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Analytical Methods

Determination of polyphenolic compounds in commercial herbal drugs and spices from Lamiaceae: thyme, wild thyme and sweet marjoram by chromatographic techniques

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Abstract

HPLC and HPTLC methods were used for a qualitative and quantitative determination of luteolin-7-*O*-β-glucuronide, lithospermic acid, rosmarinic acid and mthyl rosmarinate, together with other known compounds, in commercial herbal drugs and spices from lamiaceous species: *Thymi herba*, *Serpylli herba* and *Majoranae herba*. The contents of analyzed compounds in the studied hydrophilic extracts, prepared form herbal sources, were established using a C18 column with acetonitrile–water–formic acid as a mobile phase. The HPLC method was validated for linearity, precision and accuracy. Luteolin-7-*O*-β-glucuronide and lithospermic acid were identified as new wild thyme constituents, luteolin-7-*O*-β-glucuronide and methyl rosmarinate as new compounds in sweet marjoram. Methyl rosmarinate was isolated for the first time from thyme. The investigated herbal drugs and spices provide polyphenols in high amounts, even up to 84.3 mg per 1 g of a dried herb.

Keywords: Caffeate oligomers; Luteolin-7-*O*-β-glucuronide; Lithospermic acid; Methyl rosmarinate; Rosmarinic acid

1. Introduction

Thyme (syn. common thyme, garden thyme), wild thyme and sweet marjoram are popular spices and volatile oil-containing drugs from the Lamiaceae (previously known as Labiatae) family which are listed in current editions of the European Pharmacopoeia, United States Pharmacopoeia or national pharmacopoeias, e.g., Polish Pharmacopoeia. Their quality is generally determined by their essential oil content. However, these drugs also contain caffeic acid oligomers (known as labiataetannins), flavonoid glycosides, hydroquinone derivatives, terpenoids, biphenyls, and more. *Thymi herba* (from *Thymus vulgaris* L.) and *Serpylli herba* (from *Thymus serpyllum* L. s.l.) are officially in the European Pharmacopoeia 5 (Ph.Eur. 5). *Majoranae herba* (from *Majorana hortensis* Moench. = *Origanum majorana* L.) and *T. herba* are listed in the Polish Pharmacopoeia VI (FP VI) but wild thyme herb is not included in FP VI.

The herb of thyme and its extracts are used orally to treat dyspepsia and other gastrointestinal disturbances, cough due to cold, whooping cough, bronchitis, laryngitis and tonsillitis (as a gargle). Topical applications of thyme preparations are used in the treatment of minor wounds, the common cold, disorders of the oral cavity and in oral hygiene. In Europe, thyme is considered to be bronchospasmolytic, an expectorant, and an antimicrobial, as *Thymi extractum* and *Thymi sirupus*. It is used for catarrh of the upper respiratory tract and against symptoms of bronchitis (monographs of WHO, ESCOP, The German Commission E). The essential oil content in the drug ranges from 5 to 25 ml kg^{-1} and its composition fluctuates with chemotype. The minimum requirement for essential oil content is 1.2% v/w (Ph.Eur. 5) with a minimum 40% of thymol and carvacrol (as a sum). These isomeric monoter-

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penes are held to be largely responsible for the antiseptic, antitussive and expectorant properties of thyme. The spasmolytic effect may be due to the polymethoxyflavones (di-, tri- and tetra-methoxylated flavones), all substituted in the six-position (WHO monograph). Thyme inhibits lipid peroxidation induced *in vitro* in mitochondria and microsomes. It also partially inhibits the production of the superoxide anion (Bruneton, 1999).

The essential oil of wild thyme usually contains more carvacrol than thymol. The plant contains 1–6 ml kg^{-1} of essential oil with a highly variable composition. *S. herba* should reach a minimum 0.3% v/w of essential oil (Ph.Eur. 5). Sweet marjoram contains 7–30 ml kg^{-1} of essential oil with terpin-1-en-4-ol, α-terpineol and linalool. According to FP VI, the minimum requirement for essential oil content in *M. herba* is 0.5% v/w. Wild thyme and sweet marjoram are also traditionally administered, orally, for symptomatic treatment of gastrointestinal disturbances and cough. Their spasmolytic and antimicrobial effects are used to treat bronchial diseases. Moreover, both drugs are applied topically to relieve symptoms of the common cold, such as nasal congestion (e.g., *Majoranae unguentum*, FP VI) and in mouthwashes for oral hygiene (Bruneton, 1999).

