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Optimization of Microwave-Assisted Extraction for the Characterization of Olive Leaf Phenolic Compounds by Using HPLC-ESI-TOF-MS/IT-MS^2

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ABSTRACT: In the present work, a simple and rapid method for the extraction of phenolic compounds from olive leaves, using microwave-assisted extraction (MAE) technique, has been developed. The experimental variables that affect the MAE process, such as the solvent type and composition, microwave temperature, and extraction time, were optimized using a univariate method. The obtained extracts were analyzed by using high-performance liquid chromatography (HPLC) coupled to electrospray time-of-flight mass spectrometry (ESI-TOF-MS) and electrospray ion trap tandem mass spectrometry (ESI-IT-MS^2) to prove the MAE extraction efficiency. The optimal MAE conditions were methanol:water (80:20, v/v) as extracting solvent, at a temperature equal to 80 °C for 6 min. Under these conditions, several phenolic compounds could be characterized by HPLC-ESI-MS/MS^2. As compared to the conventional method, MAE can be used as an alternative extraction method for the characterization of phenolic compounds from olive leaves due to its efficiency and speed.

KEYWORDS: *Tunisian olive leaves, phenolic compounds, microwave-assisted extraction, high-performance liquid chromatography, time-of-flight mass spectrometry, tandem mass spectrometry*

INTRODUCTION

Polyphenols are found in plant tissues and are needed for pigmentation, growth, reproduction, resistance to pathogens, and for many other functions.[1] These compounds form one of the main classes of secondary metabolites and have received a great deal of attention in recent years for their ability to act as powerful antioxidants. Olive leaves present an easily available natural material of low cost; their extract has been used by native people of the Mediterranean basin in folk medicine. This property can be linked to the fact that the leaves are rich in polyphenols, especially in oleuropein, rutin, verbacoside, apigenin-7-glucoside, and luteolin-7-glucoside.[2,3] Recently, several studies have been focused on contents of the olive leaves and extraction of their high-added value compounds. The traditional solid−liquid extraction technique is based on the correct choice of solvents and the use of heat or/and agitation to improve the extraction efficiency; however, this technique requires longer extraction time and large amounts of solvents. In recent years, much attention has been given to the application of microwave heating in analytical and biological chemistry.[4−6] Major advantages of MAE include short-extraction time, low-energy requirement, high extraction efficiency, and minimum degradation of target components.[7] Furthermore, MAE has demonstrated its promising application in the extraction of phenolic compounds, especially thermo-sensitive ones.[8] Nevertheless, the use of microwaves for extracting phyto-constituents is still in infancy.[9]

Microwaves are electromagnetic fields in the frequency range 300 MHz to 300 GHz or between wavelengths of 1 cm and 1 m.[10] MAE is the process by which microwave energy is used to heat solvents in contact with solid samples and to partition compounds of interest from the sample into the solvent.[11] Using microwave irradiation, the thermal degradation effects can be avoided while favoring the rapid desorption from matrices.[12] Sample preparation before chromatographic separation is the most time-consuming and error-prone part of the analytical procedure.[13] Thus, optimizing an appropriate sample-preparation technique with significant advantages over conventional methods for the extraction and analysis of medicinal plants is a key factor in the overall effort of ensuring and providing high-quality herbal products. Regarding the great significance of olive leaves in obtaining high added value compounds, the purpose of this study was to obtain a new rapid and reliable extraction method based on MAE technique for the

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analysis of phenolic compounds present in olive leaf by using a combination of HPLC-ESI-TOF-MS and HPLC-ESI-IT-MS^2.

MATERIALS AND METHODS

Samples. Leaves used in this study were obtained from Tunisian olive variety "El Hor". Olive leaves were collected from different parts of the tree, so as to minimize the sun exposure effect. After collection, fresh leaves were immediately transferred to the laboratory, washed with distilled water, and ground under liquid nitrogen. Finally, samples were stored at −20 °C until use.

Chemicals and Reagents. HPLC-grade acetonitrile (ACN) methanol and ethanol were purchased from Labscan (Dublin, Ireland). Acetic acid was of an analytical grade (assay >99.5%) and purchased from Fluka (Switzerland). Water was purified by using a Milli-Q system (Millipore, Bedford, MA).

Standards compounds such as hydroxytyrosol, tyrosol, luteolin, apigenin, vanillin, quercetin, o-coumaric acid, ferulic acid, and quinic acid were purchased from Sigma−Aldrich (St. Louis, MO), (+)-pinoresinol was acquired from Arbo Nova (Turku, Finland), and oleuropein and rutin from Extrasynthèse (Lyon, France).

MAE Apparatus. MAE experiments were carried out with a START E Milestone Microwave Laboratory System (Milestone S.r.l, Sorisole (BG) Italy). The apparatus is equipped with a single magnetron system with rotating diffuser for homogeneous microwave distribution in the cavity, delivered microwave power is 1.200 W, controlled via microprocessor, allowing rapid heating of high-throughput rotors, output power up to 1200 W in 1 W increments, a Fiber-Optic Automatic Temperature Control (ATC-FO) System, which allows direct continuous monitoring and control of a reference vessel up to 300 °C, and a MPR-600/12S medium pressure segmented rotor containing 12 vessels for operating pressure up to 30 bar (435 psi). The microwave is operated via a compact Control Terminal 260 Interface with bright, touch-screen display.

