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Determination of secondary metabolites of *Origanum vulgare* subsp. *hirtum* and *O. vulgare* subsp. *vulgare* by LC-MS/MS

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Abstract: Secondary metabolites of solvent extracts of *Origanum vulgare* subsp. *hirtum* and *O. vulgare* subsp. *vulgare* were determined using liquid chromatography–mass spectrometry (LC-MS/MS). Curcumin was used as an internal standard. Rosmarinic acid was determined as the main compound of studied extracts together with other phenolic acid derivatives. In LC-MS/MS analyses, relative standard deviations (RSD %) ranged between 0.11-9.47. The correlation values were found to be greater than 0.97 for each investigated analyte.

Keywords: *Origanum;* phenolic compounds; LC-MS/MS; method validation; uncertainty assessment; rosmarinic acid.

1. Introduction

The genus *Origanum* L. is one of the most important genera in the family of Lamiaceae, the species of which are used as herbal tea and spices. It has attracted significant attention of local people in Turkey due to the aromatic properties of species. Turkey is a vital gene center of diversity of the genus. There are 21 species with three subspecies (24 taxa) and 13 hybrids, of which 25 are endemic [1-3].

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In Turkey, species of the genus *Origanum*, *Thymus* L., *Satureja* L., *Thymbra* L., *Cridothymus* L. are called as "Kekik". The *Origanum* species are known worldwide as "Oregano" and Turkey is the largest supplier of it in the world. In 2019, approximately 15000 tons of *Origanum* species were harvested and exported as raw material and essential oil [4,5]. A significant part of this quantity is obtained by cultivation of *Origanum onites* L., and relatively lower part of this amount is collected from nature.

In Turkey, seven *Origanum* species are commonly used as herbal tea and spice among 37 taxa. Wild or cultivated forms of *Origanum vulgare* L. is used as a condiment and herbal tea. The species widely distributed from Macaronesia to China and is divided into six subspecies worldwide and four subspecies are distributed in the Flora of Turkey as follows: subsp. *gracile* (K.Koch) Ietsw., subsp. *hirtum* (Link) A. Terracc., subsp. *viridulum* (Martren the-Donosti) Nyman and subsp. *vulgare*. All those species are used as herbal tea and condiments and, the most common one is *O. vulgare* subsp. *hirtum* in Turkey [6].

Apart from herbal tea and condiments, *Origanum* species have been used in the pharmaceutical, cosmetic, and food industries due to their biological activities [7]. The phytochemical studies on the essential oil of *Origanum* species have pointed out to the rich presence of thymol and carvacrol [8-10]. Differences in the composition of the essential oils of the *O. vulgare* subsp. *hirtum* and *O. vulgare* subsp. *vulgare* species have been the subject of various researchers' reports. Carvacrol, linalool, thymol, *β*-caryophyllene, and *trans*-sabinene hydrate were found to be major components of essential oils of both species [11-26]. Moreover, antimicrobial and antioxidant activities of essential oil and various extracts of *O. vulgare* subsp. *hirtum* and *O. vulgare* subsp. *vulgare* were reported [9,18, 22, 27-30]. There is a unique study about the anticholinesterase activity of essential oil of both species [17]. However, there are few studies about the phenolic contents of both species in the literature. The phenolic composition of ethanol, methanol, water, and ethyl acetate extracts of *O. vulgare* subsp. *hirtum* were investigated in a few studies [31-35]. The main phenolic contents of *O. vulgare* subsp. *hirtum* were determined as quercetin, kaemferol [31], rosmarinic acid [32-34], apigenin 7-O-beta-D-glucoside [32], caffeic acid [34], apigenin, and luteolin [35]. Total phenolic content of methanol and water extracts of *O. vulgare* subsp. *vulgare* were reported as well [21, 24]. And, two known phenolics (luteolin 7-O-beta-D-xyloside and luteolin 7-O-beta-D-glucuronide) were isolated from *O. vulgare* subsp. *vulgare n*-butanol extract [36]. To our knowledge, no report is available for the phenolic profile of *O. vulgare* subsp. *vulgare* in the literature.