Lamiaceae plants were widely studied as natural antioxidant sources because of their high contents of polyphenols (Dapkevicius et al., 2002; Dorman, Bachmayer, Kosar, & Hiltunen, 2004; Kosar, Dorman, & Hiltunen, 2005; Lu & Foo, 2001; Miura, Kikuzaki, & Nakatani, 2002; Tagashira & Ohtake, 1998; Vági et al., 2005). Biologically active ingredients from the hydrophilic extracts of dried aerial parts of thyme previously described are: caffeic acid and its oligomers (rosmarinic acid, 3′-*O*-(8″-caffeoyl)-rosmarinic acid), flavones (apigenin, luteolin, luteolin-7-*O*-β-glucuronide, luteolin-7-*O*-β-glucoside, 6-hydroxyluteolin glycosides, polymethoxyflavones), flavanones (narirutin, eriodictyol, eriocitrin, hesperidin), flavanonol (taxifolin) (Dapkevicius et al., 2002; Haraguchi et al., 1996; Kobayashi et al., 2003; Kosar et al., 2005; Miura et al., 2002; Watanabe, Shinmoto, & Tsushida, 2005), monoterpene glucosides (*p*-cymenol-9-*O*-β-glucoside, 2- and 5-*O*-β-glucosides of thymoquinol, angelicoidenol-*O*-β-glucoside) (Takeuchi, Lu, & Fujita, 2004), compounds related to hydroxyjasmone (e.g. 5′-hydroxyjasmone-5′-*O*-β-glucoside) and simple phenol glucosides (3-hydroxy-4-methoxyphenethyl-3-*O*-β-glucoside, eugenol-*O*-β-glucoside, syringin) (Kitajima, Ishikawa, & Urabe, 2004), arbutin (Takeuchi et al., 2004), acetophenone glycosides (androsin, picein, glycosides of 4-hydroxyacetophenone derivatives) (Wang et al., 1999), biphenyl compounds (e.g. 3,4,3′,4′-tetrahydroxy-5,5′-diisopropyl-2,2′-dimethylbiphenyl) (Dapkevicius et al., 2002; Haraguchi et al., 1996; Nakatani, Miura, & Inagaki, 1989; Okazaki, Kawazoe, & Takaishi, 2002), and acidic polysaccharide (Chun, Shin, Hong, Cho, & Yang, 2001). Among examined compounds, caffeic acid, rosmarinic acid, eriodictyol and luteolin glycosides effectively protected biological systems against various oxidative stresses by inhibition of superoxide anion production in the xanthine/xanthine oxidase system (Haraguchi et al., 1996; Lu & Foo, 2001; Parejo et al., 2004). A new caffeate trimer, 3′-*O*-(8″-caffeoyl)-rosmarinic acid, revealed high radical-scavenging activity in the DPPH$^{\cdot}$ assay (Dapkevicius et al., 2002). Flavones and polymethoxyflavones were evaluated as antioxidants using the linoleic acid oxidation system (Miura et al., 2002). In other research, Okazaki and coworkers (2002) showed that thymol and biphenyl inhibited *in vitro* platelet aggregation. Acetophenone glycosides exhibited a weak cytotoxicity and antitumor effect by the inhibition of DNA synthesis (Wang et al., 1999) and the purified acidic polysaccharide possessed anticomplementary activity (Chun & Jun et al., 2001; Chun & Shin, 2001).

Besides essential oil, the herb of *M. hortensis* also contains diterpenes (carnosic acid and carnosol), triterpenes (oleanolic and ursolic acids), sterols (e.g., β-sitosterol), hydroquinone derivatives (hydroquinone monomethyl ether, arbutin, methylarbutin) (Assaf, Ali, Makboul, Beck, & Anton, 1987), labiatae tannins (caffeic and rosmarinic acids) and flavones (apigenin, hispidulin, luteolin-7-*O*-β-glucoside, 6-hydroxyapigenin, 7-*O*-β-glucosides of 6-hydroxyapigenin and 6-hydroxyluteolin, and their 6″-*O*-feruloyl derivatives) (Dorman et al., 2004; Heo et al., 2002; Kawabata et al., 2003; Vági et al., 2005). The ethanolic extract from sweet marjoram showed a high inhibitory effect on AChE *in vitro*. The active component was identified as ursolic acid which also inhibited an Aβ-induced neurotoxicity (Heo et al., 2002).

Several chromatographic assays have been previously developed for determination of caffeic acid and its oligomers. A TLC-densitometric method was reported for analyzing caffeic acid and rosmarinic acid in lamiaceous species (Janicsak, Mathe, Miklossy-Vari, & Blunden, 1999). Gradient reversed-phase HPLC methods were used for the estimation of flavonoid aglycones and glycosides, both caffeic and rosmarinic acids and their methyl esters in hydrophilic extracts from aromatic herbs, such as *T. vulgaris*, *M. hortensis*, *Mentha* × *piperita*, *Mentha spicata*, *Melissa officinalis*, *Rosmarinus officinalis* and *Salvia officinalis* (Dorman et al., 2004; Kosar et al., 2005; Tagashira & Ohtake, 1998; Wang, Provan, & Helliwell, 2004). Moreover, rosmarinic, lithospermic and several salvianolic acids were determined in *Salvia miltiorrhiza* using HPLC-DAD and HPLC-MS techniques (Hu, Liang, Luo, Zhao, & Jiang, 2005; Liu et al., 2006). The quantitative estimation of arbutin in sweet marjoram was achieved by chromatospectrophotometric and reversed-phase HPLC-UV methods (Assaf et al., 1987).

In our work, we have examined hydrophilic extracts from *T. herba*, S. *herba* and *M. herba*, in order to determine the presence and content of investigated polyphenols: arbutin, flavones and flavanones, lithospermic acid, methyl rosmarinate, rosmarinic acid and free caffeic acid, using chromatographic techniques. The aim of this study was the estimation of polyphenolic compounds in commercially

available herbal drugs and spices from thyme, wild thyme and sweet marjoram.

2. Materials and methods

2.1. Solvents and chemicals

All organic solvents and reagents used in the experiment were of analytical grade. Acetonitrile was HPLC gradient grade. Water was glass-distilled and deionized. Methanol, acetonitrile and 98–100% formic acid were obtained from Merck (Germany), diisopropyl ether from Sigma–Aldrich (USA), glacial acetic acid, acetone and others from POCH (Poland).

