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Development and Validation of an Analytical Methodology Based on Liquid Chromatography–Electrospray Tandem Mass Spectrometry for the Simultaneous Determination of Phenolic Compounds in Olive Leaf Extract

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Abstract

A simple method was validated for the analysis of 31 phenolic compounds using liquid chromatography–electrospray tandem mass spectrometry. Proposed method was successfully applied to the determination of phenolic compounds in an olive leaf extract and 24 compounds were analyzed quantitatively. Olive biophenols were extracted from olive leaves by using microwave-assisted extraction with acceptable recovery values between 78.1 and 108.7%. Good linearities were obtained with correlation coefficients over 0.9916 from calibration curves of the phenolic compounds. The limits of quantifications were from 0.14 to 3.2 $\mu g\ g^{-1}$. Intra-day and inter-day precision studies indicated that the proposed method was repeatable. As a result, it was confirmed that the proposed method was highly reliable for determination of the phenolic species in olive leaf extracts.

Introduction

The relationship between diet and health has given rise to interest in natural antioxidants such as bioactive components of natural raw materials. The protective effects of diets rich in fruit and vegetables against cardiovascular diseases and certain cancers have been attributed partly to the antioxidants contained therein, particularly to phenolic compounds (1). Therefore, extensive analytical research has been carried out on the separation and determination of phenolic constituents in various fresh fruit products and environmental samples (2). The techniques previously used include thin-layer chromatography (TLC) (3), gas chromatography (GC) equipped with flame ionization detector (FID) (4) and coupled to mass spectrometer (MS) (5), high performance liquid chromatography (HPLC) equipped with photodiode array detector (DAD) (6) and coupled to mass spectrometer (MS) (7), capillary electrophoresis (CE) (8) and voltammetry (9).

Olea europaea L. is one of the most widespread fruit trees and olive leaf is a very important agricultural biomass in Turkey. On the other hand, Olive leaves (*O. europaea*) have been used as folk medicine throughout the history of civilization in the Mediterranean area. Current scientific research has shown that olive leaves contain phenolic compounds responsible for several biological activities, including antioxidant and anti-inflammatory, antimicrobial, antiviral, anti-carcinogenic, as well as beneficial cardiovascular effects (10–12). Phenolic compounds in olive leaves are numerous and of diverse nature. The major classes of phenolic compounds in olive leaf extract are phenolic acids, phenolic alcohols, flavonoids and secoiridoids, and include mainly vanillic acid, caffeic acid, hydroxytyrosol, tyrosol, rutin, verbascoside, luteolin, quercetin, oleuropein, demethyloleuropein and ligstroside (13). As a result, it is important to be able to precisely determine the phenolic compounds in olive leaves that are rich in olive biophenols.

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Numerous extraction techniques, such as superheated liquid extraction using aqueous or organic solvents at a high pressure and temperature without reaching the critical point (14), supercritical fluid extraction (15), dynamic ultrasound-assisted extraction (16), fractionation by solid phase extraction (17) and microwave-assisted extraction (18) have all been performed to extract phenolic compounds from olive leaves and olive oil mill wastewaters.

Liquid chromatography tandem mass spectrometry (LC–MS-MS) serves as a high-throughput screening and confirmatory tool, which is generally a crucial technique for analyzing phenolic components in plant samples (19). As a result of the importance of the phenolic compounds as well as interest in their identification, the aim of this study was to develop and validate a sensitive quantification method by LC–electrospray ionization (ESI)–MS/MS for the determination of phenolic compounds in olive leaf extract. Although there are several studies reporting the total phenolic content of Turkish olive leaf extracts (20–22), this study is quite original in its nature for the individual determination of such a large number of phenolic compounds in a Turkish olive leaf extract. On the other hand, the proposed method has potential to be an economical routine analytical method in laboratories studying phenolic compounds in olive leaves, after a simple sample preparation step. The work gives insight to the literature about the technique and the phenolic content of the Turkish olive leaves. Target compounds are listed in Table I. Validation of analytical parameters was carried out for 31 phenolic compounds with the proposed method. Under the optimal conditions, 24 phenolic compounds in olive leaf extract were analyzed.

Experimental

Apparatus

LC analyses were performed with an Agilent Technologies 1260 Infinity liquid chromatography system hyphenated to a 6420 Triple Quad mass spectrometer. Microwave-assisted extraction (MAE) of the samples was carried out with Cem Mars 6.

Chemicals

Methanol and formic acid of HPLC grade were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany), respectively. Ultra-pure water (18 mΩ) was obtained from a Milli-Q water purification system (Millipore Co., Ltd.)

Standards

Gallic acid, (+)-catechin, pyrocatechol, chlorogenic acid, 2,5-dihydroxybenzoic acid, 4-hydroxybenzoic acid, (−)-epicatechin, caffeic

Table I. ESI–MS/MS Parameters for the Analysis of Target Analytes by MRM Negative and Positive Ionization Mode