HPLC Apparatus. Separation of phenolic compounds from olive leave extracts was performed on an Agilent 1200 series Rapid Resolution liquid chromatographer (Agilent Technologies, CA) consisting of a vacuum degasser, autosampler, and a binary pump equipped with a C18 Eclipse Plus analytical column (4.6 × 150 mm, 1.8 μm) from Agilent Technologies. The mobile phases used were water with acetic acid (0.5%) (phase A) and acetonitrile (phase B), and the solvent gradient changed according to the following conditions: from 0 to 10 min, 95% (A):5% (B) to 70% (A):30% (B); from 10 to 12 min, 70% (A):30% (B) to 67% (A):33% (B); from 12 to 17 min, 67% (A):33% (B) to 62% (A): 38% (B); from 17 to 20 min, 62% (A):38% (B) to 50% (A):50% (B); from 20 to 23 min, 50% (A):50% (B) to 5% (A):95% (B); from 23 to 25 min, 5% (A):95% (B) to 95% (A):5% (B); from 25 to 35 min, 95% (A):5% (B) to 95% (A):5% (B). The flow rate used was set at 0.80 mL/min throughout the gradient. The column temperature was maintained at 25 °C, and the injection volume was 10 μL.

ESI-TOF-MS Detection. The HPLC system was coupled to a micrOTOF (Bruker Daltonics, Bremen, Germany), an orthogonal-accelerated TOF mass spectrometer, using an electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA). The effluent from the HPLC column was split using a T-type phase separator before being introduced into the mass spectrometer (split ratio = 1:3). Thus, in this study, the flow that arrived into the ESI-TOF-MS/IT-MS^2 detectors was 0.2 mL/min. Parameters for analysis were set using negative ion mode with spectra acquired over a mass range from m/z 50 to 1000. The optimum values of the ESI-MS parameters were: capillary voltage, +4.5 kV; drying gas temperature, 190 °C; drying gas flow, 9.0 L/min; and nebulizing gas pressure, 2 bar.

The accurate mass data of the molecular ions were processed through the newest software Data Analysis 4.0 (Bruker Daltonics, Bremen, Germany), which provided a list of possible elemental formulas by using the Smart Formula editor. The Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double bonds equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (sigma value) for increased confidence in the suggested molecular formula. The widely accepted accuracy threshold for confirmation of elemental compositions has been established at 5 ppm.

During the development of the HPLC method, external instrument calibration was performed using a Cole Palmer syringe pump (Vernon Hills, IL) directly connected to the interface, passing a solution of sodium formate cluster containing 5 mM sodium hydroxide in the sheath liquid of 0.2% formic acid in water/isopropanol 1:1 (v/v). Using this method, an exact calibration curve based on numerous cluster masses each differing by 68 Da ($NaCHO_2$) was obtained. Because of the compensation of temperature drift in the microTOF, this external calibration provided accurate mass values for a complete run without the need for a dual sprayer setup for internal mass calibration.

IT-MS^2 Detection. The identical HPLC system was coupled to a Bruker Daltonics Esquire 2000 ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray interface (Agilent Technologies, CA) in negative ion mode. The ion trap scanned at the 50−1000 m/z range at 13 000 u/s during the separation and detection. The maximum accumulation time for the ion trap was set at 200 ms, the target count at 20 000, and compound stability was set at 50%. The optimum values of the ESI-MS parameters were: capillary voltage, +3.0 kV; drying gas temperature, 300 °C; drying gas flow, 7.0 L/min; and nebulizing gas pressure, 21.7 psi. The instrument was controlled by Esquire NT software from Bruker Daltonics.

Conventional Solvent-Extraction Method. Ten milliliters of a mixture of methanol and water (80:20, v/v) was added to 1 g of fresh milled olive leaves, and the sample was maintained 24 h in the dark at room temperature. The extracts were centrifuged at 5000g for 10 min and then filtered through a 0.45 μm syringe filter prior to analysis.[14]

Microwave-Assisted Extraction Method. 1.25 g of fresh milled olive leaves was transferred into the microwave extraction vessels and suspended in 10 mL of the extraction solvent. After extraction, the vessels were cooled to room temperature before opening, using the ventilation option of the system. The obtained extracts were filtered through a 0.45 μm syringe filter prior analysis. Extraction optimization was carried out according to a univariate optimization procedure under different MAE conditions. Solvent optimization is of primary importance in MAE.[15] It is common practice to use a mixture of organic solvent and water at varying ratios to improve recovery of phenolic compounds with MAE. For this reason, different solvents such as methanol, ethanol, and their aqueous forms (40−100%, v/v) were investigated to determine the effective extraction of phenolic compounds. The extraction time was set at 8 min and the temperature at 40 °C. As in other extraction techniques, time is another parameter whose influence needs to be taken into account. The extraction time must be optimized to ensure maximum recovery in the minimum analysis time. For the extraction time optimization, samples were extracted with the most efficient solvent, optimized in the previous step, at 40 °C, and changing the irradiation time from 4 to 16 min. The high temperatures that can be achieved with microwave heating increase the solvating power of most solvents by decreasing surface tension and solvent viscosity, which improves sample wetting and matrix penetration.[16] In our study, extraction at different temperatures from 10 to 120 °C, using the optimized extraction time and solvent, was tested. The extraction efficiency is represented by a number of known phenolic compounds present in olive leaves (oleuropein (Ol), oleuropein aglycon (Ol agl), luteolin, apigenin, rutin, quercetin, apigenin-7-o-glucoside, luteolin diglucoside, and luteolin glucoside) expressed as the peak area of each one. For precision study, repeatability of the optimized method was measured as relative standard deviation (RSD %). Thus, 1.25 g of sample was extracted under the optimized MAE conditions (n = 2) on the same day (intraday precision) and on seven consecutive days (interdays precision, n = 14) and then analyzed by HPLC-MS. Each analyte was expressed as percentage of the total peak area of the identified phenolic compounds. Finally, the olive leaf extracts obtained under the optimal MAE conditions were analyzed by using HPLC coupled to ESI-TOF-MS and ESI-IT-MS^2. Peak identification was performed on

