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Determination of polyphenolic compounds by liquid chromatography–mass spectrometry in *Thymus* species

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ABSTRACT

Polyphenolic compounds represent a wide group of phytochemicals, including well-known subgroups of phenolic acids, flavonoids, natural dyes, lignans etc., which are produced by plants. These natural bioactive compounds possess a variety of beneficial effects including antioxidant and anticarcinogenic activities, protection against coronary diseases as well as antimicrobial properties. *Thymus* species have already been reported as sources of different phenolic acids and flavonoids. Moreover, the composition and content of flavonoids in *Thymus* species play important role as taxonomic markers providing distinction of species. High-performance liquid chromatography (HPLC) coupled with diode array detector (DAD) and on-line mass spectrometry (ESI-MS) method was used for analysis. The method was evaluated for a number of validation characteristics (repeatability and intermediate precision, LOD, LOQ, calibration range, and recovery). The polyphenolic pattern of five native Hungarian *Thymus* species (*T. glabrescens* Willd., *T. pannonicus* All., *T. praecox* Opiz, *T. pulegioides* L., and *T. serpyllum* L.) was characterized. The dominant compound was rosmarinic acid, which ranged between 83.49 $\mu g\,g^{-1}$ and 1.436 $mg\,g^{-1}$. Other phenolic acids (ferulic acid, caffeic acid and its other derivatives, chlorogenic acid and *p*-coumaric acids) were present in every examined *Thymus* species, as well as flavanones: naringenin, eriodictyol and dihydroquercetin; flavones: apigenin and apigenin-7-glucoside, flavonols: quercetin and rutin. The polyphenolic pattern was found to be a useful additional chemotaxonomic tool for classification purposes and determination of the locality of origin.

1. Introduction

Polyphenols are the group of substances, including well-known subgroups like phenolic acids, flavonoids, natural dyes, lignans etc., which are produced in the different part of the plants. Many health benefits have been associated with the group of flavonoids. Its pharmacological effects can be described by the following benefits [1,2]: effect on central nervous system (e.g. synthetic flavonoids possess anxiolytic properties similar to that of diazepam [3]), cardioprotective activity (prevention of atherosclerosis, antiarrhythmic and antihypertensive effect), lipid lowering activity by direct scavenging of radicals, gastrointestinal protection (antiulcer and hepatoprotective activity), antioxidant activity (via free radical scavenging), inhibition effects on expression of heat shock proteins, anti-inflammatory and analgesic activity, antineoplastic activity (via suppression of cancer cell growth), effect on blood vessels (via reduction of aggregation of erythrocytes or inhibition of aggregation of platelets), antimicrobial activity (antibacterial, antifungal and antiviral effects) and antiosteoporotic effects.

Common sources of polyphenols in human diet are fruits, vegetables, cereals, beverages (tea infusions, coffee, juice, and cider), wines and spices [4]. Shan et al. [5] considered spices and related families with the highest polyphenolic content (e.g. *Myrtaceae*, *Lauraceae*, *Lamiaceae*) as the potential sources of natural antioxidants for commercial exploitation. In several studies, different *Thymus* species have been reported as the sources of flavonoids [6–10]. Polyphenols in *Thymus* taxa possess physiological and chemical stability, and have wide structural variability. Several studies were oriented to use the unique composition of flavonoids in *Thymus* species as an important chemotaxonomic marker for the distinction of species [7,11]. For pharmacological purposes it is also important to screen, analyze and identify those substances.

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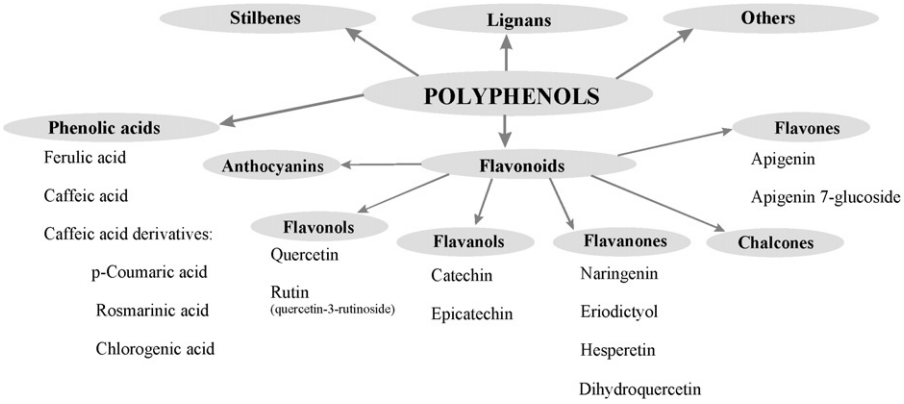

Fig. 1. Classification of the analyzed compounds within polyphenols.

