore, many studies have been reported that microwave-assisted extraction can reduce solvent requirements, extraction time and provide better extraction efficiency compared to conventional technique[9,10].

Therefore, our research team aim to investigate the influence of microwave-assisted extraction parameters (microwave power and extraction time) on the extraction yield of extract containing high hydroxytyrosol content. And taking into account the beneficial properties of hydroxytyrosol, as practical application, we proposed to enrich mayonnaise with an hydroxytyrosol rich extract in order to improve its stability during storage at room temperature.

Results and discussion

Extraction yields:

Various factors showed significant effect on the extraction yield. It can be seen from Fig. 1 that the extraction yield of the extracts rich in hydroxytyrosol using microwave increases significantly ($p < 0.05$) with time to reach its maximum value after 15 min. Using medium power (Fig. 1a), we observed that yields increased from 0.048% (0 min) to 0.157% (15 min). On the other hand, the extraction yields resulting from a treatment with high power follow the same behavior (Fig. 1b) to reach 0.206% after 15 min. Our data could be explained by Mendes *et al.*[11] results' who mentioned that an increase in microwave power influence the rupture of the cell walls and enhance the extraction due to easier penetration of the solvent into the plant matrix. In addition, Milutinovic *et al.*[12] claimed that there was a positive relationship between microwave power and extraction time.

Determination of total phenols:

The total phenol content of each extract using medium power increases over time to reach its maximum value at 26.22 mg GAE/g of extract after 15 min of treatment (Fig. 2a). On the other hand, using high power microwave treatment, we observed that total phenol content rises with increasing time to reach 28.66 mg GAE/g of extract (5 min), while a sharp decrease if olive pomace was treated for 15 min (Fig. 2b). These results were in agreement with those reported by Ju and Howard[13] who mentioned that the highest total phenolic content and the antioxidant activity of the grape extracts were obtained by extraction at 80–100°C. While, Shahidi and Naczk[14] have also found a positive relationship between the total phenolic compound content and the extraction temperature. These results were consistent with the values reported by Alu'datt *et al* .[15] who stated that total phenolic content of olive-pomace extract varied significantly ($p < 0.05$) for all heat treatments and varied from 2.2 to 4.4 mg/g of dry matter at 70°C. Consequently, the solubility of the extraction product increases with temperature[2,15].

Identification and quantification of phenolic compound:

The identification of phenolic compounds was performed by comparing both retention time and accurate mass spectra obtained from *Chemlali* olive pomace samples and stan-

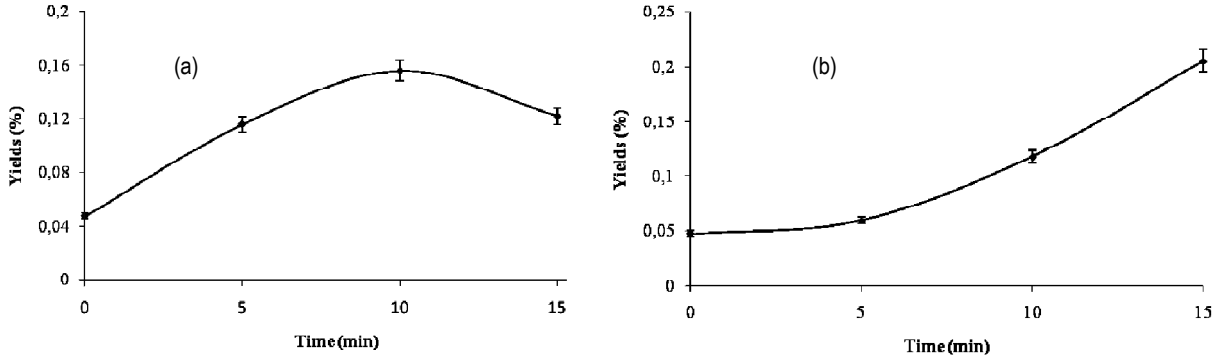

Fig. 1. Extraction yields with medium microwave power (a) and high microwave power (b). The SD values were not more than 5%.