Target compounds	R_t (min)	Precursor ion	MRM1 (CE, V)	MRM2 (CE, V)
Compounds analyzed by NI mode				
Gallic acid	8.891	168.9 $[M-H]^-$	125.0 (10)	–
Protocatechuic acid	10.818	152.9 $[M-H]^-$	108.9 (12)	–
3,4-Dihydroxyphenylacetic acid	11.224	167.0 $[M-H]^-$	123.0 (2)	–
(+)-Catechin	11.369	289.0 $[M-H]^-$	245.0 (6)	202.9 (12)
Pyrocatechol	11.506	109.0 $[M-H]^-$	90.6 (18)	52.9 (16)
2,5-Dihydroxybenzoic acid	12.412	152.9 $[M-H]^-$	109.0 (10)	–
4-Hydroxybenzoic acid	12.439	136.9 $[M-H]^-$	93.1 (14)	–
Caffeic acid	12.841	179.0 $[M-H]^-$	135.0 (12)	–
Vanillic acid	12.843	166.9 $[M-H]^-$	151.8 (10)	122.6 (6)
Syringic acid	12.963	196.9 $[M-H]^-$	181.9 (8)	152.8 (6)
3-Hydroxybenzoic acid	13.259	137.0 $[M-H]^-$	93.0 (6)	–
Vanillin	13.397	151.0 $[M-H]^-$	136.0 (10)	–
Verbascoside	13.589	623.0 $[M-H]^-$	461.0 (26)	160.8 (36)
Taxifolin	13.909	303.0 $[M-H]^-$	285.1 (2)	125.0 (14)
Sinapic acid	13.992	222.9 $[M-H]^-$	207.9 (6)	163.8 (6)
p-Coumaric acid	14.022	162.9 $[M-H]^-$	119.0 (12)	–
Ferulic acid	14.120	193.0 $[M-H]^-$	177.8 (8)	134.0 (12)
Luteolin 7-glucoside	14.266	447.1 $[M-H]^-$	285.0 (24)	–
Rosmarinic acid	14.600	359.0 $[M-H]^-$	196.9 (10)	160.9 (10)
2-Hydroxycinnamic acid	15.031	162.9 $[M-H]^-$	119.1 (10)	–
Pinoresinol	15.118	357.0 $[M-H]^-$	151.0 (12)	135.7 (34)
Eriodictyol	15.247	287.0 $[M-H]^-$	151.0 (4)	134.9 (22)
Quercetin	15.668	301.0 $[M-H]^-$	178.6 (10)	151.0 (16)
Kaempferol	16.236	285.0 $[M-H]^-$	242.8 (16)	229.1 (18)
Compounds analyzed by PI mode				
Chlorogenic acid	11.802	355.0 $[M+H]^+$	163.0 (10)	–
(−)-Epicatechin	12.458	291.0 $[M+H]^+$	139.1 (12)	122.9 (36)
Hesperidin	14.412	611.1 $[M+H]^+$	449.2 (4)	303.0 (20)
Hyperoside	14.506	465.1 $[M+H]^+$	303.1 (8)	–
Apigenin 7-glucoside	14.781	433.1 $[M+H]^+$	271.0 (18)	–
Luteolin	15.923	287.0 $[M+H]^+$	153.1 (34)	135.1 (36)
Apigenin	16.382	271.0 $[M+H]^+$	153.0 (34)	119.1 (36)

R_t, retention time; NI, negative ion; and PI, positive ion.

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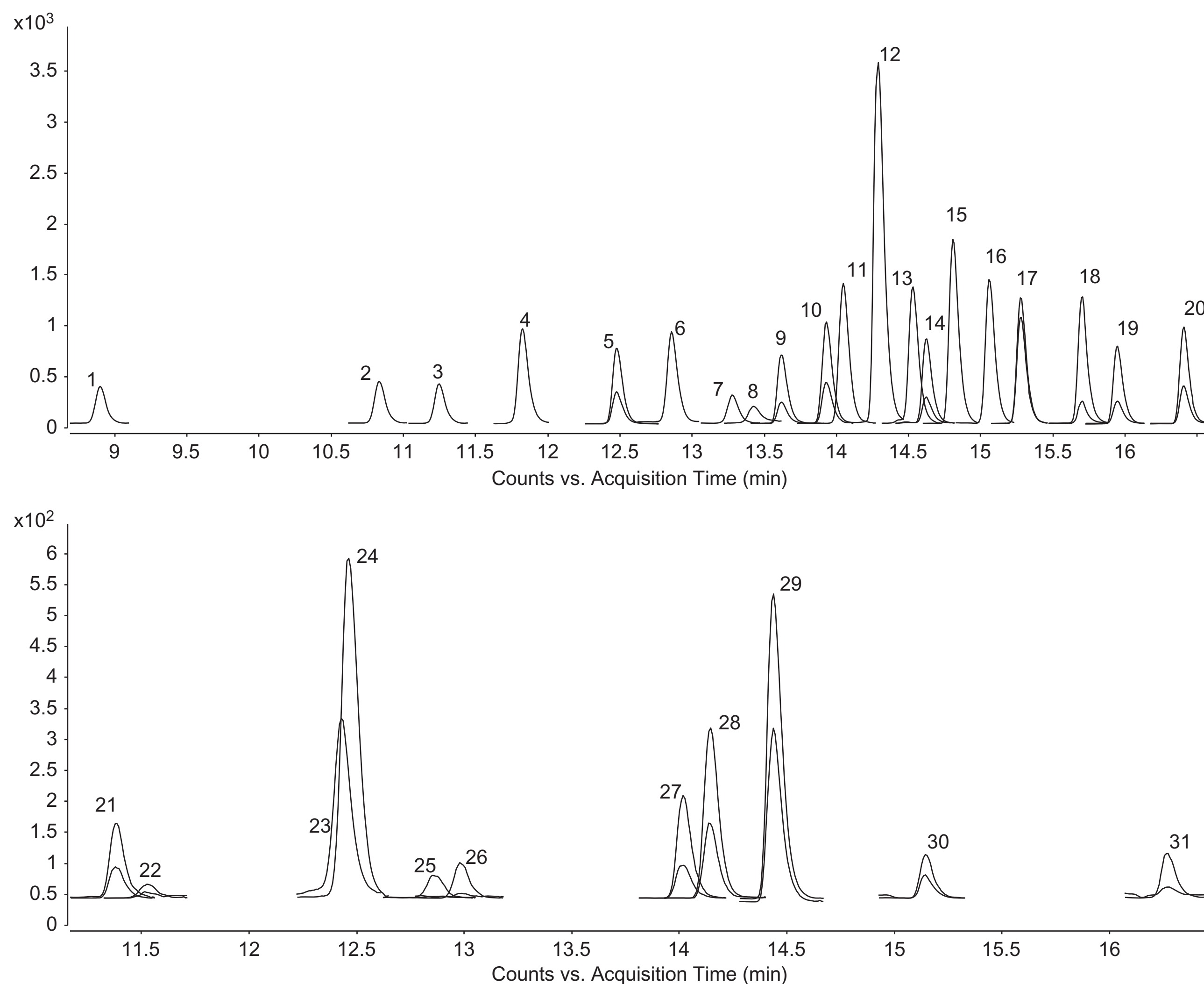