Phenolic compounds, the amount of which may differ according to the plants, are the most abundant secondary metabolites in plants. Plants produce phenolic compounds for protection under intense stress. Phenolic compounds are also called "bioflavonoids" because of their positive effects in terms of nutritional physiology. Also, phenolic compounds contribute to various health benefits of plant extracts [37-41].

The aim of this study is to investigate the phenolic profile of *O. vulgare* subsp. *hirtum* and *O. vulgare* subsp. *vulgare* by LC-MS/MS. The full method validation and uncertainty evaluation of the developed method are also discussed herein.

2. Experimental

2.1. Plant Material

O. vulgare subsp. *hirtum* collected from Muğla, (Köyceğiz, between Beyobası and Çayhisar villages, roadside) in July 2016, and *O. vulgare* subsp. v*ulgare* collected from Artvin (Borçka to Hopa, 17th km roadside, slopes) in August 2013. The species were identified by Prof. Dr. Tuncay Dirmenci at Bal,kesir University. Voucher specimens were deposited at the Herbarium of Faculty of Education, Balikesir University, Balikesir, Turkey as TD 4591 and TD 3937, respectively.

2.2. Preparation of Extracts

100 g of aerial parts of air-dried species were extracted with methanol (1 L) for 15 days. After filtration and evaporation, it was coded as Methanol-1 (12.23 g). Furthermore, another 100 g aerial parts of the species were extracted successively by chloroform (1 L) then with acetone (1 L) and methanol (1 L) for 15 days. After filtration, the solvents were evaporated using a rotary evaporator to give chloroform (2.43 g), acetone (4.16 g), and methanol extracts (10.35 g) and they were coded as Chloroform, Acetone, and Methanol-2, respectively. All the extracts were kept at -20 °C until they were used for experimental studies.

2.3 Preparation of Test Solution for LC-MS/MS

Each extract weighed (fifty to seventy mg) in a round bottom flask and 3 mL of the ethanol-water mixture (50:50 v/v) was added. In order to obtain a good solubility, the flask was gently heated at 60 ℃ on an ultrasonic bath (15 min.) until a clear solution was obtained. Then, the solutions were then transferred into a 5 mL of volumetric flask, rinsed with a 200 µL ethanol-water mixture (50:50 v/v) for three times and diluted to volume with a mobile phase. A portion of this stock solution (1 mL) was transferred into 5 mL of another volumetric flask, and 30 µL of curcumin (500 mg/L in methanol) solution was added as internal standard and diluted to the volume with the mobile phase. The solution was filtered through a 0.45 µm Millipore Millex-HV filter and the final solution (1 mL) was transferred into a capped autosampler vial and 10 mL of sample was injected to LC for each run. The samples in the auto sampler were kept at 15 ℃ during the experiment [7,10].

2.3. LC-MS Conditions

Experiments were conducted by a Zivak® HPLC (High performance liquid chromatography) and Zivak® Tandem Gold Triple quadrupole (Istanbul, Turkey) mass spectrometry equipped with a C_{18} (150 x 3 mm; 3 µm) column (Fortis Technologies, UK) at TUBITAK UME. 2.40 mTorr CID gas pressure, 5000 V ESI needle voltage, 600 V ESI (electrospray ionization) shield voltage, 300 °C drying gas temperature, 50 °C API housing temperature, 55 psi Nebulizer gas pressure and 40 psi drying gas pressure were determined as optimum ESI parameters [40,42-44]. LC-MS/MS parameters of secondary metabolites and internal standard are given in Table S1 in supporting information.