The medicinal and non-medicinal uses of different *Thymus* species are well known [12]. In the genus of *Thymus*, thyme (*Thymus vulgaris* L.) is commonly used as a culinary herb. Its drug and essential oil are official in several pharmacopoeias (e.g. European Pharmacopoeia 6th ed, Hungarian Pharmacopoeia 8th ed.) and monographs (e.g. ESCOP, German Commission E) [13]. Documented pharmacological studies support some of the traditional medicinal uses, which have been principally attributed to the volatile oil and flavonoid constituents. Other component such as caffeic acid, rosmarinic acid, oleanolic acid, saponins, and tannins may play important role in the medicinal treatment of different diseases. The plant is reported to have a wide range of biological activities, such as carminative, antispasmodic, antitussive, expectorant, bactericidal, antihelmintic and adstringent. Traditionally, it has been used for dyspepsia, chronic gastritis and diseases of the upper respiratory tract [14].

Within the genus *Thymus*, the influence of the environmental conditions on the pattern of essential oil components and flavonoids has been investigated [15,16].

Among native species involved, *Thymus pannonicus* All., *T. glabrescens* Willd. and *T. pulegioides* L. were proven to be widely dispersed, adapting to different circumstances, while *T. praecox* Opiz and *T. serpyllum* L. were linked to special habitat conditions in Hungary. Essential oil diversity of the wild-growing populations was considerable, numerous new chemotypes (*T. pannonicus*: 10; *T. glabrescens*: 8, *T. pulegioides*: 6; *T. praecox*: 6, *T. serpyllum*: 2) have been discovered [17–21].

Abiotic environmental factors (temperature, moisture, soil and climatic conditions, elevation, etc.) as well as biotic effects (human disturbance, herbivores, etc.) were proven to influence both essential oil and polyphenol production of *Thymus* species and chemical composition of coenopopulations in the course of time [16,22,23]. It was established that phenolic essential oil compounds (thymol, cavacrol) and polyphenols (e.g. flavonoids) favored warmer and drier climatic zones, while other non-phenolic substances usually accumulate in higher quantities in cooler and more humid areas. Although different chemotypes favored certain abiotic conditions, sometimes the plants of a particular chemotype grow in habitats that are less advantageous to them [24]. In addition, different chemotypes of the same species can grow in the same habitat [25,26]. These concerns prove that chemotypes are only partially dependent on the environment and support a direct relation of essential oil and flavonoid patters with the genetic features of these plants [11,15].

Several studies, focused on flavonoid investigations in *Thymus*, confirmed the occurrence of phenolic acids (e.g. ferulic acid, caffeic acid and its derivatives *p*-coumaric, rosmarinic, chlorogenic acids), flavonols (e.g. quercetin, rutin), flavanols (e.g. catechin and epicatechin), flavanones (naringenin, eriodictyol, hesperetin and dihydroquercetin), and flavones (e.g. apigenin and its glucosides, luteolin and its glucosides) [8,9,11,27].

For the polyphenolic compounds, the applicability of several effective analytical techniques has been published. Recently the following methods have been used: capillary electrophoresis (CE) [28–30], high-performance liquid chromatography (HPLC) coupled with negative atmospheric pressure chemical ionization mass spectrometry (APCI-MS) [31] or electrospray mass spectrometry (ESI-MS) [31,32], HPLC with UV detection [33–35], diode array detector [8,36–38], fluorescence detector [39], and HPTLC-densitometric technique [34,35]. Reversed-phase liquid chromatography (RPLC) coupled with electrospray mass spectrometry detection remains as the most powerful analytical method for the analysis of polyphenols, however in routine practice MS is usually replaced by more affordable UV or diode array detector [37]. Besides the speciation analysis of phenolic compounds, the scientific interest is also focused on the determination of antioxidant properties (e.g. antioxidant capacity, determination of total phenol, flavonoid and anthocyanin content) of herbs including *Lamiaceae* family [38,40,41].

The aim of the current research project has been the separation and quantification of selected polyphenols (chlorogenic acid, caffeic acid, *p*-coumaric acid, dihydroquercetin, ferulic acid, rosmarinic acid, apigenin, apigenin-7-glucoside, eriodictyol, naringenin, quercetin, hesperetin, rutin catechin and epicatechin, see Fig. 1) in extracts prepared from samples of different, approximately 5-year-old Hungarian *Thymus* taxa grown in the Experimental Station of the Corvinus University of Budapest. Three samples were obtained from the University of Medicine and Pharmacy of Târgu Mureş, Romania.

The determination of the phenolic acid and flavonoid composition of the different *Thymus* taxa was performed by HPLC–ESI-MS technique. A novel method was developed for rapid determination of fifteen components in herbal extracts, which were prepared by a simple extraction procedure. The novelty of the method is mainly in its application to a wide group of polyphenolic compounds in herbal extracts of *Thymus*. In most of the studies, usually only a few polyphenolic compounds are determined in contrast to the fifteen compounds determined here.

The efficiency of the method was characterized by a rapid evaluation of a number of validation parameters. The results of this study provide a basis for breeding programs directed at the improvement of polyphenolic composition and give valuable information for researchers of chemotaxonomy.