Figure 1. LC–ESI–MS/MS MRM chromatograms of phenolic compounds. 1–31 represent the chromatograms of gallic acid, protocatechuic acid, 3,4-dihydroxyphenylacetic acid, chlorogenic acid, (−)-epicatechin, caffeic acid, 3-hydroxybenzoic acid, vanillin, verbascoside, taxifolin, *p*-coumaric acid, luteolin 7-glucoside, hyperoside, rosmarinic acid, apigenin 7-glucoside, 2-hydroxycinnamic acid, eriodictyol, quercetin, luteolin, apigenin, (+)-catechin, pyrocatechol, 2,5-dihydroxybenzoic acid, 4-hydroxybenzoic acid, vanillic acid, syringic acid, sinapic acid, ferulic acid, hesperidin, pinoresinol and kaempferol, respectively. The phenolic concentrations are 400 μg L^{-1}.

acid, syringic acid, vanillin, taxifolin, sinapic acid, *p*-coumaric acid, ferulic acid, rosmarinic acid, 2-hydroxycinnamic acid, pinoresinol, quercetin, luteolin and apigenin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Vanillic acid, 3-hydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, apigenin 7-glucoside, luteolin 7-glucoside, hesperidin, eriodictyol and kaempferol were obtained from Fluka (St. Louis, MO, USA). Finally, verbascoside, protocatechuic acid and hyperoside were purchased from HWI Analytik (Ruelzheim, Germany).

Stock solutions at 500 mg L^{-1} were freshly prepared in methanol for all standards except luteolin 7-glucoside and hesperidin. Stock solutions of luteolin 7-glucoside and hesperidin were prepared at 100 mg L^{-1} due to their relatively low solubility in methanol. A series of standard solutions were prepared by diluting the stock solutions with methanol.

Plant material

Fresh green olive leaves (*O. europaea* L.) were harvested from the trees grown in Manisa, Turkey and were immediately transferred to the laboratory. After collecting, they were both dried and stored at ambient temperature in the dark. Finally, dry leaves were crushed and extracted via microwave-assisted extraction procedure.

LC–ESI–MS/MS method

An Agilent Technologies 1260 Infinity liquid chromatography system hyphenated to a 6420 Triple Quad mass spectrometer was used for quantitative analyses. Chromatographic separation was carried out on a Poroshell 120 EC-C18 (100 mm × 4.6 mm I.D., 2.7 μm) column. Three mobile phases were tested to obtain a complete resolution of all isomers and the highest sensitivity for all target compounds, namely: (i) 0.1% formic acid/methanol, (ii) 5 mM ammonium acetate/acetonitrile with 0.1% acetic acid and (iii) 10 mM ammonium formate with 0.1% formic acid/acetonitrile with 0.1% formic acid, respectively. The first mobile phase configuration (0.1% formic acid/methanol) was selected on the base of the better chromatographic resolution of isomeric compounds. On the other hand, the selected mobile phase

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Table II. Calibration Curves and Sensitivity Properties of the Method

Compounds	Linearity and sensitivity characteristics				
	Range (μg L^{-1})	Regression line		LOD (μg g^{-1})	LOQ (μg g^{-1})
		Linear equation	R^2		
Gallic acid	5–500	y = 4.82x − 26.48	0.9988	0.15	0.49
Protocatechuic acid	2.5–500	y = 5.65x − 9.99	0.9990	0.12	0.39
3,4-Dihydroxyphenylacetic acid	5–500	y = 5.13x − 12.39	0.9990	0.14	0.45
(+)-Catechin	10–500	y = 1.45x + 1.95	0.9974	0.40	1.3
Pyrocatechol	25–400	y = 0.11x − 0.52	0.9916	0.96	3.2
Chlorogenic acid	1–500	y = 12.14x + 32.34	0.9995	0.05	0.18
2,5-Dihydroxybenzoic acid	5–500	y = 3.79x − 14.12	0.9980	0.21	0.71
4-Hydroxybenzoic acid	5–500	y = 7.62x + 22.79	0.9996	0.17	0.57
(−)-Epicatechin	5–500	y = 9.11x − 9.99	0.9971	0.19	0.62
Caffeic acid	5–500	y = 11.09x + 16.73	0.9997	0.26	0.85
Vanillic acid	10–500	y = 0.49x − 1.61	0.9968	0.31	1.1
Syringic acid	10–500	y = 0.74x − 1.54	0.9975	0.38	1.3
3-Hydroxybenzoic acid	5–500	y = 3.69x − 12.29	0.9991	0.19	0.62
Vanillin	50–500	y = 2.02x + 135.49	0.9926	1.5	5.1
Verbascoside	2.5–500	y = 8.59x − 28.05	0.9988	0.08	0.27
Taxifolin	5–500	y = 12.32x + 9.98	0.9993	0.18	0.61
Sinapic acid	5–500	y = 2.09x − 6.79	0.9974	0.26	0.88
p-Coumaric acid	5–500	y = 17.51x + 53.73	0.9997	0.19	0.64
Ferulic acid	5–500	y = 3.32x − 4.30	0.9992	0.14	0.48
Luteolin 7-glucoside	1–500	y = 45.25x + 156.48	0.9996	0.05	0.15
Hesperidin	5–500	y = 5.98x + 0.42	0.9993	0.17	0.58
Hyperoside	2.5–500	y = 16.32x − 1.26	0.9998	0.10	0.33
Rosmarinic acid	1–500	y = 9.82x − 17.98	0.9989	0.06	0.19
Apigenin 7-glucoside	1–500	y = 21.33x − 31.69	0.9983	0.04	0.14
2-Hydroxycinnamic acid	1–500	y = 16.72x − 26.94	0.9996	0.06	0.20
Pinoresinol	10–500	y = 0.80x − 2.69	0.9966	0.39	1.3
Eriodictyol	2.5–500	y = 14.24x − 0.50	0.9998	0.08	0.27
Quercetin	5–500	y = 14.68x − 18.25	0.9997	0.12	0.41
Luteolin	5–500	y = 8.96x + 26.80	0.9992	0.13	0.45
Kaempferol	10–500	y = 0.82x − 3.06	0.9959	0.33	1.1
Apigenin	2.5–500	y = 11.29x + 38.05	0.9987	0.10	0.32

configuration also provided higher sensitivity for many of the phenolic compounds. As a result, the mobile phase was made up from solvent A (0.1%, *v/v* formic acid solution) and solvent B (methanol). The gradient profile was set as follows: 0.00 min 2% B eluent, 3.00 min 2% B eluent, 6.00 min 25% B eluent, 10.00 min 50% B eluent, 14.00 min 95% B eluent, 17.00 min 95% B and 17.50 min 2% B eluent. The column temperature was maintained at 25°C. The flow rate was 0.4 mL min^{-1} and the injection volume was 2.0 μL.