2.4. Chemicals

Kaempferol (>90%, Sigma-Aldrich), kaempferol-3-*O*-glucoside (>97%, Sigma-Aldrich), penduletin (>97%, Phytolab), quercetin (≥95%, Sigma-Aldrich), luteolin (95%, Sigma-Aldrich), luteolin-7-*O*-rutinoside (>97%, Carbosynth limited), apigenin (>97%, TRC Canada), rutin (≥99 %, Sigma-Aldrich), caffeic acid (98%, Sigma-Aldrich), *trans*-ferulic acid (≥99%, Sigma-Aldrich), rosmarinic acid (>96%, Sigma-Aldrich), fumaric acid (≥99 %, Sigma-Aldrich), isosakuranetin (>97%, Phytolab), p-coumaric acid (>98%, Sigma-Aldrich), *p*-hydroxy benzoic acid (>97%, Sigma-Aldrich), gallic acid (97.5-102.5 %, Sigma-Aldrich), pyrogallol (>98%, Sigma-Aldrich), salicylic acid (>99%, Sigma-Aldrich), vanillin (>97%, Sigma-Aldrich), syringic acid (>95%, Sigma-Aldrich), salvigenin (95 %, in house isolated), luteolin-5-*O*-glucoside (96 %, in house isolated), curcumin (97 %, isolated from *Curcuma longa*). HPLC grade methanol and ethanol were purchased from Merck (Darmstadt, Germany). Stock solutions of standards were prepared as 200 mg/L in ethanol-water (50:50, v/v). Calibration solutions were prepared in ethanol-water (50:50, v/v) in a linear range (0.1, 0.2, 0.5, 1, 5 and 10 mg/L). Dilutions were performed using automatic pipette and glass volumetric flasks (A class), which were stored at 20 ℃ in glass containers. Curcumin solution was freshly prepared, from which 300 µL was used as an Internal Standard (IS) in all LC–MS/MS experiments.

The LODs were determined to be three times larger than while LOQs were determined to be ten times larger than standard deviation [45-48]. The validation procedure and the results of the validation are discussed in the following section.

3. Results and Discussion

3.1. Optimization of LC-MS/MS

Triple quadrupole mass spectrometry was used due to its fragmented ion stability [7, 10] the parent and daughter ions patterns and collision energy optimization studied carefully by the parameters used LC-MS/MS condition. The optimized parameters are given in Table 1. One of the most critical issues of the applied method is the dissolution of plant extracts in the appropriate solvent to get clear and repeatable separation of chromatographic peaks and ionization stability. Regarding previous reports of literature [7, 10] and our findings clearly showed that the best mobile phase solution was determined to be a gradient of acidified methanol and water in ESI source for those compounds.

3.2. Method Validation

Validation of the applied method was performed using analytical standards of corresponding compounds (see section 2.4) with using the target ions (Table S1) and curcumin was used as an internal standard.

3.2.1. Linearity

Calibration curves were obtained from standard calibration solutions. The linearity of the method was assayed by analyzing the calculation of a six-point linear plot in the range of 0.1 mg/L to 10 mg/L with six replicates. The linear regression equation and the squared correlation coefficient were determined and are given in Table 1.

Table 1. Method validation parameters of LC-MS/MS for the used phenolic standards