2. Materials and methods

2.1. Chemicals

The fifteen selected phenolic compounds, standards (chlorogenic acid, caffeic acid, *p*-coumaric acid, dihydroquercetin, ferulic acid, rosmarinic acid, apigenin, apigenin-7-glucoside, eriodictyol, naringenin, quercetin, hesperetin, rutin, catechin and epicatechin) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetic acid (eluent additive for LC–MS) and water (LC–MS Chromasolv) were obtained from Fluka (Buchs, Switzerland). Methanol (LC–MS Chromasolv) was purchased from Riedel-de Haën GmbH & Co. (Seelze, Germany).

2.2. Plant material sampling

Thymus taxa (*Thymus pannonicus*, *T. glabrescens*, *T. pulegioides*, *T. praecox*, *T. serpyllum*) originating from eleven habitats (summarized in Table 1) were grown and sampled among equal environmental conditions in the Medicinal Plant Research Station at Soroksár, Corvinus University of Budapest, Hungary. Flowering shoots were collected from the Station (Soroksár) in June 2009. Plant samples were dried naturally and voucher specimens were deposited in the Herbarium of the Department of Medicinal and Aromatic Plants, Corvinus University of Budapest.

The sample codes in Table 1 were given according to the original habitats and the number in the label indicates different populations living separately in the same location (sometimes among slightly different circumstances). Seeds were collected from these populations separately and they were grown separately in Soroksár, too. These numbers can be evaluated as replicates of the same origin.

Three samples Thgla, Thpu and Thserp (*T. glabrescens* Willd., *T. pulegioides* L., *T. serpyllum* L.) were obtained from the University Botanical Garden of Medicinal Plants, University of Medicine and Pharmacy of Târgu Mureş, Romania.

2.3. LC–MS system

The qualitative and quantitative analyses of polyphenolic compounds were performed by LC–MS. The LC–MS system consisted of a liquid chromatograph (Prominence Liquid Chromatograph LC-20 AD, Shimadzu), a micro vacuum degasser (Prominence Degasser LC-20 A3, Shimadzu), an auto sampler (Prominence Auto Sampler SIL-20 ACHT, Shimadzu), a diode array detector (Prominence Diode Array Detector system SPD-M20A, Shimadzu), a column oven (Prominence Column Oven CTO-20 AC, Shimadzu), a controller (Prominence Controller CBM-20 A, Shimadzu), and an MS detector with electrospray ion source and quadrupole analyzer (Liquid Chromatograph Mass Spectrometer LCMS-2020, Shimadzu). The LabSsolutions (Shimadzu) software was used to control the LC–MS system and for data processing.

Table 2
Characteristic ions used for the ESI-MS detection of the selected polyphenolic compounds.

Polyphenolic compounds	Retention time (min)	Mass of $[M-H]^-$ ion (m/z)
Catechin	2.253	289
Caffeic acid	3.155	179
Chlorogenic acid	3.063	353
Epicatechin	4.849	289
p-Coumaric acid	5.243	163
Dihydroquercetin	6.651	303
Ferulic acid	7.290	193
Rutin	10.381	609
Apigenin-7-glucoside	11.163	431
Rosmarinic acid	11.209	359
Eriodictyol	11.258	287
Quercetin	12.697	301
Naringenin	13.071	271
Hesperetin	14.720	301
Apigenin	15.821	269

2.4. LC–MS method

Chromatographic separations were performed on an Ascentis Express C18 column (50 mm × 2.1 mm, 2.7 μm, Supelco, USA). For the separations, a gradient of mobile phase A (2% (v/v) acetic acid in water) and mobile phase B (2% (v/v) acetic acid in methanol) was used. The gradient profile was set as follows: 0.00 min 3% B eluent, 7.00 min 20% B eluent, 7.10 min 30% B eluent, 17.00 min 40% B eluent, 25.00 min 100% B eluent, 25.10 min 3% B eluent and 30.00 min 3% B eluent. The flow rate was 0.2 mL min^{-1}, the column temperature was 50 °C. The injection volume was 2 μL for *Thymus* extracts and for standard mixtures. The column effluent passed through a diode array detector before arriving in the MS interface. UV detection wavelengths were 280 nm, 320 nm, and 360 nm. The electrospray source of the MS was operated in negative mode and the interface conditions were as follows: capillary voltage of −3.5 kV, CDL voltage of 0.0 V, CDL temperature of 250 °C and deflector voltage of 0.0 V. The nebulizing gas flow rate was 1.5 L min^{-1}, the drying gas flow rate was 3.0 L min^{-1} and was obtained from a nitrogen gen-

Table 1
The origins and the labels of the *Thymus* samples.