2.2. Standards

Luteolin-7-*O*-β-glucuronide (Lgr)[1] and lithospermic acid (LA)[2] were isolated from the wild thyme herb, methyl rosmarinate (MeR)[3], naringenin (N), eriodictyol (E), luteolin (L) and luteolin-7-*O*-β-glucoside (Lg) from the thyme herb, eriocitrin (Er), and luteolin-7-*O*-rutinoside (Lr), hesperidin (Hr) and diosmin (Dr) from the peppermint leaf (Sroka, Fecka, & Cisowski, 2005).

The 50% (v/v) aq. acetone extracts of wild thyme, thyme and sweet marjoram (from 300 g of each herb) were successively subjected to a combination of adsorption and partition column chromatography (CC) using octadecyl (Bakerbond Octadecyl 40 μm Prep LC Packing, J.T. Baker, USA), Sephadex LH-20 (Pharmacia LKB, Sweden) and silica gel 60 (0.063–0.200 mm, 70–230 mesh ASTM; Merck, Germany) with various solvent systems (v/v): water–methanol (9:1, 8:2 7:3, 6:4, 5:5, 3:7, 0:10), methanol–acetone (1:9, 2:8, 5:5, 10:0) and methanol–ethyl acetate (2:8, 3:7) or methanol–methyl chloride (1:9, 2:8, 3:7, 4:6).

Rosmarinic acid (RA), naringenin-7-*O*-β-glucoside (Ng), narirutin (Nr), eriodictyol-7-*O*-β-glucoside (Eg), apigenin (A), apigenin-7-*O*-β-glucoside (Ag) and isorhoifolin (Ir) were bought from Extrasynthese (France). Chlorogenic acid (ChA), hydroquinone (Hq) and arbutin (Ab) were purchased from Fluka (Switzerland), and caffeic acid (CA) from Koch-Light Laboratories (UK). Structures of all analyzed compounds are presented in Fig. 1.

Stock standard solutions (1 mg ml^{-1}) were prepared by dissolving 2–5 mg of an individual compound in 2–5 ml of methanol and filtered through a 0.45 μm membrane filter (Millipore, USA). Working standard solutions (10–500 μg ml^{-1}) were obtained by dilution with methanol.

2.3. Plant material and sample preparation

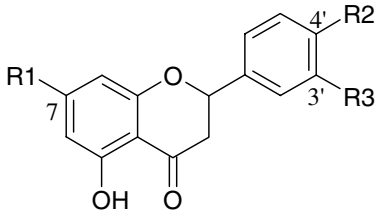
Dried plant materials used in the isolation process, i.e. *T. vulgaris* L. herb (Tv), *T. serpyllum* L. herb (Ts) and *M. hortensis* Moench. herb (Mh), were purchased from the pharmaceutical company Kawon Sp.j. (Poland), certified GMP and ISO 9002. Other samples were obtained from local chemists and markets in the years 2001–2006. Herbs of thyme, wild thyme and sweet marjoram were produced by the Polish pharmaceutical industry and food industry: Kawon Sp.j., Herbapol Lublin S.A., Herbapol Łódź S.A., Herbalux S.C., Zakład Konfekcjonowania Ziół FLOS, P.P.H Prymat Sp.zo.o., Rowita Sp.j., Kamis-Przyprawy S.A., Ziołopex Sp.zo.o., Kotanyi Polonia Sp.zo.o.

Aqueous, aq. methanolic and methanolic extracts (1:100 or 1:200) were obtained from 0.500 g of each sample of powdered herbs. To obtain hot aqueous extracts (infusions) from examined herbal drugs and spices, boiling water (50 ml or 100 ml) was poured over the powdered plant material, mixed and filtered after 15 min. Hot methanolic extracts (MeOH-Hot) were prepared using 50 ml or 100 ml of pure methanol and heated under reflux in a water bath for 15 or 30 min. after boiling. Aqueous, aq. methanolic and methanolic extracts (Aq-US, 30MeOH-US, 50MeOH-US, 70MeOH-US and MeOH-US) were obtained with the same volume of water, 30%, 50%, 70% aq. methanolic solutions (v/v) or pure methanol, using an ultrasonic bath for 15 or 30 min. All extracts were passed through paper filters (Whatman No.1) and rinsed into volumetric flasks made up to 50 ml or 100 ml with an appropriate solvent. After blending, 2 ml of each extract

[1] *Luteolin-7-O-β-glucuronide*, Lgr (807 mg) HRESIMS *m/z* (%): 461,0686 (100) $[M-H]^-$, 923.1559 (10) $[2M-H]^-$; UV (MeOH), ^1H NMR (DMSO-d_6) and ^{13}C NMR (DMSO-d_6): data in agreement with those published by Lu and Foo (2000), Dapkevicius et al. (2002), Kobayashi et al. (2003). *Naringenin*, N (38 mg), *Eriodictyol*, E (45 mg), *Luteolin*, L (40 g), *Luteolin-7-O-β-glucoside*, Lg (293 mg) UV (MeOH), ^1H NMR (DMSO-d_6) and ^{13}C NMR (DMSO-d_6): data in agreement with those published by Harborne and Mabry (1982) and Harborne (1994).