The tandem mass spectrometer was interfaced to the LC system via an ESI source. The electrospray source of the MS was operated in negative and positive multiple reaction monitoring (MRM) mode and the interface conditions were as follows: capillary voltage of −3.5 kV, gas temperature of 300°C and gas flow of 11 L min^{-1}. The nebulizer pressure was 40 psi. MRM transitions, the optimum collision energies and retention times for each species are indicated in Table I. In addition, representative LC–ESI–MS/MS chromatograms of phenolic compounds are shown in Figure 1.

Microwave-assisted extraction procedure

MAE was proposed for the first time by Japón-Luján *et al.* (18) in order to accelerate the extraction of biophenols from olive leaves. A multivariate optimization was also carried out in order to find the best working conditions for extraction and microwaves proved to be an excellent aid to the extraction of polar compounds by a polar extractant. Thus, a modified form of this MAE technique that provides high recovery values for the extraction of oleuropein (another olive biophenol) from olive leaves (9) was used in present study. 0.1 g of milled olive leaf sample and 10 mL of extractant mixture of methanol and water (80:20, *v/v*) were placed into the microwave extraction vessel and placed in the microwave-irradiation zone. The temperature of the system was raised to 80°C in ~8 min, and it remained at 80°C for 6 min. The extraction vessels were allowed to cool for 30 min at room temperature after extraction. The extracts were filtered through a 0.45 μm syringe filter prior to analysis.

Peak identification

In MRM mode the peaks of the analytes were identified by comparing the retention time, together with the monitoring ions pairs in an authentic standard solution.

Results

Linearity, limit of quantifications and selectivity

Serial standard solutions of phenolics were analyzed by LC–ESI–MS/MS, respectively. All calibration curves were generated from stock standard solutions with three replicates per level. As shown

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in Table II, the correlation coefficients (R^2) of the calibration equations were higher than 0.9916 for all analytes.

The limit of detections (LODs) and limit of quantifications (LOQs) were calculated using the formula LOD = $3S_a/b$ and LOQ = $10S_a/b$, where, S_a is the standard deviation of the response and b is the slope of the calibration curve. The results indicated that the LOQs for phenolic compounds ranged from 0.14 to 3.2 μg g^{-1} (Table II).

The selectivity was assessed through the use of MRM following the chromatographic separation. Although the chemical structures of the phenolic compounds are relatively similar, the selectivity of MS/MS to the different molecular masses is quite high. Nevertheless, many of them have different precursor ions, so there was no interference in the selected transitions, demonstrating the selectivity of the method. On the other hand, the selectivity of isomeric species with the same molecular ion was provided by chromatographic separation.

Repeatability and precision

The calibration standards at three different concentrations and an olive leaf sample (extracts from the same sample were prepared via MAE procedure) were used to determine the intra-day (three repetitions of each concentration and extract) and inter-day (three repetitions of each concentration and extract, 3 days) repeatability of proposed method. As illustrated in Table III, the intra- and inter-day precisions (RSDs) for phenolic compounds were <9.4 and 16%, respectively.

Recovery and applicability of the method

The proposed and validated method was applied for the determination of 31 phenolic compounds in an olive leaf extract. The calibration curves in the validation range defined in Table II were used for quantitation. (+)-Catechin, pyrocatechol, (−)-epicatechin, 3-hydroxybenzoic acid, vanillin, rosmarinic acid and 2-hydroxycinnamic acid were not detected in the olive leaf extract. The phenolic content of olive leaf is shown in Table IV.

The recovery studies were carried out by spiking 0.1 g of olive leaf sample at different amounts of phenolic compounds (three repetitions of each amount). Initially, the contents of the analytes in the sample were calculated according to their individual calibration curves. Each analyte was spiked into accurately weighed portion of the sample at different amounts, then was extracted via MAE procedure and finally analyzed using the proposed method. The recoveries were calculated by the formula: recovery (%) = (amount found − original amount)/amount spiked × 100%, and RSD (%) = (SD/