Samples	Region and habitat location	Year of planting (Soroksár)
Thymus glabrescens Willd.		
P2/2, P2/3	Bakony Hills (Csesznek, Castle Hill)	2005
P3/2, P3/3	Balaton Uplands (Szentbékkálla, Kő Hill)	2005
Thgla	The University Botanical Garden of Medicinal Plants, Târgu Mureş, Romania	–
Thymus pannonicus All.		
C1, C2, C4, C6	Gödöllő Hills (Ceglédbercel, Loess Hills)	2005
TS2, TS3, TS4	Buda Hills (Vörös-kővár)	2006
Ada	Backa Region (Ada)	2005
Thymus praecox Opiz		
P2	Buda Hills (Nagy-Szénás Hill)	2003
T1, T3	Buda Hills (Tétény-plateau)	2003
Thymus pulegioides L.		
B2, B3, B4	Börzsöny Hills (St. Stephan Hill)	2003
R	Zemplén Hills (Regéc)	2007
Thpu	The University Botanical Garden of Medicinal Plants, *Târgu Mureş* Romania	–
Pogány	Transylvania (Pogány)	2005
Thymus serpyllum L.		
Fenyőfő	Bakony Hills (Fenyőfő)	2005
Thserp	The University Botanical Garden of Medicinal Plants, Târgu Mureş, Romania	–

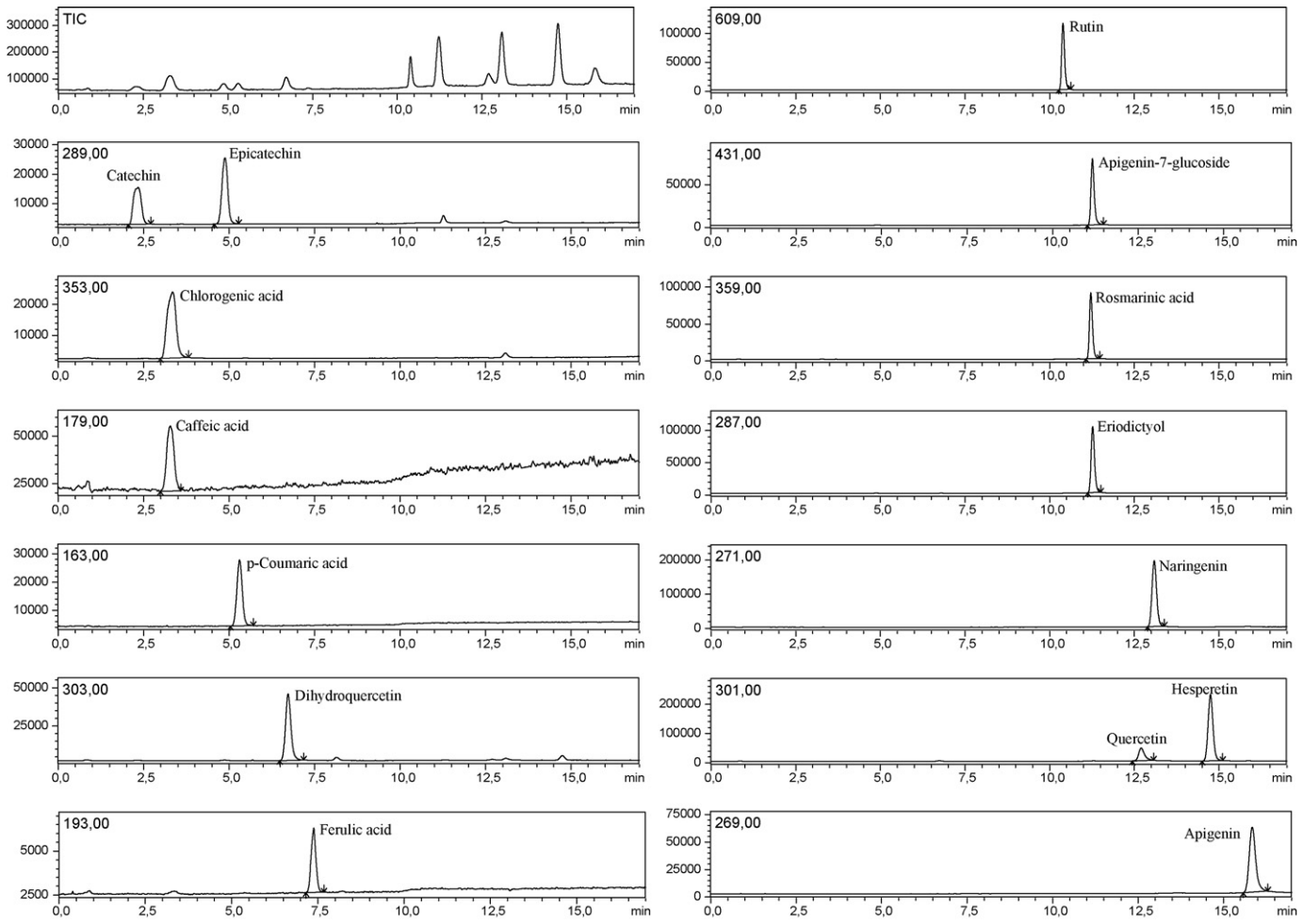

Fig. 2. LC–MS chromatograms of standards for the 15 phenolic compounds obtained in SIM mode and the total ion chromatogram (TIC).

erator. The detector voltage was 0.95 kV. Under these conditions, as illustrated in Fig. 2, no baseline separation was observed for all fifteen phenolic compounds in the total ion chromatogram. The ions chosen for each compound and their retention times are shown in Table 2.