No	Compounds	Linear regression equation	R^2	LOD (mg/L)	LOQ (mg/L)	Recovery %	RSD %
1	Kaempferol	y=0.0230x+0.0116	0.984	0.002	0.008	101.0	5.47
2	Kaempferol-3-*O*-rutinoside	y=0.1080x+0.0135	0.997	0.014	0.045	95.0	8.15
3	Salvigenin	y=0.0355x+0.8620	0.991	0.036	0.119	97.4	5.21
4	Penduletin	y=0.1630x+0.0262	0.996	0.089	0.297	100.8	9.47
5	Quercetin	y=0.1150x+0.0078	0.993	0.001	0.002	99.0	0.11
6	Luteolin	y=0.2120x+0.0699	0.993	0.062	0.207	98.9	0.16
7	Luteolin-7-*O*-glucoside	y=0.1350x+0.0246	0.995	0.022	0.072	106.3	8.56
8	Luteolin-5-*O*-glucoside	y=0.2300x+0.0413	0.992	0.01	0.034	98.2	1.12
9	Apigenin	y=0.1780x+0.0850	0.996	0.15	0.501	99.6	4.01
10	Rutin	y=0.0232x+0.0008	0.996	0.01	0.034	98.4	7.9
11	Isoquercetin	y=0.0115x+0.0215	0.995	0.199	0.665	106.4	9.42
12	*p*-Coumaric acid	y=0.2670x+0.1810	0.977	0.006	0.021	101.2	6.39
13	Caffeic acid	y=0.3300x+0.0036	0.992	0.028	0.093	102.3	8.04
14	*trans*-Ferulic acid	y=0.0655x+0.0266	0.992	0.047	0.158	100.3	5.21
15	Chlorogenic acid	y=0.2620x+0.0674	0.998	0.445	1.483	99.9	5.45
16	Rosmarinic acid	y=0.1960x+0.0043	0.998	0.022	0.072	97.3	3.73
17	Fumaric acid	y=0.0569x+0.0177	0.991	0.003	0.01	97.3	5.44
18	*p*-Hydroxy benzoic acid	y=0.1230x+0.0280	0.993	0.002	0.007	99.7	4.78
19	Gallic acid	y=0.0569x+0.0177	0.991	0.002	0.008	100.4	4.85
20	Pyrogallol	y=0.0438x+0.0073	0.980	0.001	0.002	101.5	5.47
21	Salicylic acid	y=0.0255x+0.1780	0.970	0.211	0.704	101.3	0.21
22	Vanillin	y=0.0982x+0.0158	0.998	0.019	0.064	99.6	6.57
23	Syringic acid	y=0.0305x+0.0079	0.997	0.022	0.073	99.9	8.39

3.2.2. LOD and LOQ

The limit of detection (LODs) of the method for each compound was determined according to the following equation: LOD or LOQ = κSDa/b, where 3 for LOQ and κ= 3 for LOD, SDa represents the standard deviation of the intercept, and b represents the slope (Table 1).

3.2.3. Recovery, Repeatability, and Intermediate Precision

Before the evaluation recovery, repeatability, and intermediate precision of the method used and screening measurements were done on Methanol-1 and Chloroform extracts. Then, according to the detected levels of compounds in the extracts, we spiked the extracts to reach the final concentrations as 0.1 mg/L and 0.5 mg/L and 1 mg/L in 5 mL volumetric flasks. Unspiked plant extracts were also analyzed to determine the target compounds concentrations in the blank sample. The recovery of each component at each fortification levels was calculated according to the following formula.

$$Recovery\ \% = \frac{Measured\ conncentration - endogeneous\ concentration}{spiked\ concentration} x100 \quad (1)$$

The recoveries of measured components were ranged from 95.0 to 106.4 % (Table 1). Mean relative standard deviations (RSD) were found to be 0.1-10 % for all and those data was added to the uncertainty budget of all reported compounds.

The repeatability of the developed method was assessed at three concentration levels of the recovery studies. For the intermediate (reproducibility) precision, a set of spiked samples having three concentration levels were analyzed twice a week for a period of 3 weeks and repeatability of method evaluated.

3.3.Estimation of Uncertainty

3.3.1. Identification of Uncertainty Sources

The bottom-up approach was applied to obtain the measurement of the uncertainty value for the developed method. The sources for uncertainty were determined as were weighing the sample, calibration graph, and repeatability. Detailed evaluation procedure corresponding equations are given in our previous papers [45-47] and to avoid repetition, we summarized the calculation of combined standard measurement uncertainty of target compounds in plant extracts in equation 2. The expanded measurement uncertainty was obtained by multiplying the combined standard measurement uncertainty value with 2 (coverage factor) at 95% confidence level. The uncertainty value of measurement results are given Table 1.