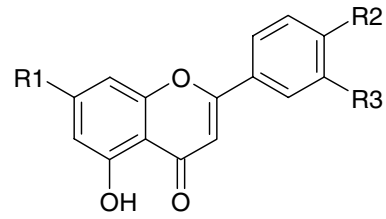
[2] *Lithospermic acid*, LA (495 mg) ^1H NMR (D_2O-Acetone d_6): 8.38 (OH), 7.88–7.83 (d $J_{trans} = 16.0$, 1H, 7), 7.07–7.05 (d $J = 8.5$, 1H, 6), 6.92 (d $J = 2.4$, 1H, 2″), 6.78–6.74 (d $J = 8.5$, 1H, 5), 6.75 (s, 1H, 2′), 6.78–6.60 (m, 4H, 5′, 5″, 6′, 6″), 6.21–6.16 (d $J_{trans} = 16.0$, 1H, 8), 5.91–.89 (d $J = 4.8$, 1H, 7″), 4.95–4.92 (d → dd $J = 8.0$, 1H, 8′), 4.20–4.12 (d $J = 4.8$, 1H, 8″), 3.03–2.94 (ddd, $^2J = 14.0$, $^3J = 8.0$, 2H, 7′); ^{13}C NMR (D_2O-acetone d_6): 179.14 (COOH 9″), 178.72 (COOH 9′), 168.37 (COOR, 9), 147.85 (3), 145.42 (4), 145.23 (3″), 144.98 (3′), 143.98 (4′), 143.74 (4″), 143.41 (7), 134.22 (1″), 130.55 (1′), 129.80 (2), 123.91 (1), 121.18 (6′), 120.99 (6), 117.39 (2C, 5, 6″), 117.03 (2′), 116.37 115.93 (5′, 5″), 115.65 (8), 113.48 (2″), 88.65 (7″), 77.06 (8′), 60.12 (8″), 37.55 (7′); HRESIMS *m/z* (%): 559.0940 (5) $[M-2H+Na]^-$, 493.12110 (100) $[M-44-H]^-$, 295.0602 (10) (Hu et al., 2005); ^1H NMR (D_2O-acetone d_6) and ^{13}C NMR (D_2O-acetone d_6) data in agreement with those published by Kelley et al. (1975, 1976), Kohda et al. (1989).

[3] *Methyl rosmarinate*, MeR (55 mg) ^1H NMR (D_2O-aetone d_6): 8.37 (OH), 7.46–7.43 (d $J_{trans} = 15.9$, 1H, 7), 7.04 (br s, 1H, 2), 6.91–6.89 (dd $^3J = 8.1$, $^4J = 1.0$, 1H, 6), 6.79–6.78 (d $J = 8.1$, 1H, 5), 6.69 (br s, 1H, 2′), 6.68–6.66 (d $J = 8.0$, 1H, 5′), 6.52–6.50 (dd $^3J = 8.0$ $^4J = 1.5$, 1H, 6′), 6.21–6.17 (d $J_{trans} = 15.9$, 1H, 8), 5.10–5.07 (q → dd $^3J = 4.6$ & 8.0, 1H, 8′), 3.58 (s, 3H, CH_3), 2.98–2.94 (dd $^2J = 14.3$, $^3J = 4.6$, 1H, 7′eq), 2.92–2.87 (dd $^2J = 14.3$, $^3J = 8.0$, 1H, 7′ax); ^{13}C NMR (D_2O-acetone d_6): 171.42 (COOR, 9′), 167.52 ($COOCH_3$, 9), 148.53 (4), 147.18 (7), 145.50 (3), 144.86 (3′), 143.97 (4′), 128.05 (1′), 126.50 (1), 122.77 (6), 121.34 (6′), 117.06 (2′), 116.24 (5), 115.93 (5′), 114.94 (8), 113.48 (2), 73.70 (8′), 52.63 (CH_3), 36.67 (7′); HRESIMS *m/z* (%): 373.0929 (100) $[M-H]^-$, 179.0379 (5) [caffeic acid-H]$^-$; ^1H NMR (D_2O-acetone d_6) and ^{13}C NMR (D_2O-acetone d_6) data in agreement with those published by Kelley et al. (1975, 1976), Kohda et al. (1989), Tagashira and Ohtake (1998) and Parejo et al. (2004).



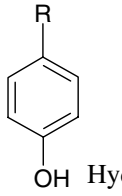
Flavanones

	R1	R2	R3
Naringenin	OH	OH	H
Naringenin–7–*O*–β–glucoside	OH	OH	H
Narirutin	*O* – rutinoside	OH	H
Eriodictyol	OH	OH	OH
Eriodictyol–7–*O*–β–glucoside	OH	OH	OH
Eriocitrin	*O* – rutinoside	OH	OH
Hesperidin	*O* – rutinoside	OCH_3	OH

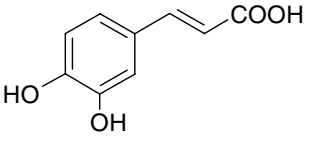


Flavones

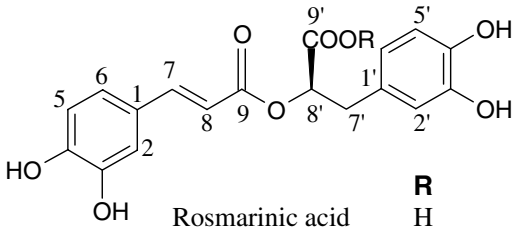
	R1	R2	R3
Apigenin	OH	OH	H
Apigenin –7 – *O*–β–glucoside	OH	OH	H
Isorhoifolin	*O* – rutinoside	OH	H
Luteolin	OH	OH	OH
Luteolin – 7 – *O*–β–glucoside	OH	OH	OH
Luteolin – 7 – *O*–β–glucuronide	OH	OH	OH
Luteolin – 7 – *O* – rutinoside	OH	OH	OH
Diosmin	*O* – rutinoside	OCH_3	OH



	R
Hydroquinone	OH
Arbutin	*O*–β–glucoside



Caffeic acid



	R
Rosmarinic acid	H
Methyl rosmarinate	CH_3

Lithospermic acid

Fig. 1. Structures of polyphenolic compounds analyzed in Lamiaceae.

were filtrated through a 0.45 μm membrane filter (Millipore, USA).