2.5. Procedures

2.5.1. Standard solutions

For each phenolic compound, a stock solution of 500 ppm was prepared by dissolving 5 mg of product in 10 mL of pure methanol. To ensure the complete dissolution, ultrasonication was applied

Table 3
Intraday precision of the retention time of the polyphenolic compounds in the HPLC–MS analysis of a standard solution (concentration of each compound was 334 ng mL^{-1}) and intraday precision of the concentration of the polyphenolic compounds in the HPLC–MS analysis of a *Thymus* extract sample (B3).

Polyphenolic compounds	Standard solution		*Thymus* extract (B3)	
	Retention time		Concentration	
	min[a]	RSD %	ng mL^{-1} [a]	RSD %
Catechin	2.253	0.44	<QL	–
Caffeic acid	3.155	0.47	5975.75	1.88
Chlorogenic acid	3.063	0.90	17,671.64	1.54
Epicatechin	4.849	0.13	26.76	3.99
p-Coumaric acid	5.243	0.62	665.31	3.77
Dihydroquercetin	6.651	0.24	1502.95	2.75
Ferulic acid	7.290	0.36	1261.26	4.84
Rutin	10.381	0.11	2838.84	1.91
Apigenin-7-glucoside	11.163	0.09	13.25	0.77
Rosmarinic acid	11.209	0.08	1,181,145.03	0.74
Eriodictyol	11.258	0.10	250.60	8.25
Quercetin	12.697	0.22	341.07	1.52
Naringenin	13.071	0.22	544.56	4.64
Hesperetin	14.720	0.26	22.65	1.53
Apigenin	15.821	0.22	3397.64	4.03

[a] Data are mean values from six determinations.

Table 4
Interday precision of the retention time of the polyphenolic compounds in the HPLC–MS analysis of a standard solution (concentration of each compound was 334 ng mL^{-1}) and interday precision of the concentration of the polyphenolic compounds in the HPLC–MS analysis of a *Thymus* extract sample (B3).

Polyphenolic compounds	Standard solution					*Thymus* extract (B3)				
	Retention time					Concentration				
	Day 1[a] (min)	Day 2[a] (min)	Day 3[a] (min)	Mean (min)	RSD %	Day 1[a] (ng mL^{-1})	Day 2[a] (ng mL^{-1})	Day 3[a] (ng mL^{-1})	Mean (ng mL^{-1})	RSD %
Catechin	2.253	2.302	2.274	2.276	1.08	<QL	<QL	<QL	<QL	–
Caffeic acid	3.155	3.176	3.159	3.163	0.35	5975.75	5867.01	6102.18	5981.65	1.97
Chlorogenic acid	3.063	3.102	3.068	3.078	0.69	17,671.64	18,020.32	17,452.4	17,714.79	1.62
Epicatechin	4.849	4.865	4.789	4.834	0.83	26.76	27.87	25.45	26.69	4.54
p-Coumaric acid	5.243	5.199	5.222	5.221	0.42	665.31	696.83	650.24	670.79	3.54
Dihydroquercetin	6.651	6.679	6.664	6.665	0.21	1502.95	1460.76	1478.42	1480.71	1.43
Ferulic acid	7.290	7.192	7.315	7.266	0.89	1261.26	1302.50	1291.40	1285.05	1.66
Rutin	10.381	10.386	10.372	10.380	0.07	2838.84	2805.39	2905.26	2849.83	1.78
Apigenin-7-glucoside	11.163	11.171	11.186	11.173	0.10	13.25	14.44	12.71	13.47	6.57
Rosmarinic acid	11.209	11.215	11.235	11.220	0.12	1,181,145.0	118,278.1	117,157.6	117,860.2	0.52
Eriodictyol	11.258	11.275	11.264	11.266	0.08	250.60	229.91	236.19	238.90	4.44
Quercetin	12.697	12.703	12.668	12.689	0.15	341.07	334.99	345.00	340.35	1.48
Naringenin	13.071	13.105	13.126	13.101	0.21	544.56	516.99	530.48	530.68	2.60
Hesperetin	14.720	14.718	14.745	14.728	0.10	22.65	23.13	22.30	22.69	1.84
Apigenin	15.821	15.832	15.819	15.824	0.04	3397.64	3242.93	3279.52	3306.70	2.45

[a] Data are mean values from six determinations.

for 10 min. Samples used for LC–MS analyses were prepared by dilution of the stock solutions with 2% (v/v) acetic acid in water: methanol = 4:6 (v/v). All the stock solutions were stored in amber flask at 4 °C, and under these conditions no degradation was observed for at least two weeks.