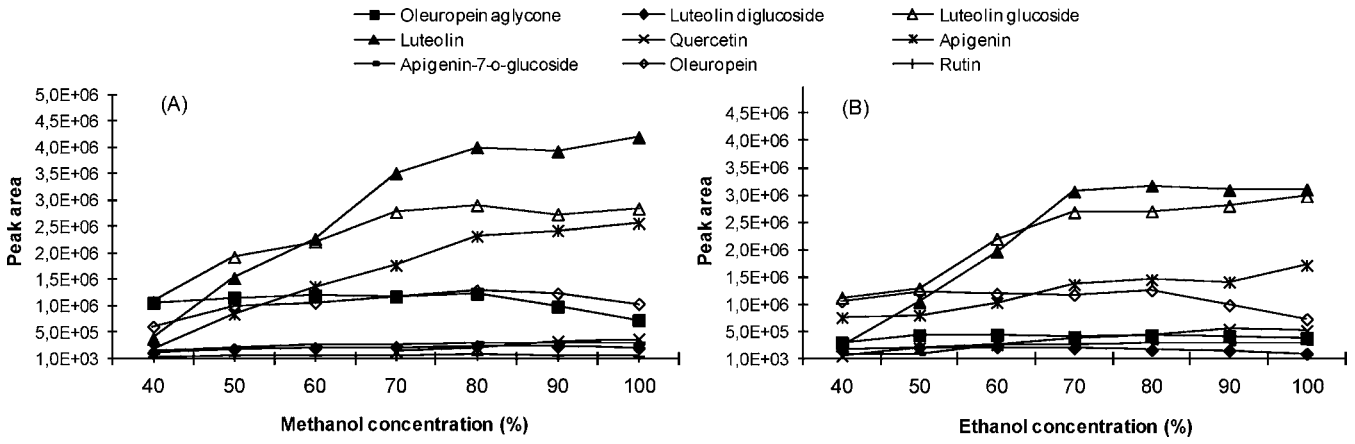

Figure 1. Influence of solvent composition (ethanol and methanol) on the recovery of the main phenols in olive leaves (A and B).

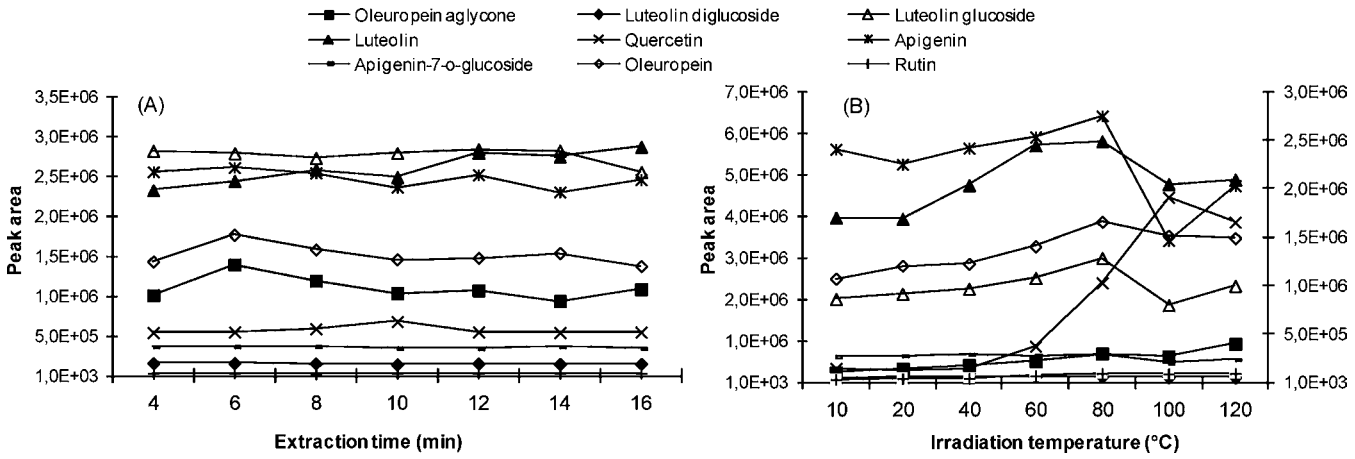

Figure 2. Influence of extraction time and temperature when using methanol/water (80/20) on the recovery of the main phenols in olive leaves (A and B). (B) Right axis, apigenin, oleuropein, apigenin-7-o-glucoside; left axis, luteolin, quercetin, luteolin glucoside, oleuropein aglycon, rutin, and luteolin diglucoside.

the basis of their relative retention time values, TOF-MS and IT-MS^2 data, comparison with authentic standard solutions when available, and using the information previously reported in the literature.[17−20]

Statistical Analysis. To see the difference between the optimized MAE and conventional methods, the one-way ANOVA test at a confidence level of 95% was performed using SPSS 13.0 for windows software.