The detailed quantitative and qualitative analyses of Tv, Ts and Mh extracts were performed with both HPTLC and HPLC methods.

2.4. Apparatus and chromatographic conditions

UV spectra were recorded on a Perkin Elmer UV/VIS Lambda 20 spectrometer. ^{1}H NMR (300 MHz), ^{13}C NMR (75 MHz) and 2D NMR experiments were recorded on a Bruker 300 spectrometer using the residual solvent peaks as internal standard. HRESI MS data were recorded in MeOH on a Mariner API-TOF™ mass spectrometer with Mariner Data Explorer™ version 3.2.

Planar chromatography was performed on 10 × 20 cm HPTLC NH_2 and HPTLC Si60 plates (MERCK, Germany). In HPTLC methods, extracts (10–50 μl) and standards (5–20 μl) were manually applied to the plates as 5–10 mm bands. Chromatograms were developed in horizon-

tal Teflon DS-chambers (Chromdes, Poland). HPTLC NH_2 plates were developed with mobile phases of: acetone – formic acid (85:15, v/v) and acetone–acetic acid (85:15, v/v), at a distance of 9 cm from the origin, while HPTLC Si60 plates were developed with a mobile phase, diisopropyl ether–acetone–formic acid–water (50:30:10:10, v/v/v/v), at the same distance. Plates were dried in a stream of warm air for few minutes after development. Compounds were detected under UV light at 365 nm before and after spraying with 2% methanolic $AlCl_3$, a natural product/polyethylene glycol reagent (NP/PEG; yellow, orange and green bands) or in visible light after treatment with bis-diazotized sulfanilamide (yellow, orange, red and brown bands).

The HPLC separation of polyphenolic compounds was performed on a Knauer system (Germany) equipped with two pumps (type 64), a sample injector and variable wavelength UV detector (type 87.00). The column used was a Beta Basic-18, 5 μm C_{18} (250 × 4.6 mm ID) type from Thermo Hypersil (UK). The fingerprint profiles were recorded at an optimized wavelength of 280 nm. The flow rate was set to 0.9 ml/min. Polyphenolic compounds were analyzed using an acetonitrile–water gradient with a formic acid addition (0.2%, 1.5%, 3% and 5% FA) according to the solvent programme: solvent A, 5% formic acid in acetonitrile; solvent B, 5% formic acid in water, commencing with 10% A in B, rising to 40% A after 25 min, then to 70% A after 30 min. The injection volume for all samples was 20 μl. Solvent solutions were vacuum-degassed with ultrasonication prior to usage.

All chromatographic experiments were performed at 20 °C.

2.5. Methods validation

To determine a linearity for HPLC calibration, plots of analyzed compounds were constructed on the basis of peak areas (y) using six concentration solutions (x). All plots were linear in the examined range (10–500 μg ml^{-1}). Linear ranges have been shown as an amount of standard (ng) applied with a single injection. The limits of detection (LOD) and quantification (LOQ) for HPLC analyses were calculated from calibration equations using a signal-to-noise ratio ($S/N \geqslant 3{:}1$ and $S/N \geqslant 10{:}1$, respectively) and have been expressed as an amount (ng) of an examined polyphenol per injection. Calibration equations, ranges, correlation coefficients (r) of estimated compounds, their LOD and their LOQ values are listed in Table 1.

Precision was expressed as coefficient of variation (%CV) of multiple independent determinations and was defined as repeatability (injection precision) and intermediate precision. Both parameters were performed using mixtures of standards at a concentration of 25 μg ml^{-1} (500 ng of each standard per injection) to measure peak areas. To

Table 1
Validation parameters of the HPLC method with 5% FA addition to the solvent system