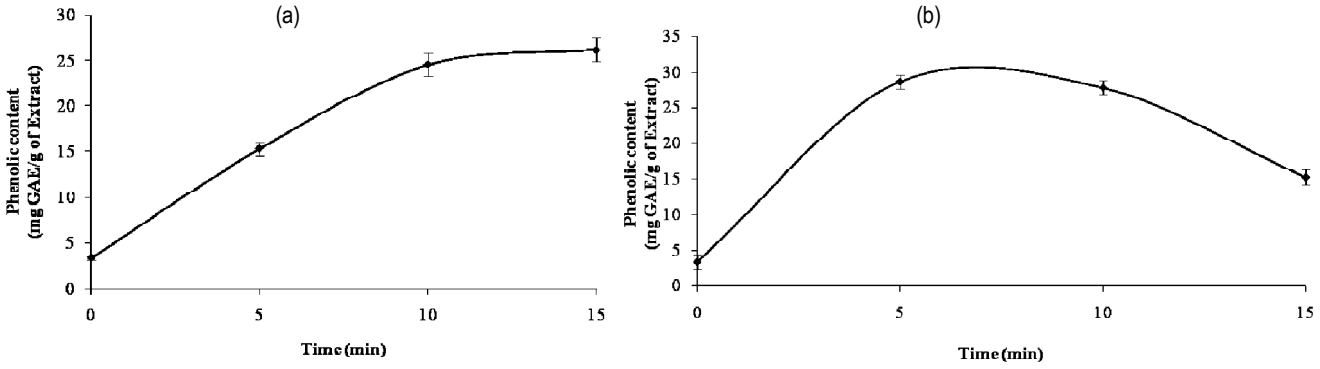

Fig. 2. Total phenol content obtained with medium microwave power (a) and high microwave power (b). The SD values were not more than 5%.

dards of the most important compounds via GC-MS. For example, the compound eluted at t_R= 12.87 min, the compound's mass spectra (data not shown) showed a high-intensity ion at *m/z* = 73 and interesting fragment ions at *m/z* = 267,135 and 91 which suggested the presence of a salicylic acid. On the other hand, the compound eluted at t_R= 11.54 min, its mass spectra (data not shown) displayed a molecular ion at *m/z* = 282 and the most intense ion at *m/z* = 267, 193, 179 and 73. This compound is tyrosol[6]. However, the mass spectra of the compound eluted at t_R = 15.03 min (data not shown) displayed an intense peak at *m/z* = 267 which generate the fragment ion at *m/z* = 193 after a loss of three methyl sililate unit and other important fragments at *m/z* = 179 and *m/z* = 73. This compound was identified as hydroxytyrosol[6].

Moreover, the results of the present study have demonstrated that the recovery of hydroxytyrosol from two-phase *Chemlali* olive pomace was affected by treatment time and microwave treatment power conditions. According to Table 1a, using high microwave power, an important hydroxytyrosol degradation reaction has occurred which confirms the results obtained when we have studied the yield of the obtained extracts. While, from Table 1b, we can reveal that the olive pomace treatment with microwave medium power caused a significant increase in the content of hydroxytyrosol. As we concluded, the extract obtained after 15 min of treatment with medium power contained the highest level of hydroxytyrosol (≈1.9 g/kg of two phase *Chemlali* olive pomace). As it was previously reported, hydrodroxytyrosol could be obtained from many compounds such as hydroxytyrosol-4-β-D-glucoside, nuzhenide, oleuroside, verbascoside, dimethyl oleuropein, oleuropein.

Pre-industrial application: Stability of mayonnaise after enrichment with hydroxytyrosol rich extract:

Many natural antioxidants show great potential for improving the oxidation stability of food products. These compounds also have a wide range of additional health benefits[16]. To the best of our knowledge, no study has been carried out the effect of polyphenols on the stability of the mayonnaise during the storage period. Therefore, in order to improve its stability during storage, we proposed to study the addition of hydroxytyrosol rich extract as natural antioxidant. In fact, various contents (65–195 μg) of hydroxytyrosol were added to 100 g of fresh prepared mayonnaise. And, we studied several quality parameters such as: free acidity, peroxide value and concentration of conjugated dienes), as well as the content of polyphenols and squalene in mayonnaise's lipids fraction.

Changes in free acidity: Acidity measured the amount of free fatty acids present in a lipid fraction[17], expressed as a percentage of oleic acid, within each category. According to Fig. 3a, we can conclude that the enrichment of the mayonnaise with an extract rich in hydroxytyrosol greatly affects the free acid of the lipid fraction. On the other hand, the addition of some milligrams of cooking salt increased remarkably the acid number during the four weeks of storage. This increase could be explained by triglyceride hydrolysis reactions. Torres *et al.*[18] and Kishk *et al.*[19] studied the effect of ginger powder on the stability of mayonnaise and reported that an inverse relationship between ginger powder concentration and the free acid value during the storage period.