RESULTS AND DISCUSSION

Optimization of MAE Conditions. *Solvent Optimization.* By comparing the extraction efficiency of both ethanol and methanol (Figure 1A and B), it can be seen that at fixed time and temperature increasing the concentration of the solvent has a benefit in terms of increasing the extraction efficiency for the majority of the phenolic compounds. The highest recovery of phenolic compounds using methanol or ethanol was obtained at a mixture solvent:water (80:20, v/v). Nevertheless, ethanol did not provide as much yield as methanol-based extraction solvents. Thus, a mixture methanol:-water (80:20) was selected as the most efficient solvent composition. It has been reported that aqueous methanol is often the solvent of choice for recovery of a wide range of phenolic compounds from diverse types of samples including fruit, vegetables, and olive oil.[21] A small amount of water in the extracting solvent can penetrate easily into the cells of the plant matrix and facilitate better heating of the plant matrix. This in turn increases the mass transfer of the active constituents into the extracting solvent.[15] A recent study carried out on the optimization of solvent type in the solid−liquid extraction showed that a mixture of methanol−water (80:20) was found to be the best solvent for olive leaf extracts with high levels of flavonoids and important antioxidant activity.[14]

Extraction Time Optimization. The obtained results showed (see Figure 2) that after 6 min there was no remarkable increase of phenolic compound extraction with the increase in extraction time; therefore, 6 min was selected as an appropriate extraction time.

Temperature Optimization. The obtained results are shown in Figure 2B. The extraction efficiency of the phenolic compounds increased with the rise of temperature. Nevertheless, up to 80 °C, the extraction efficiency began to decrease for most of the phenolic compounds under study. It can be explained by the thermal degradation of some of the selected phenolics. Thus, the optimal temperature was chosen at 80 °C.

Precision Study. The RSD % values for the yield and the major and well-known phenolic compounds of the extract are represented in Table 1. Intraday repeatability of the developed method was between 1.30% and 2.49%, whereas the interday repeatability was from 3.01% to 8.47%. Intraday precision was higher than the interday precision, and the method showed a good overall repeatability.

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Table 1. Precision Study of the Optimized Method Results Expressed as Relative Standard Deviation (RSD%) for Yield and Each Analyte

compound	RSD[a] %	RSD[b] %
yield	0.99	3.76
quinic acid	1.75	4.49
HyTy	1.86	5.48
Ol agl derivative	1.48	7.59
10-Hy-Ol	1.73	3.01
Ol	1.52	4.57
apigenin-7-o-glucoside	1.30	4.69
apigenin rutinoside	1.89	8.47
luteolin glucoside isomer 1	1.77	6.42
luteolin glucoside isomer 3	1.73	5.02
diosmetin	2.49	7.82
chryseriol-7-o-glucoside	2.39	5.57

[a]Intraday precision. [b]Interday precision.

Analysis of Olive Leaf Extracts by HPLC-ESI-TOF-MS/IT-MS^2. TOF-MS instrumentation can provide excellent mass resolution and mass accuracy and, in combination with measurement of true isotopic pattern, is the perfect choice for molecular formula determination using the Smart Formula editor. Furthermore, IT-MS^2 is suitable for obtaining fragments ions of structural relevance for identifying target compounds in complex matrix.

The HPLC-ESI-TOF-MS profiles of the analyzed extracts showed several peaks that could be characterized as shown in Figure 3. The identified compounds are summarized in Table 2 along with their retention time, molecular formula, m/z experimental and calculated, tolerance, σ value, error (ppm), and classification order in the list of possibilities (sorted with respect to σ value).

As shown in Table 2, the phenolic compounds identified were from different families: simple phenols (vanillin and hydroxytyrosol (HyTy)), secoiridoids (2-(2-ethyl-3-hydroxy-6-propionylcyclohexyl) acetic acid glucoside, elenolic acid glucoside isomer 1 (EA glucoside isomer 1), EA glucoside isomer 2, Ol agl derivative, 10-hydroxy-oleuropein (10-Hy-Ol), Ol, 2″-methoxyoleuropein isomers, Ol, Ol isomer, Ol glucoside, and secologanoside), flavonoids in aglycone form (luteolin, quercetin, apigenin, diosmetin, and taxifolin) and in glycosylated form (luteolin rutinoside isomer 1, luteolin rutinoside isomer 2, apigenin-7-o-glucoside, rutin, luteolin glucoside with 4 isomers, luteolin diglucoside isomer 1, luteolin diglucoside isomer 2, chryseriol-7-o-glucoside, apigenin rutinoside, diosmin isomer 1, and diosmin isomer 2), and lignans (syringaresinol, pinoresinol, and acetoxypinoresinol (Ac-pinoresinol)). Another polar compound has been identified: quinic acid.

In this work, the ESI-IT-MS^2 was focused on some phenolic compound showing peaks at m/z 701, 607, 609, 593, 577, and 569 when their TOF-MS spectra were not enough to confirm their identity (Figure 4).

The ESI-IT-MS^2 spectrum showed a peak at m/z 701 with fragments (539, 377, 307, and 275 m/z) (Figure 4A). The ion at m/z 539 arises from the loss of a glucosyl unit (162 Da), which, by the loss of another glucosyl moiety, produces the fragment at m/z 377. The fragment ion at m/z 307 is explained by the loss of a C_4H_6O from the latter fragment, while the fragment at m/z 275 derives from the loss of CH_3OH from the fragment at m/z 307. Thus, the peak was identified as oleuropein glucoside.

The ion at m/z 607.1668 obtained by ESI-TOF-MS showed two peaks (RT 11.9 and 12.24 min) with identical molecular formula (Table 2). The ESI-IT-MS^2 spectra showed fragments at m/z 299 and 284 (Figure 4B). The fragment ion at m/z 299, typical mass in the negative mode of diosmetin, indicates the loss of rutinose (m/z 308), and the fragment ion at m/z 299 showed a loss of a methyl group (15 Da) producing the fragment ion at m/z 284. Therefore, these two peaks were identified as diosmin and its isomer. To our knowledge, this is the first time that this compound has been identified in olive leaves.