Table III. Intra-day and Inter-day Precision

Compounds	Intra-day (RSD%, $n = 3$)				Inter-day (RSD%, $n = 9$)			
	Low level	Med. level	High level	Extract	Low level	Med. level	High level	Extract
Gallic acid	2.9	0.6	0.5	1.8	9.9	2.0	3.4	13
Protocatechuic acid	1.9	1.0	0.7	1.3	9.5	1.5	2.6	6.4
3,4-Dihydroxyphenylacetic acid	4.3	1.7	0.7	1.1	9.6	3.1	2.7	14
(+)-Catechin	5.6	3.8	2.6	nd	7.9	7.7	3.9	nd
Pyrocatechol	9.4	6.2	3.5	nd	15	9.5	9.1	nd
Chlorogenic acid	1.7	0.3	0.3	6.0	9.5	2.6	1.7	13
2,5-Dihydroxybenzoic acid	7.4	1.5	1.1	2.9	10	2.2	1.8	12
4-Hydroxybenzoic acid	4.3	1.9	1.4	3.9	11	3.3	2.5	5.7
(−)-Epicatechin	4.0	9.0	0.4	nd	9.3	9.8	5.0	nd
Caffeic acid	4.1	1.0	0.7	1.9	11	2.0	1.4	9.7
Vanillic acid	7.3	5.3	1.9	4.0	11	6.7	4.5	9.7
Syringic acid	5.6	3.2	1.3	3.5	8.4	4.0	4.3	14
3-Hydroxybenzoic acid	5.7	2.0	1.0	nd	9.2	2.4	1.5	nd
Vanillin	6.2	2.5	2.2	nd	8.1	3.7	4.8	nd
Verbascoside	3.5	2.6	2.2	5.6	15	3.0	3.0	16
Taxifolin	4.6	0.8	0.2	2.6	8.4	1.9	1.4	9.3
Sinapic acid	3.1	2.9	2.2	2.9	9.0	2.9	4.3	7.3
p-Coumaric acid	3.0	0.4	0.8	2.3	15	3.1	2.1	8.8
Ferulic acid	7.3	1.2	0.4	3.9	9.2	3.8	1.9	9.8
Luteolin 7-glucoside	2.3	0.3	0.5	2.9	6.9	2.8	2.0	12
Hesperidin	5.5	1.3	0.7	3.5	9.6	3.0	2.4	6.9
Hyperoside	1.8	1.4	0.5	5.1	11	2.7	3.7	8.4
Rosmarinic acid	2.3	1.9	0.5	nd	9.2	2.7	2.4	nd
Apigenin 7-glucoside	1.5	0.7	0.5	2.9	6.4	4.0	4.2	6.8
2-Hydroxycinnamic acid	5.1	1.9	0.6	nd	11	2.5	1.6	nd
Pinoresinol	3.6	3.1	2.3	5.5	15	6.8	4.6	13
Eriodictyol	3.7	1.9	0.8	2.8	11	2.3	1.4	9.2
Quercetin	2.3	0.7	0.7	4.7	7.4	1.5	1.0	9.8
Luteolin	2.9	0.3	0.1	0.9	6.3	1.8	2.8	4.4
Kaempferol	7.9	2.6	0.8	3.7	9.7	4.0	4.1	10
Apigenin	6.7	1.4	0.4	4.9	9.0	4.6	5.3	5.8

Low level means, 2.5 μg L^{-1} for chlorogenic acid, apigenin 7-glucoside, luteolin 7-glucoside, rosmarinic acid and 2-hydroxycinnamic acid; 5 μg L^{-1} for protocatechuic acid, verbascoside, hyperoside, eriodictyol and apigenin; 10 μg L^{-1} for gallic acid, 3,4-dihydroxyphenylacetic acid, 2,5-dihydroxybenzoic acid, 4-hydroxybenzoic acid, (−)-epicatechin, caffeic acid, 3-hydroxybenzoic acid, taxifolin, sinapic acid, *p*-coumaric acid, ferulic acid, hesperidin, quercetin and luteolin; 25 μg L^{-1} for (+)-catechin, vanillic acid, syringic acid, pinoresinol and kaempferol; 50 μg L^{-1} for pyrocatechol; 75 μg L^{-1} for vanillin. Medium and high level mean 200 and 400 μg L^{-1}, respectively. nd, not determined in olive leaf extract. RSD, relative standard deviation.

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Table IV. Quantification of Phenolic Compounds in an Olive Leaf Extract and Recovery Data of the Proposed Method

Compounds	Content (μg g^{-1})	Low level (n = 3)			High level (n = 3)		
		Spiked (μg g^{-1})	Found (μg g^{-1})	Recovery (%)	Spiked (μg g^{-1})	Found (μg g^{-1})	Recovery (%)
1	2.58 ± 0.56	5.00	7.63 ± 0.34	101.1 ± 5.3	50.00	55.65 ± 1.38	106.1 ± 2.4
2	17.48 ± 3.70	5.00	20.54 ± 2.91	97.1 ± 0.9	50.00	66.75 ± 1.35	98.5 ± 5.0
3	0.47 ± 0.06	5.00	4.80 ± 0.02	86.5 ± 1.4	50.00	48.01 ± 4.17	95.1 ± 8.4
4	nd	5.00	5.15 ± 0.29	103.0 ± 5.9	50.00	50.96 ± 0.66	101.9 ± 1.3
5	nd	5.00	5.33 ± 0.31	106.7 ± 6.1	50.00	48.33 ± 2.42	96.7 ± 4.8
6	2.67 ± 0.08	5.00	7.30 ± 0.25	92.7 ± 6.3	50.00	51.19 ± 1.60	97.0 ± 3.4
7	5.54 ± 1.00	5.00	10.50 ± 0.76	99.1 ± 5.3	50.00	56.68 ± 1.23	102.3 ± 2.1
8	15.31 ± 0.98	5.00	20.23 ± 1.02	98.4 ± 7.6	50.00	65.83 ± 0.69	101.0 ± 0.7
9	nd	5.00	4.49 ± 0.08	89.8 ± 1.6	50.00	48.22 ± 0.55	96.4 ± 1.1
10	15.34 ± 3.11	5.00	19.92 ± 3.29	91.7 ± 3.7	50.00	66.59 ± 2.98	102.5 ± 1.6
11	15.87 ± 4.59	5.00	20.63 ± 5.00	95.1 ± 8.5	50.00	70.23 ± 4.24	108.7 ± 3.8
12	2.72 ± 0.98	5.00	7.92 ± 0.51	104.1 ± 9.5	50.00	50.70 ± 2.21	95.9 ± 6.3
13	nd	5.00	5.14 ± 0.27	102.9 ± 5.5	50.00	49.98 ± 1.49	100.0 ± 3.0
14	nd	5.00	4.94 ± 0.32	98.9 ± 6.5	50.00	50.01 ± 0.34	100.0 ± 0.7
15	2,912.4 ± 183.9	–	–	–	2,500.0	4,774.1 ± 57.3	78.1 ± 7.7
16	8.21 ± 1.18	5.00	11.92 ± 1.77	85.1 ± 15.7	50.00	54.50 ± 0.74	92.6 ± 2.9
17	4.89 ± 1.24	5.00	9.34 ± 1.87	88.9 ± 13.2	50.00	53.59 ± 4.05	97.4 ± 6.0
18	81.07 ± 9.21	5.00	81.12 ± 6.63	91.5 ± 3.5	50.00	123.93 ± 6.54	85.7 ± 7.6
19	45.53 ± 9.78	5.00	50.19 ± 9.62	93.3 ± 6.1	50.00	94.57 ± 9.22	98.1 ± 9.2
20	2,247.2 ± 90.9	100.0	2,342.7 ± 132.8	94.9 ± 4.3	1,000.0	3,171.5 ± 31.2	97.0 ± 3.2
21	304.05 ± 9.52	35.00	337.49 ± 9.49	95.6 ± 1.7	350.00	602.98 ± 27.95	85.4 ± 6.3
22	66.92 ± 5.72	5.00	68.63 ± 3.60	89.2 ± 17.7	50.00	114.04 ± 2.65	94.2 ± 9.2
23	nd	5.00	4.88 ± 0.15	97.7 ± 3.1	50.00	49.46 ± 1.36	98.9 ± 2.7
24	346.89 ± 17.11	30.00	369.42 ± 18.88	94.9 ± 1.6	300.00	610.66 ± 26.89	87.9 ± 3.6
25	nd	5.00	4.67 ± 0.22	93.4 ± 4.3	50.00	47.09 ± 1.21	94.2 ± 2.4
26	4.01 ± 0.66	5.00	8.36 ± 1.62	87.3 ± 13.6	50.00	46.74 ± 4.35	85.5 ± 9.3
27	6.77 ± 0.58	5.00	11.41 ± 0.33	94.2 ± 9.3	50.00	50.45 ± 2.69	87.3 ± 6.5
28	40.40 ± 3.33	5.00	42.96 ± 0.87	89.5 ± 5.5	50.00	87.25 ± 2.68	93.7 ± 1.7
29	219.60 ± 3.93	5.00	223.63 ± 5.19	94.6 ± 1.8	50.00	263.39 ± 6.72	87.6 ± 6.7
30	2.86 ± 0.77	5.00	7.55 ± 0.42	93.8 ± 10.7	50.00	50.25 ± 1.64	94.8 ± 3.3
31	92.51 ± 25.98	5.00	107.52 ± 27.39	100.4 ± 0.5	50.00	154.88 ± 30.87	104.8 ± 6.9