Table 1a. Phenolic composition of studied two-phase olive pomace treated by microwaves at high power

Name of compound	Percentage (%) (min)			
	0	5	10	15
Salicyclic acid	0.41	1.82	–	–
Hydroxytyrosol	–	1.81	–	–
Tyrosol	–	1.42	–	3.85
Cafeic acid	–	–	1.30	0.18
m-Coumaric acid	–	2.09	1.24	1.54
Homovanillic alcohol	–	1.18	–	-
3,4-Dihydroxymandelic acid	–	3.52	–	6.20
3,4-Dihydroxybenzoic acid	–	1.72	5.53	–
2,4-Dihydroxybenzoic acid	–	–	–	0.47
m-Hydroxyphenyl acetic acid	0.18	0.47	–	–
Ferulic acid	–	–	0.88	–
2,6-Dihydroxybenzoic acid	–	2.9	–	–
4-Hydroxybenzoic acid	–	0.49	–	–
o-Hydroxyphenyl acetic acid	–	–	–	0.93

Changes in peroxide value: Peroxide values (PV) were used for an estimation of oxidative degradation of lipid fraction. The values of the peroxide indices increased in the mayonnaise samples prepared with the progress of the storage period reaching their highest values after 4 weeks. The enrichment with 130 and 195 μg of hydroxytyrosol rich extraction may enhance the increase in peroxide indices in the prepared mayonnaise samples (Fig. 3b). On the other hand, the peroxide value of the lipid portion of mayonnaise containing cooking salt exceeds 20 meq.O_2/kg after storage for four weeks. Our results were confirmed by those reported by Stoilova *et al.*[20] and Wettasinghe and Shahidi[21] who have illustrated that hydrophobic antioxidants are directed to the interface of the lipid portion and water and prevent the lipid portion on the oxidation. The antioxidant activity that appeared in the prepared mayonnaise was due to the hydroxytyrosol content as well as the other phenolic compounds. More recently, Timm-Heinrich *et al.*[22] report that some phenolic compounds show a strong intermediate inhibitory effect on the indices of peroxides. The antioxidant activities of phenolic compounds can occur from three mechanisms: By inhibiting the formation of free radicals, chelating the catalyst metals and trapping oxygen[23,24].

Table 1b. Phenolic composition of studied two-phase olive pomace treated by microwaves at medium power

	Percentage (%) (min)			
	0	5	10	15
Salicyclic acid	0.41	0.77	2.7	4.40
Hydroxytyrosol	–	2.29	3.74	7.18
Tyrosol	–	2.37	5.17	6.26
Cafeic acid	–	–	0.88	–
m-Coumaric acid	–	–	1.46	–
Homovanillic alcohol	–	0.96	2.96	–
3,4-Dihydroxymandelic acid	–	2.53	–	–
3,4-Dihydroxybenzoic acid	–	–	3.51	3.11
2,4-Dihydroxybenzoic acid	–	1.26	–	–
m-Hydroxyphenyl acetic acid	0.18	0.74	–	–
Ferulic acid	–	–	–	1.95
2,5-Dihydroxybenzaldehyde	–	0.78	–	–
3,4-Dihydroxyphenyl glycol	–	–	–	1.46
p-Hydroxyphenyl acetic acid	–	–	2.62	0.53

Determination of conjugated dienes: Primary products of lipid oxidation containing conjugated double bonds can be quantified by UV spectrometry. Indeed, the oxidation of the polyunsaturated fatty acids is accompanied by a moving of the double bonds which pass from the malonic position to the conjugate position. Conjugated dienes absorb at 232–233 nm, they can be determined by measuring absorbance at these wavelengths[25]. Conjugated diene values obtained for samples stored at room temperature ranged from 1.24 to 3.77 (Fig. 3c). Conjugated dienes could be present during the early stages of lipid oxidation. During storage at 20°C in the dark, the oxidation reactions of the lipids appear to be initiated. On the other hand, it seems that the presence of conjugated dienes during the storage of mayonnaise prepared by enriching with 195 μg of hydroxytyrosol could delay the formation of the primary products of lipid oxidation. In general, the relevance of the assay of conjugated dienes for lipid extracts of mayonnaise raises questions. In fact, measurements were carried out on the lipid extract, or degradation of hydroperoxides and conjugated dienes, which are particularly unstable products in the case of long chain polyunsaturated fatty acids, may occur during extraction. Finally, conjugated dienes derived from the oxidation of long-chain polyunsaturated fatty acids have complex structures which can interfere with absorbance measurements[26].