$$\frac{u_c\,(A)}{C_A} = \sqrt{(\frac{u\,(W_{SS})}{W_{ss}})^2 + (\frac{u\,(C_0)}{C_0})^2 + u(R_m)^2 + u(r)^2} \quad (2)$$

where,
$u_c(A)$: Combined standard measurement uncertainty of the analyte
C_A: Concentration of the target analyte
$u(W_{SS})$: Combined standard measurement uncertainty of the sample intake
W_{SS}: Weight of the starting sample
$u(c_0)$: Combined standard measurement uncertainty of the calibration curve
c_0: Determined concentration of the sample by using the calibration curve
$u(R_m)$: Combined standard measurement uncertainty of recovery
$u(r)$: Standard measurement uncertainty of repeatability

3.4. Secondary Metabolites of O. vulgare subsp. *hirtum and O. vulgare* subsp. *vulgare*

There are a few studies reporting the phenolic content of various extracts of *O. vulgare* subsp. *hirtum.* The presence of quercetin, kaempferol, and apigenin was reported in *O. vulgare* subsp. *hirtum* methanol, water and ethanol extracts [31]. Furthermore, *O. vulgare* subsp. *hirtum* considered as a good source of rosmarinic acid [32,33]. Moreover, the total phenolic content of methanol extract of *O. vulgare* subsp. *hirtum* were determined [14,49]. However, no study reported about the comprehensive phenolic composition of *O. vulgare* subsp. *vulgare*

Table 2. Secondary metabolites of *O. vulgare* subsp. *vulgare* by LC-MS/MS (mg/kg)

Compounds	**Extracts***			
	Methanol-1	**Chloroform**	**Acetone**	**Methanol-2**
Flavonoids				
Kaempferol	408.95±28.86	-	80.56±5.69	135.27±9.55
Kaempferol-3-*O*-Rutinoside	3.08±0.28	-	-	-
Salvigenin	1.28±0.09	-	-	-
Penduletin	116.5±11.81	-	-	-
Isoquercetin	2.13±0.61	-	-	-
Luteolin	163.14±41.91	-	18.75±4.82	50.11±12.87
Luteolin-7-*O*-Glucoside	74.27±7.56	-	4.07±0.41	30.61±3.12
Luteolin-5-*O*-Glucoside	103.75±6.68	-	6.48±0.42	41.44±2.67
Apigenin	-	-	-	46.29±3.73
Rutin	9.58±0.63	-	2.44±0.16	4.08±0.27
Phenolic acids				
p-Hydroxy benzoic acid	-	-	-	528.98±42.03
Caffeic acid	743.15±147.06	-	113.53±22.47	292.09±57.80
p-Coumaric acid	28.3±4.36	-	-	-
(E)-Ferulic acid	1256.36±87.79	-	36.53±2.55	753.37±52.64
Chlorogenic acid	132.35±18.33	-	7.85±1.09	44.24±6.13
Rosmarinic acid	7944.48±609.18	-	852.97±65.41	3235.73±248.11
Simple phenolics				
Pyrogallol	18.66±1.24	-	-	13.91±0.93
Vanillin	-	3.46±0.32	2.61±0.24	-
Dicarboxylic acid				
Fumaric acid	1425.35±98.85	-	6.00±0,42	362.74±25.16
Total identified metabolites (mg/kg dried herba)	**12431**	**3.46**	**1051**	**5538**

***Methanol-1:** direct methanol extract, **Chloroform:** chloroform extract, **Acetone:** acetone extract, **Methanol-2:** methanol extract; The uncertainty value was not considered for the total identified metabolites

In this study, the phenolic compounds of *O. vulgare* subsp. *vulgare* and *O. vulgare* subsp. *hirtum.* were examined under four groups: flavonoids, phenolic acids, simple phenolics, and the dicarboxylic acids. Rosmarinic acid was determined as the major compound of the species. While the mass fractions of rosmarinic acid were found to be 7944.48 ± 609.18, 852.97 ± 65.41 and 3235.73 ± 248.11 mg/kg in the Methanol-1, Acetone, and Methanol-2 extracts of *O.vulgare* subsp. *vulgare*, respectively (Table 2), it was determined as 78.26 ± 6.00, 175.13 ± 13.43 and 373.91 ± 28.67 mg/kg, in the extracts *O. vulgare* subsp. *hirtum*, respectively (Table 3). Chloroform extract of *O. vulgare* subsp. *hirtum* was identified as rich in flavonoids and penduletin was determined as the major compound (125.28±10.22 mg/kg). In addition, Methanol-1 extract of *O. vulgare* subsp. *hirtum* was identified as rich in caffeic acid (247.28±46.13 mg/kg) (Table 3). Methanol-2 extracts of both species were identified as the richest extracts for phenolics whereas chloroform extracts of both species were identified as the poorest one. Other contents of secondary metabolites and their concentrations are given in Table 2 and Table 3. The