1–31 represent gallic acid, protocatechuic acid, 3,4-dihydroxyphenylacetic acid, (+)-catechin, pyrocatechol, chlorogenic acid, 2,5-dihydroxybenzoic acid, 4-hydroxybenzoic acid, (−)-epicatechin, caffeic acid, vanillic acid, syringic acid, 3-hydroxybenzoic acid, vanillin, verbascoside, taxifolin, sinapic acid, *p*-coumaric acid, ferulic acid, luteolin 7-glucoside, hesperidin, hyperoside, rosmarinic acid, apigenin 7-glucoside, 2-hydroxycinnamic acid, pinoresinol, eriodictyol, quercetin, luteolin, kaempferol and apigenin, respectively. nd, not determined in olive leaf extract.

mean) × 100%. Quantitative recoveries were obtained in all cases, varying from 78.1 to 108.7% (Table IV).

Matrix effect

One significant drawback in ESI MS quantitative analysis is the matrix effect. ESI is susceptible to matrix components that may lead to signal suppression or, less frequently, to an enhancement of the signal of the analyte (23–27). For this purpose, it is necessary to examine the matrix effect of the proposed analytical technique. Due to absence of blank matrices for the olive leaves, the matrix effect of the method was determined at 10 μg g^{-1} concentration of phenolic compounds. The matrix effects were calculated relatively for phenolic compounds by comparing the response of three areas of chromatographic peak (19). The Eq. (1) is given as follows:

$$\mathrm{ME}(\%) = \left[\frac{\mathrm{A(matrix + std) - A(matrix)}}{\mathrm{A(std)}} - 1\right] \times 100(\%) \quad (1)$$

where A(matrix + std) is the response for the matrix with the addition of standard; A(matrix) is the response for the matrix only; and A(std) is the response for the standard only.

An enhancement in analytical signal was only observed for quercetin (9.8%). As expected, the matrix effect usually resulted in signal suppression of the analytes. Signal suppression is believed to occur when matrix components compete with the analyte ions for access to the droplet surface for gas phase emission (27). High signal suppressions sometimes lead to erroneous results. In this case, the matrix effect needs to be reduced. There are several strategies to reduce matrix effect, e.g., selective extraction, effective sample cleanup after the extraction, or improvement of the chromatographic separation. Sometimes, these approaches are not the appropriate solutions because they could lead to analyte losses as well as long analysis times (27). Here, the matrix effect in the proposed method was not generally greater than the RSD values obtained for inter-day reproducibility (ranging from −17 to 9.8%) of the phenolic compounds. As a result, generally slight signal suppressions were observed when the sample preparation was carried out by the proposed MAE method (Table V).

Stability

The stability of the sample extract was evaluated by injecting the same olive leaf extract at 0, 2, 4, 6 and 8 h (five injections) after

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Table V. Matrix Effect of Phenolic Compounds in Olive Leaf Extract and Stability of the Proposed Method

Compounds	Matrix effect (%, $n = 3$)	Stability (RSD%, $n = 3$)
Gallic acid	−4.5 (1.1)[a]	8.0
Protocatechuic acid	−5.6 (1.7)	3.5
3,4-Dihydroxyphenylacetic acid	−6.5 (1.4)	13
(+)-Catechin	−3.3 (1.5)	nd
Pyrocatechol	−5.6 (0.3)	nd
Chlorogenic acid	−3.2 (0.9)	3.4
2,5-Dihydroxybenzoic acid	−5.4 (0.3)	5.8
4-Hydroxybenzoic acid	−5.9 (1.5)	4.7
(−)-Epicatechin	−7.3 (0.9)	nd
Caffeic acid	−7.5 (1.7)	8.2
Vanillic acid	−11 (1.9)	13
Syringic acid	−8.1 (2.9)	11
3-Hydroxybenzoic acid	−9.7 (1.8)	nd
Vanillin	−0.6 (0.3)	nd
Verbascoside	−9.2 (2.6)	5.4
Taxifolin	−4.2 (1.8)	9.1
Sinapic acid	−10 (1.8)	9.9
p-Coumaric acid	−2.4 (1.0)	3.3
Ferulic acid	−10 (0.4)	2.9
Luteolin 7-glucoside	−8.5 (1.6)	7.8
Hesperidin	−3.6 (1.8)	2.1
Hyperoside	−7.3 (1.6)	3.7
Rosmarinic acid	−4.3 (1.8)	nd
Apigenin 7-glucoside	−7.6 (2.5)	4.1
2-Hydroxycinnamic acid	−11 (1.9)	nd
Pinoresinol	−17 (3.5)	17
Eriodictyol	−16 (1.6)	3.3
Quercetin	9.8 (2.3)	2.7
Luteolin	−16 (3.1)	8.0
Kaempferol	−11 (2.3)	16
Apigenin	−2.7 (0.8)	2.4

nd, not determined in olive leaf extract.
[a]Standard deviation (SD).