2.5.2. Sample preparation

Air-dried *Thymus* samples were pulverized, and the herbal powder was weighted in amounts of 200 mg to eppendorf tubes. Extraction was ensured with ultrasonication after addition of 1.5 mL solvent mixture (methanol: water = 6:4 (v/v)) to each sample. Purification of extracts was done by centrifugation and filtration through a 0.45 μm pore size Syringeless filter (Mini-Uniprep, Whatman). Samples were stored in the dark at 4 °C until the LC–MS analysis was carried out.

3. Results and discussion

3.1. Rapid method validation

To assert that the LC–MS method had performances compatible with those required for routine analysis of polyphenolic compounds from *Thymus* samples, a rapid validation of this method (without ruggedness study) was carried out.

3.1.1. Selectivity

For the fifteen compounds, the minimum resolution was observed between the peaks of caffeic acid and chlorogenic acid; rosmarinic acid, eriodictyol and apigenin-7-glucoside. In the field of the analysis of polyphenolic compounds, the MS detection is generally preferred because of its inherent high selectivity, especially in single ion monitoring mode (SIM). Fig. 2 shows fifteen MS-SIM chromatograms of the studied phenolic compounds besides the total ion chromatogram (TIC).

3.1.2. Repeatability and intermediate precision

A reversed phase Ascentis Express C18 column – a high-speed HPLC column – packed with fused core particles was used for the separations. The fused core particle consists of a thin porous shell (0.5 μm) of high-purity silica and a solid silica core (1.7 μm diameter). The retention time of phenolic compounds was observed to be between 2.25 min and 16.2 min, respectively. The total time of analysis was less than 30 min.

Repeatabilities (intra- and interday precision) of the method were evaluated by assaying six replicate injections of a standard solution (334 ng mL^{-1} for each polyphenolic compound), and a *Thymus* (B3) extract sample. The responses measured on each chromatogram were the retention time of each peak of polyphenolic compounds and the corresponding area of the peak of the $[M-H]^-$ ion. Very small variation of the retention times was observed; the RSD values for the intraday precision ranged from 0.08% to 0.9%, which could allow an easy identification of the compounds. The RSD values for the area of the $[M-H]^-$ ion peaks ranged from 0.5% to 9.8% depending on the compound, with a pooled RSD of 5%. The RSD values that indicate the interday precision ranged from 0.52% to 6.57% for the concentration of polyphenolic compounds. The mean values and standard deviations of the retention times, and the concentrations of phenolic compounds (from the standard solution of 334 ng mL^{-1} and a *Thymus* extract sample – B3) are listed in Tables 3 and 4. The standard deviations proved the precision and the repeatability of the retention time to be rather good.

Table 5
LOD and LOQ values of the HPLC–MS method for analysis of the polyphenolic standards.

Polyphenolic compounds	LOD [ng mL^{-1}]	LOQ [ng mL^{-1}]
Catechin	16.70	55.61
Caffeic acid	66.79	222.40
Chlorogenic acid	5.02	16.70
Epicatechin	5.02	16.70
p-Coumaric acid	16.70	55.61
Dihydroquercetin	2.51	8.35
Ferulic acid	11.12	33.40
Rutin	1.25	4.18
Apigenin-7-glucoside	1.25	4.18
Rosmarinic acid	1.25	4.18
Eriodictyol	1.25	4.18
Quercetin	1.25	4.18
Naringenin	0.84	2.77
Hesperetin	1.25	4.18
Apigenin	1.25	4.18

Table 6
Content of polyphenolic compounds on dry-weight basis in the *Thymus* species.