Changes in phenolic content: Polyphenols were the major compounds of plants that have significant antioxidant activity, but they were not the only ones. The antioxidant

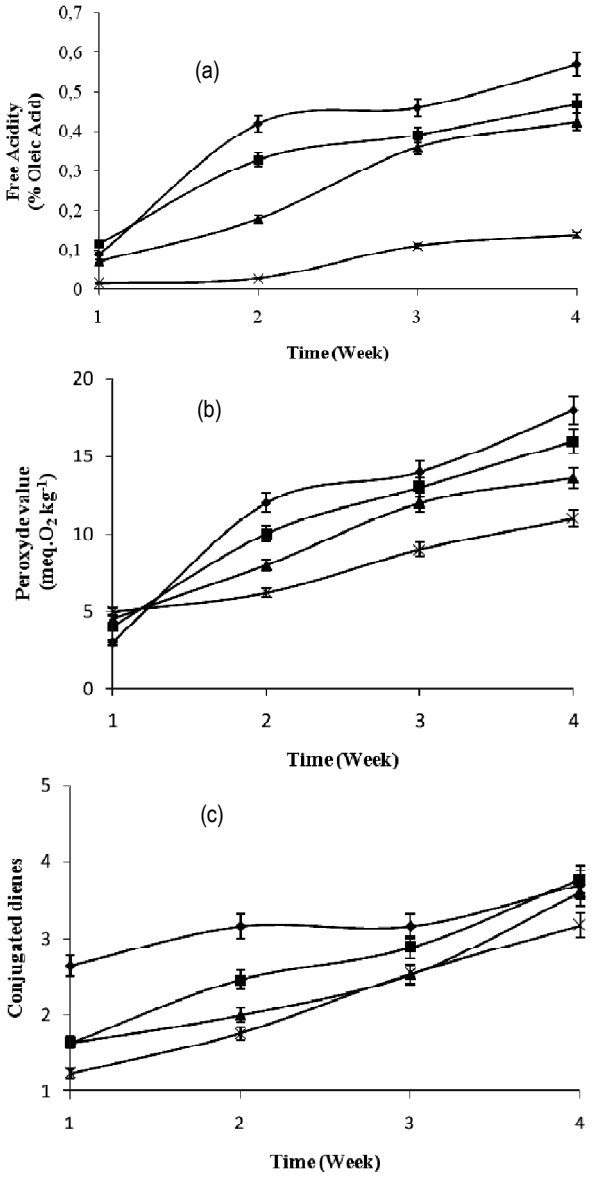

Fig. 3. Changes in free acidity (a), peroxide value (b) and conjugated dienes (c) of mayonnaise stored at 20°C in the dark. The SD values were not more than 5%: (—◆—) salt (500 mg), (—■—) hydroxytyrosol (65 μg), (—▲—) hydroxytyrosol (130 μg), (—×—) hydroxytyrosol (195 μg).

activity of the phenolic compounds is mainly due to their redox property. They play an important role in the human diet as agents for preventing several diseases by protecting the body's tissues against oxidative stress[14,26]. Total phenolic content of the lipid fraction of mayonnaise showed several decreases to reach ~57% of the salt samples and a further decrease ~86% of the rich extract hydroxytyrosol samples during the first week (Fig. 4a). Frankel[27] has shown that the reduction capacity is essentially related to the *ortho* or *para* position of the hydroxyl groups in the phenolic compounds.

Changes in squalene content: In order to complete the study on the importance of antioxidant compounds, we would like to study the changes in the content of squalene present in the lipid fraction of the prepared mayonnaise. The identification of the squalene was carried out by comparing their retention time and their UV spectra[28]. From Fig. 4b, during the first two weeks of storage at room temperature, we could conclude that the content of squalene varied significantly ($p < 0.05$) according to the prepared mayonnaise. In this context, the content of squalene in mayonnaise stored with cooking salt decreases from 7.69 to ~4 mg/kg of lipid fraction from the first week of storage. Whereas; the loss rate of squalene content in mayonnaise samples rich with 65–195 μg of hydroxytyrosol was lower than when we used salt; it is