Several peaks were detected with m/z 609.1461 obtained by ESI-TOF-MS, among which two peaks (RT 9.2 and 10.13 min)

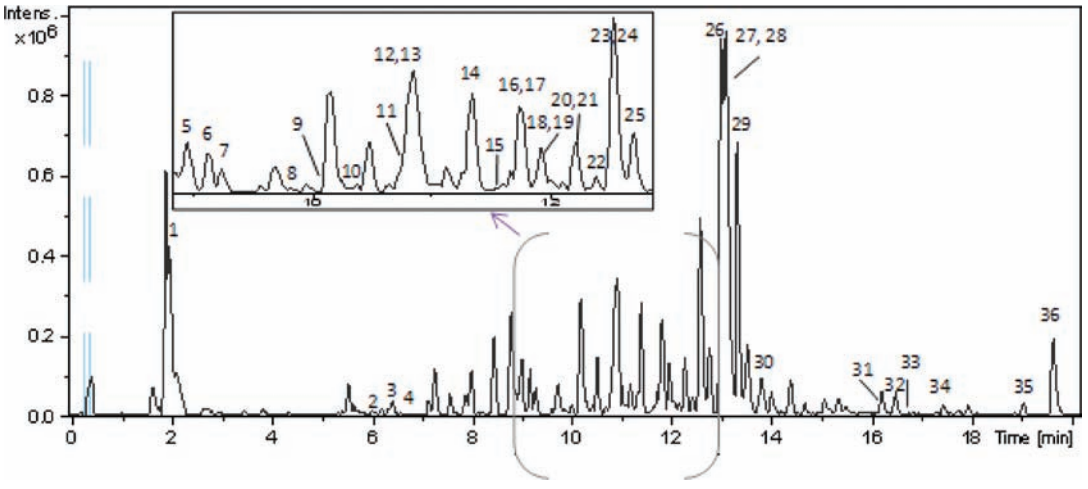

Figure 3. BPC of an olive leaf extract obtained under the optimized condition of MAE. (1) quinic acid, (2) secologanoside, (3) vanillin, (4) HyTy, (5) E A glucoside isomer 1, (6) Ol agl derivative, (7) luteolin diglucoside, (8) E A glucoside isomer 2, (9) luteolin diglucoside isomer 1, (10) 2-(2-ethyl-3-hydroxy-6-propionylcyclohexyl)Ac Ac glucoside, (11) rutin, (12) luteolin rutinoside isomer 1, (13) 10-Hy-Ol, (14) luteolin glucoside isomer 1, (15) Ol glucoside, (16) apig rutinoside, (17) syringaresinol, (18) diosmin isomer 1, (19) luteolin rutinoside isomer 2, (20) diosmin isomer 2, (21) taxifolin, (22) luteolin glucoside isomer 2, (23) apigenin-7-glucoside, (24) luteolin glucoside isomer 3, (25) chryseriol-7-o-glucoside, (26) 2″-methoxyoleuropein isomer 1, (27) luteolin glucoside isomer 4, (28) 2″-methoxyoleuropein isomer 2, (29) Ol, (30) Ol isomer, (31) luteolin, (32) quercetin, (33) pinoresinol, (34) Ac-pinoresinol, (35) apigenin, (36) diosmetin.

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Table 2. Characterization of Olive Leaf Extract Obtained under Optimal Conditions of MAE by HPLC-ESI-TOF/IT-MS (Selected Ion: $[M - H]^-$)[a]