LC-MS/MS chromatograms of phenolic compounds are given in Figure S1 and Figure S2 in the supplementary material.

The difference of the measured amount of substances in Methanol 1, Methanol 2 and sum of the successive solvent extracts, might be due to the acidity of chloroform and atmospheric conditions in acetone and methanol. We know that, "when plant extracts are stored in organic solvents for a long period of time at room temperature and/or under sunlight, there is a risk of artifact formation and decomposition/isomerization of the components. In conclusion, heat, light, air, and pH are the other factors that may lead to the degradation of natural compounds and differentiate their amount in plant extracts [49]". Thus, we recommend the use of the extractions directly, rather than successive solvent extraction for screening. This study is the most comprehensive data reported for the screening of twenty-three different secondary metabolites and nineteen secondary metabolites were identified in the extracts of *O. vulgare* subsp. *hirtum* and thirteen for *O. vulgare* subsp. *vulgare.*

This study is the most comprehensive data reported for the screening of twenty-three different secondary metabolites. Nineteen secondary metabolites were investigated for *O. vulgare* subsp. *hirtum* and thirteen for *O. vulgare* subsp. *vulgare.*

Table 3. Secondary metabolites of *O. vulgare* subsp. *hirtum* by LC-MS/MS (mg/kg)

Compounds	**Extracts***			
	Methanol-1	**Chloroform**	**Acetone**	**Methanol-2**
Flavonoids				
Kaempferol	25.02±1.51	-	-	17.69±1.30
Penduletin	38.32±4.23	125.28±10.22	-	59.21±5.22
Quercetin	84.18±5.32	83.21±5.31	178.58±5.14	110.08±7.02
Luteolin	-	-	6.97±0.35	-
Apigenin	30.56±0.88	-	10.39±0.92	-
Phenolic acids				
Gallic acid	-	-	-	6.65±0.36
Salicylic acid	-	-	84.25±3.22	-
Syringic acid	65.11±3.55	-	-	154.33±12.25
Caffeic acid	247.28±46.13	56.58±11.20	-	140.28±27.45
p-Coumaric acid	-	-	35.67±3.88	-
Rosmarinic acid	78.26±6.00	-	175.13±13.43	373.91±28.67
Simple phenolics				
Pyrogallol	-	-	25.36±2.08	-
Dicarboxylic acid				
Fumaric acid	39.64±2.97	35.35±2.45	79.31±5.50	12.24±0.74
Total identified metabolites (mg/kg dried herba)	**608**	**300**	**495**	**874**

***Methanol-1:** direct methanol extract, **Chloroform:** chloroform extract, **Acetone:** acetone extract, **Methanol-2:** methanol extract; The uncertainty value was not considered for the total identified metabolites

In conclusion, the phenolic composition of the chloroform, acetone, and methanol extracts of *O. vulgare* subsp. *hirtum* and *O. vulgare* subsp. *vulgare* were successfully analyzed by validated LC-MS/MS method. The method performances are reported for the first time herein. Rosmarinic acid, caffeic acid, and penduletin were determined as the major phenolic compounds of both species. Methanol extracts of both species are the richest extracts in terms of phenolic compounds. This study supported that *O. vulgare* subsp. *hirtum* and *O. vulgare* subsp. *vulgare* species could become a source of potentially bioactive compounds in pharmaceutical and food industries, and the validated method can be used for the screening of secondary metabolites of plant species for the standardization of extracts and dietary supplements.

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Supporting Information

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