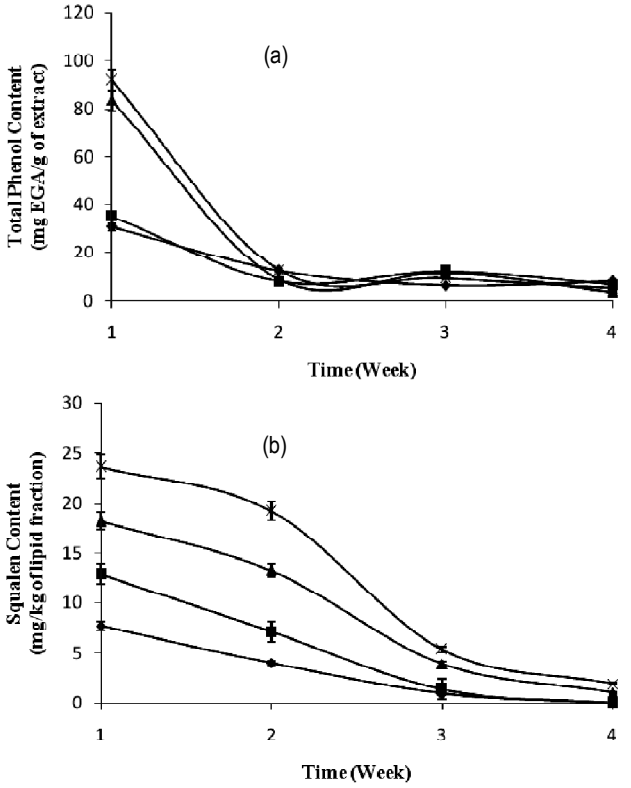

Fig. 4. Changes in total phenol content (a) and squalene (b) during storage of mayonnaise. The SD values were not more than 5%: (—◆—) salt (500 mg), (—■—) hydroxytyrosol (65 μg), (—▲—) hydroxytyrosol (130 μg), (—×—) hydroxytyrosol (195 μg).

worth noting that using 195 μg of hydroxytyrosol could reduce the loss of squalene (~18.50%). This small reduction in squalene is due to the synergy between the roles of phenolic compounds, specifically hydroxytyrosol, and squalene, which are characterized by their antioxidant properties[29].

Experimental

Plant material and sample preparation:

Preparation plant material of waste of olive: The two-phase *Chemlali* olive pomace used in the experiment was collected from an olive oil factory located in Sfax, Tunisia, and kept at a temperature of –20°C in the dark until its use. Residual oil was extracted from olive cake using a centrifuging apparatus at 4°C for 30 min (8500 rpm).

Description of samples: Mayonnaise control sample was prepared using the formula contained the following ingredients: 250 g corn oil, one egg, 15 g vinegar. The preparation was carried out by mixing egg and vinegar together using electric mixer and then corn oil has been added. All ingredients were homogenized for 15 min. The obtained mayonnaise was divided into four series: one was considered as sample control (0.5 g of salt), and the three others were enriched with 65,130,195 μL of weighed quantities of hydroxytyrosol rich extract (dissolved in 1 ml acetic acid) to obtain the desired final quantities. Then, they were added to mayonnaise and mixed in the darkness at room temperature. The samples were stored in the dark in full filled dark bottles for 4 weeks at room temperature and analysed every 7 days of storage. All samples were analysed three times.

Treatment conditions:

A domestic microwave oven was used for sample treatment (Perfect Combo MW 651, De Longhi, Treviso, Italy). Six samples were prepared using different conditions. Briefly, each sample was prepared as follow: 250 mL of distillated water were added to 50 g of two-phase olive pomace in a sealed vial and placed on the rotatory turntable plate of the oven at equal distance and exposed at medium or high power. For each microwave power, the samples were subjected to microwaving for 5, 10 and 15 min. After microwave treatment, each obtained sample was centrifuged for 3 min at 8500 rpm (Centrifuge MF 20, Awel Industries, France). After each treatment, the hydrophobic fraction was extracted 3 times by a separator funnel with 150 mL ethyl acetate, which was subsequently dried by evaporation before being analyzed using GC-MS.

Gas chromatography-Mass spectrometry:

The GC-MS analysis was carried out with an inert MSD 5975B model, equipped with an HP5MS capillary column (30 m in length, internal diameter 0.25 mm, 0.25 μm film thickness). The carrier gas was used at a flow rate of 1 mL min^{-1}. The temperature program of the furnace was as follows: 1 min at 100°C, 100–260 min at 4°C. and 10 min at 260°C[6]. For the silylation procedure, a mixture of pyridine (20 μL) and BSTFA (100 μL) was added and swirled in screw-cap glass tubes and consecutively placed in a water bath at 80°C for 45 min. Only, 1 μL of the silylated mixture was directly analysed using GC-MS apparatus[6].