Standard	Calibration equation	r	Linear range (μg ml^{-1})	LOD (ng)	LOQ (ng)	Repeatability (%CV)[a]	Intermediate precision (%CV)[a]	Recovery (% ±SD)[b]
Hydroquinones								
Arbutin	$y = 2622.72x - 9.04$	0.9992	25–500	91.8	145	3.6	4.03	100 ± 2.53
Hydroquinone	$y = 20306.42x - 24.39$	0.9999	25–200	27.0	33.9	4.95	5.4	102 ± 1.01
Flavanones								
Naringenin	$y = 64101.6x - 37.66$	0.9998	25–150	12.7	14.9	1.9	2.73	102 ± 2.16
Naringenin-7-*O*-β-glucoside	$y = 31375.95\ x - 15.57$	0.9999	10–150	11.8	16.3	1.32	2.42	99.3 ± 0.89
Narirutin[c]	$y = 46035x + 16.72$	0.9999	10–150	13.0	43.5	1.25	2.21	97.9 ± 2.19
Eriodictyol	$y = 27201.92x - 11.63$	0.9999	10–100	10.8	15.9	3.77	4.07	100 ± 1.7
Eriodictyol-7-*O*-β-glucoside	$y = 38174x - 40.5$	0.9999	10–150	22.8	26.5	1.13	2.18	99.4 ± 1.13
Eriocitrin	$y = 28687.33x - 37.38$	0.9997	10–300	28.2	33.0	1.09	2.18	99.7 ± 2.49
Hesperidin[d]	$y = 28402.06x - 7.24$	0.9991	10–150	8.27	15.7	2.76	3.75	98.9 ± 1.68
Flavones								
Apigenin	$y = 57640.51x + 21.22$	0.9995	12.5–125	8.85	29.5	2.19	2.63	99.2 ± 1.56
Luteolin	$y = 27991.2x - 0.94$	0.9999	10–100	2.82	7.82	2.49	4.15	99.4 ± 1.82
Luteolin-7-*O*-β-glucuronide[e]	$y = 23434.31x - 6.73$	0.9999	10–150	5.8	12.0	1.58	2.27	98.0 ± 1.88
Luteolin-7-*O*-rutinoside	$y = 17086.80x - 19.96$	0.9997	25–200	26.9	35.1	1.91	2.45	101 ± 0.77
Caffeic acid derivatives								
Lithospermic acid	$y = 8456.8x - 16.42$	0.9997	25–300	45.9	62.5	1.87	2.55	102 ± 1.3
Rosmarinic acid	$y = 33050.48x - 28.06$	0.9994	10–150	18.8	23.0	1.66	2.56	99.0 ± 1.38
Methyl rosmarinate	$y = 21164.4x + 19.32$	0.9995	10–150	5.84	62.0	2.17	3.22	96.3 ± 3.49
Chlorogenic acid	$y = 28572.83x - 12.36$	0.9997	25–200	10.8	15.7	2.87	3.69	98.0 ± 0.98
Caffeic acid	$y = 57950x - 3.06$	0.9997	10–150	2.09	4.51	2.38	2.89	102 ± 1.11

($n = 6$).

[a] %CV value of standard peak area ($n = 7$) for concentration 25 μg ml^{-1} (500 ng per injection).

[b] Recovery test ($n = 3$).

[c] Under these chromatographic conditions narirutin migrates together with isorhoifolin.

[d] Hesperidin with diosmin.

[e] luteolin-7-*O*-β-glucuronide with luteolin-7-*O*-β-glucoside.

measure repeatability, the same sample was independently analyzed seven times according to the same HPLC procedure. Intermediate precision was examined using seven samples which were prepared with the same sample preparation procedure and analyzed with the same chromatographic conditions on three different days (inter-day precision).

Accuracy of the method was evaluated with a recovery test. Samples of extracts were prepared to contain an analyzed compound at about 50–100 μg ml^{-1}. An equal volume of a standard solution with the same amount of tested compound was then added.

Results from the determination of precision and accuracy are presented in Table 1.

2.6. Content measurement

The quantitative estimation of polyphenols was performed by referring to standardized curves of authentic samples. A concentration of analyzed compounds was measured in triplicate as the mean of three independent solutions, using the HPLC method and solvent system with 5% formic acid addition. We counted amounts (mg g^{-1}) of each individual polyphenol in a dried herb (Tv, Ts, Mh). Total polyphenols content (TPP) was calculated as the sum of all detected polyphenolic compounds which were present in examined species from their average values. Total contents of hydroquinones (THQ), flavonoids (TF) and caffeic acid derivatives (TC) were similarly calculated. Mean values, standard deviations (SD), medians, and both minimum and maximum contents of all achieved results have also been determined (Tables 2–4).

3. Results and discussion

3.1. Chromatographic conditions

Good separation conditions should be satisfactory when analyzed peaks have a baseline separation with adjacent peaks within an acceptable analysis time. According to literature data, an acid addition allows better separation of polyphenols since it reduces the ioniza-

Table 2
Contents of determined polyphenolic compounds (mg g^{-1}) in *Thymus vulgaris* herb