peak	compound	RT (min)	molecular formula	m/z experimental	m/z calculated	tolerance (ppm)	σ value	error (ppm)	classification order in GMF
1	quinic acid	2.00	$C_7H_{11}O_6$	191.0562	191.0561	4	0.0098	−0.7	first (1)
2	secologanoside	6.02	$C_{16}H_{21}O_{11}$	389.1089	389.1089	5	0.0034	0.2	first (1)
3	vanillin	6.45	$C_8H_7O_3$	151.0402	151.0401	4	0.0052	−0.7	first (1)
4	HyTy	6.62	$C_8H_9O_3$	153.0561	153.0557	5	0.0515	−2.2	first (1)
5	E A glucoside isomer 1	8.90	$C_{17}H_{23}O_{11}$	403.1241	403.1246	4	0.0139	1.3	first (1)
6	Ol agl derivative	9.14	$C_{16}H_{25}O_{10}$	377.1453	377.1453	4	0.0062	0.2	first (1)
7	luteolin diglucoside isomer 1	9.20	$C_{27}H_{29}O_{16}$	609.1463	609.1461	4	0.0060	−0.3	first (2)
8	E A glucoside isomer 2	9.80	$C_{17}H_{23}O_{11}$	403.1249	403.1246	4	0.0544	−0.7	first (1)
9	luteolin diglucoside isomer 2	10.13	$C_{27}H_{29}O_{16}$	609.1465	609.1461	4	0.0317	−0.6	first (3)
10	2-(2-ethyl-3-hydroxy-6-propionylcyclohexyl)Ac Ac glucoside	10.27	$C_{19}H_{31}O_9$	403.1965	403.1974	4	0.0056	2.1	first (1)
11	rutin	10.70	$C_{27}H_{29}O_{16}$	609.1438	609.1461	4	0.0414	3.7	first (2)
12	luteolin rutinoside isomer 1	10.80	$C_{27}H_{29}O_{15}$	593.1469	593.1512	8	0.0127	7.2	first (3)
13	10-Hy-Ol	10.87	$C_{25}H_{31}O_{14}$	555.1703	555.1719	4	0.0090	2.9	first (2)
14	luteolin glucoside isomer 1	11.37	$C_{21}H_{19}O_{11}$	447.0930	447.0933	4	0.0096	0.7	first (1)
15	Ol glucoside	11.50	$C_{31}H_{41}O_{18}$	701.2282	701.2298	4	0.0495	2.3	first (1)
16	apig rutinoside	11.79	$C_{27}H_{29}O_{14}$	577.1534	577.1563	5	0.0064	4.9	first (2)
17	syringaresinol	11.80	$C_{22}H_{25}O_8$	417.1548	417.1555	4	0.0061	1.8	first (1)
18	diosmin isomer 1	11.90	$C_{28}H_{31}O_{15}$	607.1674	607.1668	4	0.0150	−0.9	first (2)
19	luteolin rutinoside isomer 2	11.94	$C_{27}H_{29}O_{15}$	593.1518	593.1512	4	0.0309	−1	first (2)
20	diosmin isomer 2	12.24	$C_{28}H_{31}O_{15}$	607.1684	607.1668	4	0.0227	−2.6	first (3)
21	taxifolin	12.30	$C_{15}H_{11}O_7$	303.0491	303.051	4	0.0145	3.6	first (1)
22	luteolin glucoside isomer 2	12.42	$C_{21}H_{19}O_{11}$	447.0936	447.0933	4	0.0066	−0.6	first (1)
23	apigenin-7- glucoside	12.51	$C_{21}H_{19}O_{10}$	431.0974	431.0984	4	0.006	2.2	first (1)
24	luteolin glucoside isomer 3	12.56	$C_{21}H_{19}O_{11}$	447.0920	447.0933	4	0.0049	2.9	first (1)
25	chryseriol-7-o- glucoside	12.72	$C_{22}H_{21}O_{11}$	461.1090	461.1089	4	0.0113	2.2	first (1)
26	2″-methoxyoleuropein isomer 1	12.96	$C_{26}H_{33}O_{14}$	569.1902	569.1876	5	0.4840	−4.6	second (4)
27	luteolin glucoside isomer 4	13.03	$C_{21}H_{19}O_{11}$	447.0928	447.0933	4	0.0010	1.2	first (1)
28	2″-methoxyoleuropein isomer 2	13.04	$C_{26}H_{33}O_{14}$	569.1912	569.1876	10	0.4900	−6.4	third (4)
29	Ol	13.28	$C_{25}H_{31}O_{13}$	539.1745	539.177	5	0.0076	4.7	first (2)
30	Ol isomer	13.74	$C_{25}H_{31}O_{13}$	539.1754	539.177	4	0.0067	3	first (2)
31	luteolin	16.15	$C_{15}H_9O_6$	285.0410	285.0405	4	0.0004	−2.7	first (1)
32	quercetin	16.40	$C_{15}H_9O_7$	301.0351	301.0354	4	0.0083	1.1	first (1)
33	pinoresinol	16.87	$C_{20}H_{21}O_6$	357.1332	357.1344	4	0.0206	3.1	first (1)
34	Ac-pinoresinol	17.51	$C_{22}H_{23}O_8$	415.1397	415.1398	5	0.0212	0.2	first (1)
35	apigenin	19.02	$C_{15}H_9O_5$	269.0451	269.0455	4	0.0098	1.7	first (1)
36	diosmetin	19.61	$C_{16}H_{11}O_6$	299.0551	299.0561	4	0.0059	3.4	first (1)

[a]Retention time (RT), oleuropein (Ol), hydroxytyrosol (HyTy), elenolic acid (EA), oleuropein aglycon (Ol agl), 10-hydroxy-oleuropein (10-Hy-Ol), acetoxypinoresinol (Ac-pinoresinol), generated molecular formula (GMF).

had identical molecular formulas and yielded the same fragments (m/z 285 and 447) by ESI-IT-MS^2 (Figure 4C). Diagnostic fragments at m/z 447 and 285 suggested the removal of one and two glucosyl units, respectively, with m/z 285 representing the aglycon form. Thus, these peaks were identified as luteolin diglucoside and its isomer.

With the same m/z at 609.1461, a peak with different molecular formula was detected at 10.7 min. Its ESI-IT-MS^2 yielded fragments 301 and 447 (Figure 4D). The fragment at m/z 301 is diagnostic of quercetin derivatives resulting from the loss of a rutinosyl moiety (m/z 308), and the fragment at m/z 447 could correspond to the pseudomolecular ion of quercetin-3-rhamnoside (quercetrin) indicating the loss of a rhamnose unit. Therefore, this peak was identified as rutin (quercetin 3-o-rutinoside).

The m/z at 593.1512 showed the presence of two peaks with identical molecular formula (RT 10.8 and 11.94 min). The ESI-IT-MS^2 applied to these ions showed the presence of the fragment at m/z 285 (Figure 4E), which corresponds to the aglycon luteolin, indicating the loss of a rutinosyl moiety. Thus, these peaks were identified as luteolin rutinoside and its isomer. To our knowledge, the luteolin rutinoside isomer is reported for the first time in olive leaf extract.

The ESI-IT-MS^2 spectrum of the ion at m/z 577 showed the presence of a fragment at m/z 269 (Figure 4F) that corresponds to the aglycon form apigenin, which indicates the loss of a rutinose unit (308 Da). Thus, this peak was identified as apigenin rutinoside.