Extraction of oil phase from mayonnaise:

The separation of oil from mayonnaise was done by freezing mayonnaise for a minimum of 24 h at –80°C. The mayonnaise was defrosted and centrifuged (1620 g, 10 min, 4°C).The oil phase was then collected and used for the different analyses[30].

Analytical methods:

Free acidity: The method used was that described by the standard NF. T 60-240. This method is to put a test socket in a mixture of solvent (ethanol-diethyl ether (25/25 (v/v)), and then titration of free fatty acids using a ethanolic solution of potassium hydroxide (KOH) in the presence of phenolphthalein (colored indicator)[31].

Peroxide value: The peroxide value has been determined by the method corresponding to the Standard International Olive Council[32]. Briefly, 1 g of lipid fraction from mayonnaise have been dissolved in 30 mL of a mixture acetic acid/chloroform (60:40, v/v), then 0.5 mL of a saturated solution of potassium iodide have been added to the mixture, and then finally 30 mL of distilled water after exactly one minute of agitation. This solution was then titrated with sodium thiosulphate (0.01 *N*) using starch as the colored indicator.

Concentration of conjugated diene: 50 mg of the lipid phase from mayonnaise was mixed with 5 mL of cyclohexane and the absorbance was measured at λ = 234 nm.

F-C Test for measurement of total phenol concentration:

The amount of total polyphenols was determined using the Folin-Ciocalteu's method according to the methods described previously by Rigane *et al.*[33]. Total phenol content was expressed as mg equivalent gallic acid per gram of extracts (mg GAE/g of extract). All samples were analyzed in triplicate.

Analysis of squalene:

Chromatographic purification of squalene: The optimized procedure was as follows: Virgin olive oil (1 g) was weighed and dissolved in 1 mL *n*-hexane. The silica cartridge (150×25 mm, 0.06–0.2 mm, 70–230 mesh ASTM, Scharlau Chemie S.A., Spain) was conditioned with 75 mL of *n*-hexane before the application of oil solution. The squalene was eluted with 2×80 mL of *n*-hexane. The collected fraction was evaporated under reduced pressure at room temperature. The dry residues were dissolved in the appropriate solvent (*n*-hexane) for HPLC analysis to verify the purity of squalene fraction. Method validation was performed according to Grigoriadou *et al.*[34].

HPLC analysis of squalene: The squalene content in the lipid fraction extracted from each mayonnaise samples was determined by diluting about 0.5 mL of each fraction in 1 mL of *n*-hexane. Squalene determination was carried out on a reversed phase Nucleosil C_{18} column (particle size 5 µm, 125 4.0 mm i.d.) (Macherey-Nagel, Duren, Germany) maintained at 26°C. The elution solvent was 100% acetonitrile, the flow rate 1.2 mL/min and the injection volume 10 µL. Detection was achieved with a UV-Vis detector at 208 nm. Quantification was accomplished with the use of standard curves calculated by linear regression analysis[34]. Quantitative evaluation (167–600 mg/kg) was performed by means of a 4-point regression curve ($y = 18490x$, $r^2 = 1$).

Statistical analysis:

Results of the analytical determinations were expressed as mean ± standard deviation (SD) of 3 measurements. Statistical differences were calculated using a one-way analysis of variance (ANOVA), employing the Student's *t*-test. Differences were considered significant at $p < 0.05$.

Conclusion

All independent variables (microwave power and extraction time) demonstrated a significant effect on microwave-assisted extraction from alperujo. High-quality extracts were obtained in a short time using a new technology due to its fast and effective extraction technique. This study revealed that the highest hydroxytyrosol content obtained after 15 min of two phase *Chemlali* olive pomace using medium power of a domestic microwave apparatus (solvent: water). In addition, the positive results proved that this low-cost procedure could be an alternative to the conventional extraction method for obtaining an hydroxytyrosol rich extract from alperujo. Furthermore, we have demonstrated that hydroxytyrosol could stabilize mayonnaise during storage at room temperature and in the dark for 4 weeks. It inhibits its thermal deterioration by improving its hydrolytic stability, inhibiting double bond conjugation and reducing the formation of second products of oxidation. The results of this study could be interesting for mayonnaise packagers and marketers to estimate the expiry date of mayonnaise.

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