No	*Ab/THQ*	Er	Lr	Eg	Lgr	Nr	Ng	Hr	E	L	N	*TF*	CA	RA	MeR	*TC*	*TPP*
1	*0.89*	1.05	0.74	0.43	10.85	0.16	0.25	0.85	0.03	0.33	0.18	*14.87*	0.52	25.19	0.36	*26.07*	*41.83*
2	*0.79*	0.82	1.07	0.4	11.66	0.14	0.35	0.86	1.17	0.31	0.33	*17.11*	0.57	22.84	0.36	*23.77*	*41.67*
3	*0.72*	1.01	0.99	0.37	11.23	0.13	4.4	0.81	1.21	0.33	0.52	*21*	0.36	17.08	0.32	*17.76*	*39.48*
4	*0.78*	1.05	0.79	0.46	10.12	0.24	0.4	0.95	1.36	0.71	0.52	*16.6*	0.47	33.06	0.32	*33.85*	*51.23*
5	*0.73*	0.93	0.72	0.43	9.04	0.25	0.53	1.22	1.32	0.66	0.51	*15.61*	0.45	35.31	0.31	*36.07*	*52.41*
6	*0.78*	1	0.77	0.45	12.06	0.31	0.84	1.34	1.38	0.67	0.29	*19.11*	0.53	27.24	0.33	*28.1*	*47.99*
7	*0.92*	1.15	0.83	0.48	14.47	0.34	0.32	1.18	1.65	0.78	0.44	*21.64*	0.55	27.07	0.28	*27.9*	*50.46*
8	*0.7*	1.31	0.67	0.41	12.47	0.19	0.57	1.25	0.98	0.24	0.28	*18.37*	0.52	18.31	nd	*18.83*	*37.9*
9	*0.71*	1.35	0.69	0.4	12.57	0.18	0.33	1.08	1.01	0.2	0.27	*18.08*	0.57	18.36	nd	*18.93*	*37.72*
10	*0.8*	1.48	2.28	0.4	11.54	0.19	0.27	1.65	1.03	0.3	0.27	*19.41*	0.61	17.83	0.32	*18.76*	*38.97*
11	*0.78*	1.26	1.87	0.35	9.65	0.17	0.34	1.67	1.03	0.2	0.23	*16.77*	0.47	16.76	0.33	*17.56*	*35.11*
12	*1.13*	1.28	1.94	0.48	10.29	0.07	0.45	0.91	0.82	0.27	0.24	*16.75*	0.39	15.75	0.31	*16.45*	*34.33*
13	*0.9*	1.36	1.8	0.48	11.65	0.1	0.45	1.08	0.9	0.57	0.23	*18.62*	0.58	15.72	0.34	*16.64*	*36.16*
14	*1.14*	0.71	0.59	0.31	9.22	0.21	0.35	0.87	0.5	0.25	0.64	*13.65*	0.68	15.41	0.18	*16.27*	*31.06*
15	*1.07*	0.79	0.67	0.35	9.4	0.25	0.58	0.87	0.6	0.21	0.32	*14.04*	0.7	15.5	0.2	*16.4*	*31.51*
16	*0.75*	1.87	1.04	0.58	8.14	0.49	0.58	0.84	2.01	0.81	0.45	*16.81*	0.45	27.23	1.41	*29.09*	*46.65*
17	*0.87*	1.9	0.82	0.48	7.68	0.41	0.49	0.81	1.93	0.96	0.54	*16.02*	0.41	27.94	1.52	*29.87*	*46.76*
18	*0.89*	1.83	1.03	0.56	8.06	0.4	0.57	0.57	1.84	1.19	0.53	*16.58*	0.39	24.8	1.2	*26.39*	*43.86*
19	*0.65*	1.07	2.11	0.61	3.85	0.26	0.48	0.48	2.73	0.67	0.65	*12.91*	0.34	13.81	0.46	*14.61*	*28.17*
20	*0.66*	1.25	1.99	0.62	3.91	0.36	0.6	0.8	2.78	0.76	0.33	*13.4*	0.37	11.56	0.58	*12.51*	*26.57*
21	*0.64*	1.21	1.91	0.64	3.91	0.32	0.51	1.14	2.56	0.68	0.45	*13.33*	0.36	11.04	0.4	*11.8*	*25.77*
22	*0.75*	1.26	1.24	0.96	5.54	0.32	0.51	0.57	2.13	0.43	0.36	*13.32*	0.51	18.76	1	*19.27*	*33.34*
23	*0.76*	1.33	1.31	1.08	4.69	0.31	0.71	0.8	2.25	0.49	0.54	*13.51*	0.5	20.82	0.84	*22.16*	*36.43*
24	*0.9*	1.23	1.26	1.03	4.54	0.27	0.7	1.24	2.14	0.55	0.8	*13.76*	0.46	21.83	0.9	*23.19*	*37.85*
25	*0.78*	1.35	1.45	1.41	3.33	0.35	0.72	0.74	1.55	0.66	0.38	*11.94*	0.35	33.26	1.16	*34.77*	*47.49*
26	*0.79*	1.31	1.71	1.47	3.67	0.34	0.45	0.72	1.46	0.71	0.68	*12.52*	0.38	29.86	1.17	*31.41*	*44.72*
27	*0.8*	1.38	1.55	1.21	3.25	0.3	0.62	0.73	1.84	0.81	0.66	*12.35*	0.38	30.06	1.44	*31.88*	*45.03*
Mean	*0.82*	1.24	1.25	0.62	8.4	0.26	0.64	0.96	1.49	0.55	0.43	*15.85*	0.48	21.94	0.63	*22.97*	*39.65*
SD	*0.13*	0.3	0.53	0.33	3.47	0.1	0.76	0.3	0.69	0.26	0.17	*2.72*	0.10	6.95	0.45	*7.167*	*7.55*
Median	*0.78*	1.26	1.07	0.48	9.22	0.26	0.51	0.87	1.38	0.57	0.44	*16.02*	0.47	20.82	0.36	*22.16*	*38.97*
Min	*0.64*	0.71	0.59	0.31	3.25	0.07	0.25	0.48	0.03	0.2	0.18	*11.94*	0.34	11.04	0.18	*11.8*	*25.77*
Max	*1.14*	1.9	2.28	1.47	14.47	0.49	4.4	1.67	2.78	1.19	0.8	*21.64*	0.7	35.31	1.52	*36.07*	*52.41*

nd, not detected; *THQ*, Total Hydroquinones; *TF*, Total Flavonoids; *TC*, Total Caffeic acid derivatives; *TPP*, Total Polyphenols; Ab, arbutin; Er, eriocitrin; Lr, luteolin-7-*O*-rutinoside; Eg, eriodictyol-7-*O*-β-glucoside; Lgr, luteolin-7-*O*-β-glucuronide; Nr, narirutin; Ng, naringenin-7-*O*-β-glucoside; Hr, hesperidin; E, eriodictyol; L, luteolin; N, naringenin; CA, caffeic acid; RA, rosmarinic acid; MeR, methyl rosmarinate.

tion of both hyroxyl and carboxyl groups. It was also suggested that separation was better when column temperature was kept at 20 °C (Liu et al., 2006; Parejo et al., 2004; Hu et al., 2005). We examined the effects of both acetonitrile and formic acid concentrations on the resolution of a chromatographic system used to optimize separation conditions. Optimal chromatographic conditions were first determined for a mixture of analyzed polyphenols, followed by application to the analysis of Tv, Ts and Mh extracts. The most suitable flow rate was found to be 0.9 ml min^{-1}. Based on the UV absorption maximum of all analyzed compounds, we recorded our chromatograms at 280 nm since arbutin and hydroquinone were invisible at 325 nm, characteristic for caffeic acid derivatives. For caffeate oligomers (LA, RA), the octadecyl phase of a Beta Basic-18 column from Thermo Hypersil was better than both LiChrospher and LiChrosorb columns from Merck.