preparation using the proposed MAE technique. The peak areas of the analytes were recorded at each five injection. Finally, variations were expressed by relative standard deviations of five individual responses of each analyte to assess the sample extract (Table V). Consequently, the sample extracts would be stable within 8 h according to the RSD values of the analytes for repeatability assay.

Discussion

There are many studies suggested for the identification and determination of phenolic compounds in olive leaf extracts. However, the existing determination methods have been validated for the quantification of a relatively small number of phenolic compounds compared to the proposed method. Comparison of the proposed method with the techniques previously reported for the determination of phenolic compounds in olive leaf extracts is listed in Table VI. According to the table, the proposed method is able to perform the determination of a large number of phenolic compounds by a single run.

Proposed method addressed some important issues. Chromatographic separation of the isomeric species (3-hydroxybenzoic acid and 4-hydroxybenzoic acid, 2,5-dihydroxybenzoic acid and protocatechuic acid, 2-hydroxycinnamic acid and *p*-coumaric acid, vanillic acid and 3,4-dihydroxyphenylacetic acid, (+)-catechin and (−)-epicatechin, luteolin and kaempferol) was achieved successfully by the proposed method. On the other hand, it is well known that chlorogenic acid is the ester of caffeic acid and (−)-quinic acid and this ester bond might easily be fragmented. The other important issue with the proposed method was the separation of caffeic acid and chlorogenic acid.

Table VI. Comparison of the Quantitation Techniques Reported for the Determination of Phenolic Compounds in Olive Leaf Extracts

Analytical technique	Number of phenolic compounds validated for quantification	Run time (min)	Ref.
HPLC-DAD	2	30	(28)
HPLC-DAD	7	60	(29)
LC–ESI–MS	6	60	(30)
LC–ESI–MS-MS	7	30	(31)
HPLC-DAD	6	35	(32)
HPLC-DAD	5	37	(33)
HPLC-DAD	14	60	(34)
LC–ESI–MS-MS	31	17.5	This work

HPLC, high performance liquid chromatography; DAD, diode array detector; LC, liquid chromatography; ESI, electrospray ionization; MS, mass spectrometer.

Conclusion

In this article, a simple, rapid, reproducible and sensitive method was developed and validated for the simultaneous determination of 31 phenolic compounds using LC–ESI–MS/MS. The MAE technique in this study is able to combine high phenolic compounds recovery with slight signal suppressions of the target analytes. Applicability of the method was verified on a real sample of olive leaf extract and 24 phenolic compounds were quantified. Consequently, analytical properties of the validated method satisfied the routine analysis of phenolic compounds in olive leaves samples.

Supplementary data

Supplementary data are available at *Journal of Chromatographic Science* online.

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References

1. Hertog, M.G.L., Feskens, E.J.M., Hollman, P.C.H., Katan, M.B., Kromhout, D.; Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study; *Lancet*, (1993); 342: 1007–1011.
2. Chen, H., Zuo, Y., Deng, Y.; Separation and determination of flavonoids and other phenolic compounds in cranberry juice by high-performance