Two peaks were detected at 12.96 and 13.04 min showing the same m/z at 569. The Smart Formula editor provided the same molecular formula for these two peaks, which showed the same fragmentation pattern. These compounds were tentatively identified as 2″-methoxyoleuropein and its isomer. This secoiridoid glycoside was reported in other specie of the Oleaceae family as *Jasminum officinale*.[22] The ESI-IT-MS^2 spectrum is represented in Figure 4G, and a proposed fragmentation pathway for 2″-methoxyoleuropein is represented in Figure 5. The fragment at m/z 537 could be

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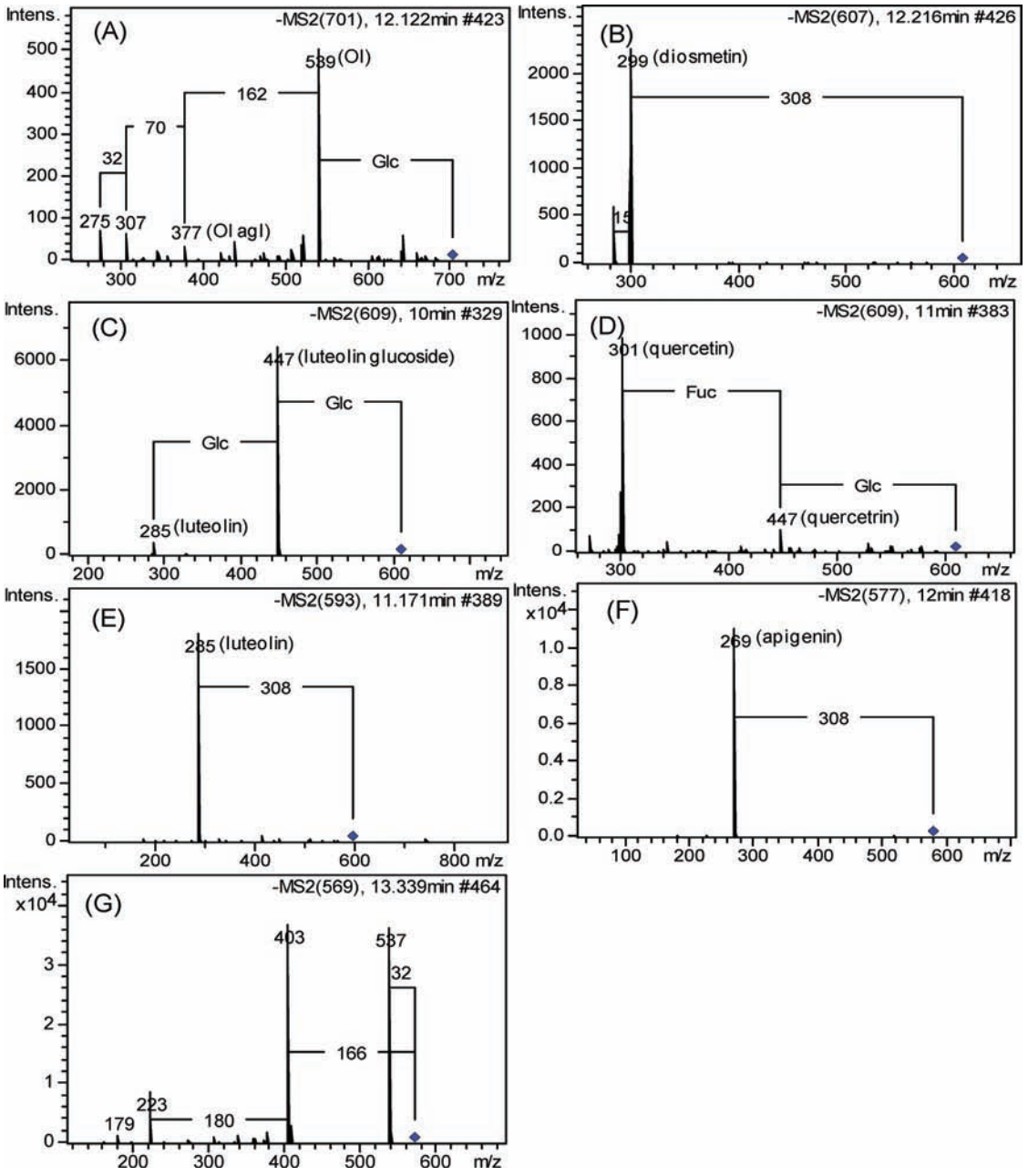

Figure 4. ESI-IT-MS^2 spectra of m/z 701 (A), 607 (B), 609 (C), 609 (D), 593 (E), 577 (F), and 569 (G).

attributed to the loss of a methoxyl group, while the fragment at m/z 403 is due to the cleavage of the phenolic moiety, and it could undergo an elimination of the glucose moiety (fragment at m/z 223) or methoxyl group (fragment at m/z 179). The fragment at m/z 337 could be due to the cleavage of the elenolic ring from the precursor ion.

Efficency of the Optimized MAE Method. To improve the efficiency of the optimized MAE method, it was compared to the conventional extraction method. With respect to the extraction time, MAE was the fastest, requiring just 6 min giving the highest yield (16.7% of fresh weight), whereas in the extract obtained by the conventional extraction the yield did not exceed 10% (Table 3). Qualitatively, the examination of the profiles of the extracts obtained under the optimized conditions of MAE and the conventional method revealed that three compounds were not detected in the extracts obtained by conventional method, which were HyTy, secologanoside, and luteolin glucoside isomer 2. Quantitatively, the statistical analysis showed significant differences between both methods ($p < 0.05$). The main significant observation was that the major detected secoiridoids and flavonoids in the extracts (2″-methoxyoleuropein, oleuropein, 10-Hy-Ol, apigenin-7-o-glucoside, and luteolin glucoside) showed better recoveries with MAE. Being the major compounds identified in the extract under study, these compounds are represented in Table 3 and expressed as percentage of the total peak areas. 2″-Methoxyoleuropein and its isomer represented the highest percentages (30.23% and 26.08%, for MAE and conventional extraction, respectively). Apigenin-7-glucoside was much better extracted using MAE (7.42%) than using the conventional one (1.42%). Ol and its isomers also showed important percentage of 12.51% with MAE, whereas they presented 6.30% in the extract obtained by the conventional method. It has been frequently reported that oleuropein is of the major phenolic