Thymus species	Sample name	Catechin [$\mu g\,g^{-1}$]	Caffeic acid [$\mu g\,g^{-1}$]	Chlorogenic acid [$\mu g\,g^{-1}$]	Epicatechin [$ng\,g^{-1}$]	*p*-Coumaric acid [$\mu g\,g^{-1}$]	Dihydroquercetin [$\mu g\,g^{-1}$]	Ferulic acid [$\mu g\,g^{-1}$]	Rutin [$\mu g\,g^{-1}$]	Apigenin-7-glucoside [$\mu g\,g^{-1}$]	Rosmarinic acid [$\mu g\,g^{-1}$]	Eriodictyol [$\mu g\,g^{-1}$]	Quercetin [$\mu g\,g^{-1}$]	Naringenin [$\mu g\,g^{-1}$]	Hesperetin [$ng\,g^{-1}$]	Apigenin [$\mu g\,mg^{-1}$]
Thymus glabrescens Willd.	P2/2	1.26	49.07	2.28	115.05	1.15	39.87	4.18	3.18	0.15	1078.01	6.45	1.88	9.57	ND	26.06
Thymus glabrescens Willd.	P2/3	5.55	51.59	15.21	55.92	0.70	4.97	5.89	2.38	0.19	1062.76	1.26	0.46	4.18	ND	9.50
Thymus glabrescens Willd.	P3/2	1.52	51.63	310.57	ND	12.57	11.20	8.43	21.99	23.41	1131.30	1.87	0.95	2.79	ND	30.18
Thymus glabrescens Willd.	P3/3	6.37	59.44	11.72	171.67	1.21	19.97	4.03	0.63	ND	1075.62	5.01	1.75	13.44	ND	64.83
Thymus glabrescens Willd.	Thgla	3.60	100.60	4.12	192.63	1.23	41.96	3.58	2.15	28.25	1436.36	16.08	0.47	9.96	ND	69.17
Thymus pannonicus All.	C1	ND	48.76	26.32	37.20	0.84	35.91	4.40	ND	ND	1273.59	12.61	1.78	20.56	ND	35.96
Thymus pannonicus All.	C2	ND	52.03	107.97	53.25	0.74	21.62	4.87	5.66	ND	1118.33	6.20	2.05	7.88	ND	33.24
Thymus pannonicus All.	C4	1.75	43.10	207.51	60.49	0.81	30.35	5.15	31.15	0.11	1170.48	9.62	2.03	23.60	ND	58.79
Thymus pannonicus All.	C6	7.76	45.94	73.89	104.00	1.95	28.83	4.27	13.77	0.12	1090.89	11.59	1.95	15.26	ND	54.23
Thymus pannonicus All.	TS2	2.56	50.78	38.04	24.13	1.83	6.49	11.04	67.58	ND	948.51	1.54	1.19	4.59	ND	44.86
Thymus pannonicus All.	TS3	3.79	50.41	14.12	57.13	1.07	48.06	3.42	1.80	ND	1060.57	14.53	2.67	16.24	ND	65.56
Thymus pannonicus All.	TS4	5.71	52.63	206.18	ND	0.77	40.93	2.70	29.87	ND	1050.52	13.71	3.36	18.37	ND	120.69

Table 6 (*Continued*)

Thymus species	Sample name	Catechin [μg g^{-1}]	Caffeic acid [μg g^{-1}]	Chlorogenic acid [μg g^{-1}]	Epicatechin [ng g^{-1}]	*p*-Coumaric acid [μg g^{-1}]	Dihydroquercetin [μg g^{-1}]	Ferulic acid [μg g^{-1}]	Rutin [μg g^{-1}]	Apigenin-7-glucoside [μg g^{-1}]	Rosmarinic acid [μg g^{-1}]	Eriodictyol [μg g^{-1}]	Quercetin [μg g^{-1}]	Naringenin [μg g^{-1}]	Hesperetin [ng g^{-1}]	Apigenin [μg mg^{-1}]
Thymus pannonicus All.	Ada	1.08	41.41	22.17	ND	0.60	28.51	3.89	3.63	ND	1048.95	6.17	1.40	8.54	ND	45.57
Thymus praecox Opiz	P2	1.84	35.33	2.56	5249.81	3.07	2.67	11.08	8.77	0.26	948.81	0.76	1.26	0.96	24.7	12.63
Thymus praecox Opiz	T1	ND	43.95	224.11	ND	3.56	3.09	7.09	171.00	5.50	83.49	1.14	4.45	3.63	ND	11.78
Thymus praecox Opiz	T3	2.99	50.78	38.04	24.13	1.83	6.49	11.04	67.58	0.04	948.51	1.54	9.61	7.56	210.2	28.38
Thymus pulegioides L.	B2	ND	54.21	250.28	240.81	2.49	3.67	12.50	67.73	ND	948.26	0.73	3.46	2.76	46.0	8.34
Thymus pulegioides L.	B3	ND	44.82	132.54	200.73	4.99	11.27	9.46	21.29	0.10	886.09	1.88	2.56	4.08	169.9	25.48
Thymus pulegioides L.	B4	ND	63.97	60.29	63.83	2.00	13.28	4.75	7.64	ND	1042.77	3.01	1.29	24.64	ND	20.42
Thymus pulegioides L.	R	1.99	59.95	45.97	0.31	0.76	15.60	3.56	20.01	0.19	1148.74	5.54	2.59	9.53	ND	104.54
Thymus pulegioides L.	Thpu	2.66	105.28	7.39	ND	1.48	93.73	3.66	2.14	4.94	1412.81	30.82	5.32	17.46	ND	121.44
Thymus pulegioides L.	Pogány	ND	61.43	678.08	ND	2.04	2.46	6.32	28.67	0.84	1010.68	0.68	0.73	4.03	ND	9.04
Thymus serpyllum L.	Fenyőfő	0.06	61.08	26.98	132.26	0.94	2.70	2.10	6.70	ND	1121.31	0.48	1.29	3.08	92.2	30.42
Thymus serpyllum L.	Thserp	2.18	109.59	13.03	ND	1.60	88.57	3.64	3.84	3.90	1372.76	31.13	6.58	13.48	ND	124.44

Footnote: ND – not detected.