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liquid chromatography; *Journal of Chromatography A*, (2001); 913: 387–395.
3. Ragazzi, E., Veronese, G.; Quantitative analysis of phenolic compounds after thin-layer chromatographic separation; *Journal of Chromatography A*, (1973); 77: 369–375.
4. Zhou, F., Li, X., Zeng, Z.; Determination of phenolic compounds in wastewater samples using a novel fiber by solid-phase microextraction coupled to gas chromatography; *Analytica Chimica Acta*, (2005); 538: 63–70.
5. Zafra, A., Juárez, M.J.B., Blanc, R., Navalón, A., González, J., Vílchez, J. L.; Determination of polyphenolic compounds in wastewater olive oil by gas chromatography–mass spectrometry; *Talanta*, (2006); 70: 213–218.
6. Shui, G., Leong, L.P.; Separation and determination of organic acids and phenolic compounds in fruit juices and drinks by high-performance liquid chromatography; *Journal of Chromatography A*, (2002); 977: 89–96.
7. Alarcón-Flores, M.I., Romero-González, R., Martínez Vidal, J.L., Garrido Frenich, A.; Multiclass determination of phenolic compounds in different varieties of tomato and lettuce by ultra high performance liquid chromatography coupled to tandem mass spectrometry; *International Journal of Food Properties*, (2016); 19: 494–507.
8. Deng, Y., Fan, X., Delgado, A., Nolan, C., Furton, K., Zuo, Y., *et al.*; Separation and determination of aromatic acids in natural water with preconcentration by capillary zone electrophoresis; *Journal of Chromatography A*, (1998); 817: 145–152.
9. Cittan, M., Koçak, S., Çelik, A., Dost, K.; Determination of oleuropein using multiwalled carbon nanotube modified glassy carbon electrode by adsorptive stripping square wave voltammetry; *Talanta*, (2016); 159: 148–154.
10. Lee, O.-H., Lee, B.-Y.; Antioxidant and antimicrobial activities of individual and combined phenolics in *Olea europaea* leaf extract; *Bioresource Technology*, (2010); 101: 3751–3754.
11. Poudyal, H., Campbell, F., Brown, L.; Olive leaf extract attenuates cardiac, hepatic, and metabolic changes in high carbohydrate-, high fat-fed rats; *Journal of Nutrition*, (2010); 140: 946–953.
12. Taamalli, A., Arráez-Román, D., Barrajón-Catalán, E., Ruiz-Torres, V., Pérez-Sánchez, A., Herrero, M., *et al.*; Use of advanced techniques for the extraction of phenolic compounds from Tunisian olive leaves: phenolic composition and cytotoxicity against human breast cancer cells; *Food and Chemical Toxicology*, (2012); 50: 1817–1825.
13. Talhaoui, N., Taamalli, A., Gómez-Caravaca, A.M., Fernández-Gutiérrez, A., Segura-Carretero, A.; Phenolic compounds in olive leaves: analytical determination, biotic and abiotic influence, and health benefits; *Food Research International*, (2015); 77: 92–108.
14. Japón-Luján, R., Luque de Castro, M.D.; Superheated liquid extraction of oleuropein and related biophenols from olive leaves; *Journal of Chromatography A*, (2006); 1136: 185–191.
15. Le Floch, F., Tena, M., Ríos, A., Valcárcel, M.; Supercritical fluid extraction of phenol compounds from olive leaves; *Talanta*, (1998); 46: 1123–1130.
16. Japón-Luján, R., Luque-Rodríguez, J., de Castro, L.; Dynamic ultrasound-assisted extraction of oleuropein and related biophenols from olive leaves; *Journal of Chromatography A*, (2006); 1108: 76–82.
17. De Marco, E., Savarese, M., Paduano, A., Sacchi, R.; Characterization and fractionation of phenolic compounds extracted from olive oil mill wastewaters; *Food Chemistry*, (2007); 104: 858–867.
18. Japón-Luján, R., Luque-Rodríguez, J.M., Luque de Castro, M.D.; Multivariate optimisation of the microwave-assisted extraction of oleuropein and related biophenols from olive leaves; *Analytical and Bioanalytical Chemistry*, (2006); 385: 753–759.
19. Wu, R., Ma, F., Zhang, L., Li, P., Li, G., Zhang, Q., *et al.*; Simultaneous determination of phenolic compounds in sesame oil using LC–MS/MS combined with magnetic carboxylated multi-walled carbon nanotubes; *Food Chemistry*, (2016); 204: 334–342.
20. Altıok, E., Bayçın, D., Bayraktar, O., Ülkü, S.; Isolation of polyphenols from the extracts of olive leaves (*Olea europaea* L.) by adsorption on silk fibroin; *Separation and Purification Technology*, (2008); 62: 342–348.
21. Erbay, Z., Icier, F.; Optimization of hot air drying of olive leaves using response surface methodology; *Journal of Food Engineering*, (2009); 91: 533–541.
22. Şahin, S., Şamlı, R.; Optimization of olive leaf extract obtained by ultrasound-assisted extraction with response surface methodology; *Ultrasonics Sonochemistry*, (2013); 20: 595–602.
23. Pascoe, R., Foley, J.P., Gusev, A.I.; Reduction in matrix-related signal suppression effects in electrospray ionization mass spectrometry using on-line two-dimensional liquid chromatography; *Analytical Chemistry*, (2001); 73: 6014–6023.
24. Zrostlíková, J., Hajslová, J., Poustka, J., Begany, P.; Alternative calibration approaches to compensate the effect of co-extracted matrix components in liquid chromatography-electrospray ionisation tandem mass spectrometry analysis of pesticide residues in plant materials; *Journal of Chromatography A*, (2002); 973: 13–26.
25. Hajšlová, J., Zrostlíková, J.; Matrix effects in (ultra)trace analysis of pesticide residues in food and biotic matrices; *Journal of Chromatography A*, (2003); 1000: 181–197.
26. Reemtsma, T.; Liquid chromatography–mass spectrometry and strategies for trace-level analysis of polar organic pollutants; *Journal of Chromatography A*, (2003); 1000: 477–501.
27. Stüber, M., Reemtsma, T.; Evaluation of three calibration methods to compensate matrix effects in environmental analysis with LC-ESI-MS; *Analytical and Bioanalytical Chemistry*, (2004); 378: 910–916.
28. Lalas, S., Athanasiadis, V., Gortzi, O., Bounitsi, M., Giovanoudis, I., Tsaknis, J., *et al.*; Enrichment of table olives with polyphenols extracted from olive leaves; *Food Chemistry*, (2011); 127: 1521–1525.
29. Pereira, A.P., Ferreira, I.C.F.R., Marcelino, F., Valentão, P., Andrade, P. B., Seabra, R., *et al.*; Phenolic compounds and antimicrobial activity of olive (*Olea europaea* L. Cv. Cobrançosa) leaves; *Molecules (Basel, Switzerland)*, (2007); 12: 1153–1162.
30. Goulas, V., Exarchou, V., Troganis, A.N., Psomiadou, E., Fotsis, T., Briasoulis, E., *et al.*; Phytochemicals in olive-leaf extracts and their anti-proliferative activity against cancer and endothelial cells; *Molecular Nutrition & Food Research*, (2009); 53: 600–608.
31. Tóth, G., Alberti, Á., Sólyomváry, A., Barabás, C., Boldizsár, I., Noszál, B.; Phenolic profiling of various olive bark-types and leaves: HPLC–ESI/MS study; *Industrial Crops and Products*, (2015); 67: 432–438.
32. Savournin, C., Baghdikian, B., Elias, R., Dargouth-Kesraoui, F., Boukef, K., Balansard, G.; Rapid high-performance liquid chromatography analysis for the quantitative determination of oleuropein in *Olea europaea* leaves; *Journal of Agricultural and Food Chemistry*, (2001); 49: 618–621.
33. Talhaoui, N., Gómez-Caravaca, A.M., León, L., De la Rosa, R., Segura-Carretero, A., Fernández-Gutiérrez, A.; Determination of phenolic compounds of “Sikitita” olive leaves by HPLC-DAD-TOF-MS. Comparison with its parents “Arbequina” and “Picual” olive leaves; *LWT Food Science and Technology*, (2014); 58: 28–34.
34. Benavente-García, O., Castillo, J., Lorente, J., Ortuño, A., Del Rio, J.; Antioxidant activity of phenolics extracted from *Olea europaea* L. leaves; *Food Chemistry*, (2000); 68: 457–462.

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