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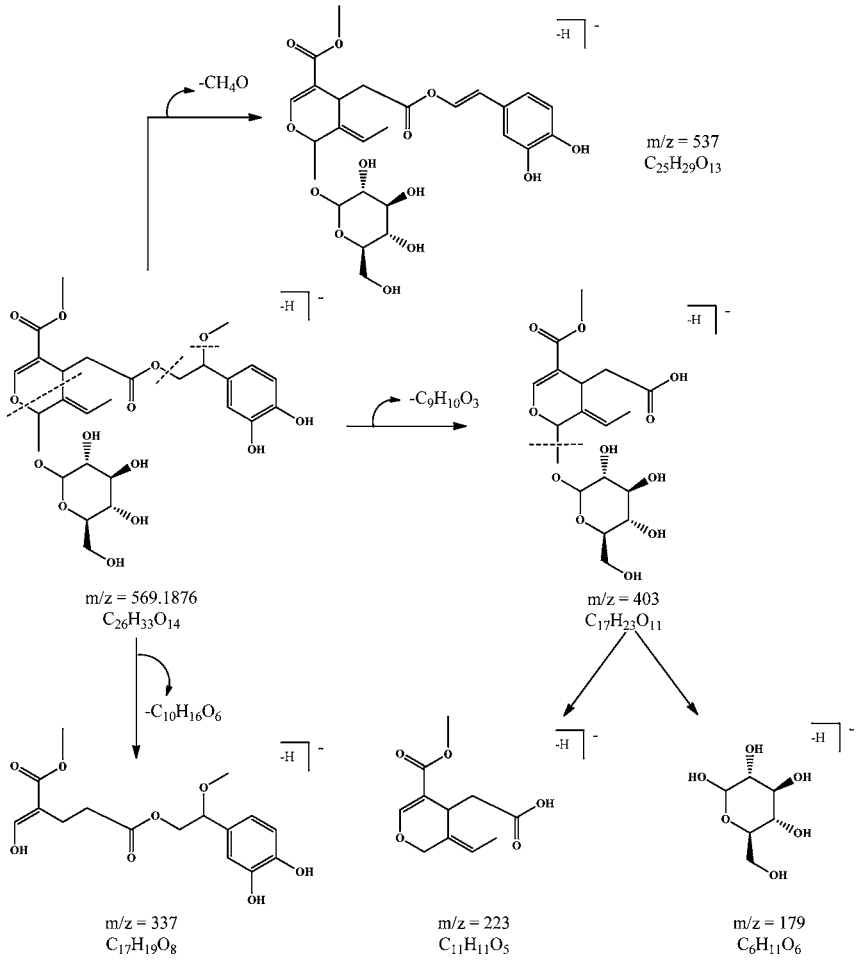

Figure 5. Fragmentation pathway for the secoiridoid 2″-methoxyoleuropein.

Table 3. Comparison between the Optimized Microwave-Assisted Extraction and the Conventional Method

extraction process (conditions: solvent, *T*, time)	yield[a](% ± SD)	10-Hy-Ol[b] (% ± SD)	Ol and isomers[b](% ± SD)	apigenin-7-o-glucoside[b] (% ± SD)	2″-methoxyoleuropein and isomers[b]	luteolin glucoside and isomers[b](% ± SD)
MAE (methanol:water 80:20, 80 °C, 6 min)	16.70 ± 0.24	7.35 ± 0.11	12.51 ± 0.07	7.42 ± 0.11	30.23 ± 0.13	12.63 ± 0.09
conventional (methanol:water 80:20, room temp, 24 h)	9.40 ± 0.41	4.16 ± 0.06	6.30 ± 0.38	1.42 ± 0.09	26.08 ± 0.12	12.05 ± 0.11

[a]Expressed as % of leaf fresh weight. [b]Expressed as % of the total peak areas; SD, standard deviation.

compounds in olive leaf.[23,24] Nevertheless, the phenolic composition of olive leaf would be affected by several factors.

In conclusion, in this study, a new precise and effective time-saving extraction method, based on the use of microwave energy, has been optimized for the analysis of phenolic compounds from Tunisian olive leaves. The characterization of the extracts obtained under the optimized MAE conditions, by using a combination of HPLC-ESI-TOF-MS and HPLC-ESI-IT-MS^2, revealed the existence of a large number of phenolic compounds from different classes; among them, the secoiridoid 2″-methoxyoleuropein, the flavonoids diosmin and its isomer, luteolin diglucoside isomer, and luteolin rutinoside isomer are reported for the first time in olive leaves. The proposed MAE method allows the extraction of these compounds in a shorter time (6 min) with higher efficiency when compared to the conventional solvent method. Therefore, MAE proved to be an attractive alternative to conventional extraction methods for the extraction of phenolic compounds from olive leaves.

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■ ABBREVIATIONS

MAE, microwave-assisted extraction; HPLC, high-performance liquid chromatography; ESI-TOF-MS, electrospray time-of-flight mass spectrometry; ESI-IT-MS^2, electrospray ion trap tandem mass spectrometry; Ol, oleuropein; HyTy, hydroxytyrosol; EA, elenolic acid; Ol agl, oleuropein aglycon; 10-Hy-Ol, 10-hydroxy-oleuropein; Ac-pinoresinol, acetoxypinoresinol

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