3.1.3. Determination of LOD, LOQ, and calibration range

The limit of detection (LOD) was determined experimentally, and was taken as the concentration that produced a detector signal that could be clearly distinguished from the baseline (larger than three times the baseline noise). The limit of quantitation (LOQ) was taken as the concentration that produced a detector signal 10 times larger than the baseline noise. A calibration curve was prepared for each polyphenolic compound by measuring six different concentrations of the standard solution in the concentration range of 16.7–16,700 ng mL^{-1}. All injections were repeated three times ($n = 3$). The calibration ranges adequately covered the variations in the amounts of polyphenols in the samples. The correlation coefficients ($r2$) ranged from 0.9994 to 1.0000. The LOD and LOQ values are summarized in Table 5.

3.1.4. Recovery

The recovery of the method was determined by the standard addition method applied on a selected *Thymus* extract sample (B3). In spiked samples ($n = 3$), the concentrations of the phenolic compounds were increased by 50%, 100%, and 150%. These spiked *Thymus* extract samples were analyzed ($n = 3$) and the amount of analyte recovered was calculated. The recoveries for the polyphenols were between 93.5% and 101.2%, (mainly between 95% and 98%).

3.2. Evaluation of polyphenolic pattern in the samples

The content of polyphenolic compounds on dry-weight basis in *Thymus* extract is summarized in Table 6. The dominant compound in all examined species was rosmarinic acid, which ranged from 83.49 μg g^{-1} to 1.436 mg g^{-1}. These results correspond to the investigations of Clifford [42] and Kulišić et al. [38]. After the quantitative analysis, we have established that the occurrence of rosmarinic acid was general, with rather low variability within and among species (approx. 0.9–1.2 mg g^{-1}). On the basis of the rosmarinic acid concentration, the species cannot be distinguished, but the locality can be determined. The amount of chlorogenic acid has changed in a wide range (approx. 2.3–310 μg g^{-1}), where the sample of *T. serpyllum* accumulated the lowest quantity. As far as the phenolic acids are concerned, caffeic acid was the less variable compound (approx. 35–64 μg g^{-1}), however in samples from Romania, *Thymus* species produced outstanding values (100–110 μg g^{-1}).

Other phenolic acids (ferulic acid and *p*-coumaric acid) were present in all examined *Thymus* species, as well as flavanones naringenin, eriodictyol and dihydroquercetin. Hesperetin was found only in few samples of *T. praecox* Opiz, *T. pulegioides* L., and *T. serpyllum* L. From the group of flavones, both apigenin and apigenin-7-glucoside were present in all cases. Flavonols, quercetin and rutin were also found in all species, but quercetin was present only in low levels in comparison with rutin. The highest concentration of rutin was found in the sample T1 (*T. praecox* Opiz). Vila [11] published that quercetin was found only in *T. vulgaris*, but our results showed that quercetin was present in all samples of the examined *Thymus* species in the range between 0.4648 μg g^{-1} and 9.606 μg g^{-1}.

Considering the quantity of polyphenols according to the locality of growth, we found a similarity between the samples originating from Romania. The highest contents of some polyphenols (caffeic acid, apigenin-7-glucoside, rosmarinic acid and eriodictyol) were present always in the Romanian samples (Thgla, Thpu, and Thserp) within these species (*T. glabrescens* Willd., *T. pulegioides* L., and *T. serpyllum* L.). Thpu and Thserp had the highest levels of catechin, and quercetin, but they had the lowest epicatechin, rutin, and chlorogenic acid concentrations within the samples of the same species, as well.

The quantity of polyphenols differed within one species, but the most significant differences were noticed between samples coming from the remote localities of origin. The flavanone content (naringenin, eriodictyol and dihydroquercetin) of Thpu and Thserp grown at Târgu Mureș differed significantly from the samples of the same *Thymus* taxa collected at Soroksár.

When the studied *Thymus* species of different taxa were planted at the same place (Soroksár, Hungary), the difference in their flavonoid profile depended mainly on their genetic feature, but also some microdiversity could occur. We could conclude that the concentration of polyphenols could be used as a chemotaxonomic tool for the identification of the origin within one *Thymus* species.

Further investigations are needed to determine the taxonomic value of flavonoids and phenolic acids in the case of Hungarian native *Thymus* taxa, where luteolin and its derivatives should also be involved in the analysis.

4. Conclusions

A liquid chromatography method coupled with electrospray mass spectrometric detection was developed for the determination of fifteen polyphenolic compounds in *Thymus* extract sample. The method requires a very simple sample preparation prior to analysis. The results showed that the technique is very sensitive, reproducible, and accurate. The LOD range for this method using ESI-MS detection is 0.84–66.79 ng mL^{-1} for the polyphenolic compounds.

The flavonoid profiles found in species of *Thymus* provide useful additional taxonomic characters at different levels of classification. Our results showed that the flavonoid profile depended mainly on genetic feature of *Thymus* species, when they are planted on the same locality. The individuals of the same species originated from remote localities, however, can differ in the amount of polyphenols, therefore these differences could be useful tools for the identification of the origin within one *Thymus* species. Further genetic investigations are required in order to determine the correlation between chemical and genetic relationships in the *Thymus* genus.

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