The optimal separation for a mixture of eighteen polyphenolic compounds (Mix18) was obtained by a 5% formic acid addition to the solvent system (solution A and B). In this chromatographic condition we achieved well separated peaks of rosmarinic acid and lithospermic acid, flavone and flavanone aglycones, rutinosides of eriodictyol and luteolin, but luteolin-7-*O*-β-glucuronide migrated together with luteolin-7-*O*-β-glucoside and hesperidin with diosmin (Fig. 2). These glycosides were better divided when we used a 0.2% or 1.5% concentration of formic acid.

3.2. Sample extraction

In order to obtain quantitative extraction of a dried plant material, variables involved in the procedure, such as solvent, extraction method and extraction time, were optimized. Water, hot water, 30% aq. methanol, 50% aq. methanol, 70% aq. methanol and pure methanol were employed as extraction solvents. The same five powdered herbs from the examined species were extracted with water, aq. methanol and pure methanol using an ultrasonic bath within 15 or 30 min, with pure methanol under reflux using a water bath, as well as infusing with boiling water. Water, 30% aq. methanol and pure methanol could not completely extract analyzed polyphenols using an ultrasonic bath. However, the content of arbutin achieved for water at room temperature was relatively

Table 3
Contents of determined polyphenolic compounds (mg g^{-1}) in *Thymus serpyllum* herb

No	Er	Lr	Lgr	E	L	*TF*	CA	LA	RA	*TC*	*TPP*
1	0.83	1.03	24.94	0.38	2.75	*29.93*	0.43	3.39	13.16	*16.98*	*46.91*
2	0.78	0.89	24.31	0.37	2.73	*29.08*	0.42	3.52	13.11	*17.05*	*46.13*
3	0.81	0.94	23.58	0.38	2.84	*28.55*	0.44	3.36	12.98	*16.78*	*45.33*
4	0.65	0.8	19.45	0.32	3.41	*24.63*	0.47	4.05	12.41	*16.93*	*41.56*
5	0.72	0.82	22.25	0.32	3.7	*27.81*	0.44	3.98	14.04	*18.46*	*46.27*
6	0.73	0.82	22.25	0.37	3.7	*27.87*	0.44	4.05	14	*18.49*	*46.36*
7	1.94	0.88	12.16	0.57	0.86	*16.41*	0.47	4.55	8.72	*13.74*	*30.15*
8	1.86	0.79	12.11	0.6	0.92	*16.28*	0.52	4.96	8.19	*13.67*	*29.95*
9	1.82	0.75	11.95	0.61	0.89	*16.02*	0.44	5.21	8.18	*13.83*	*29.85*
10	1.61	0.73	11.73	0.46	0.72	*15.25*	0.37	5.68	7.8	*13.85*	*29.1*
11	1.71	0.8	13.32	0.53	0.78	*17.14*	0.42	5.77	8.4	*14.59*	*31.73*
12	1.59	1.3	11.23	0.57	0.83	*15.52*	0.8	37.54	20.14	*58.48*	*74*
13	1.58	1.24	12.37	0.56	0.69	*16.44*	0.81	32.58	18.18	*51.57*	*68.01*
14	1.55	1.26	10.52	0.57	0.75	*14.65*	0.77	35.1	19.34	*55.21*	*69.86*
15	1.27	1.63	24.85	0.63	0.47	*28.85*	0.42	11.7	17.12	*29.24*	*58.09*
16	1.26	1.63	24.31	0.56	0.42	*28.18*	0.39	10.35	17.75	*28.49*	*56.67*
17	1.23	1.59	23.98	0.51	0.4	*27.71*	0.41	11.08	14.8	*26.29*	*54*
18	1.4	2.86	7.37	0.99	2.33	*14.95*	0.33	5.79	9.75	*15.87*	*30.82*
19	1.35	2.87	7.05	1.42	2.23	*14.92*	0.21	6.33	7.63	*14.17*	*29.09*
20	0.58	2.87	7.46	1.2	2.3	*14.41*	0.26	8.18	10.09	*18.53*	*32.94*
21	0.63	2.9	5.74	0.79	0.41	*10.47*	0.37	4.15	21.79	*26.31*	*36.78*
22	0.6	3.25	6.41	0.8	0.71	*11.77*	0.33	3.14	18.18	*21.65*	*33.42*
23	0.97	1.99	7.57	0.81	0.51	*11.85*	0.32	4.54	13.1	*17.96*	*29.81*
24	0.75	1.03	7.24	0.74	0.5	*10.26*	0.87	42.85	24.45	*68.17*	*78.43*
25	0.68	0.74	7.04	0.6	0.47	*9.53*	0.83	41.54	23.98	*66.35*	*75.88*
26	1.8	1.25	13.54	0.7	1.4	*18.69*	0.45	11.65	19.1	*31.2*	*49.89*
Mean	1.189	1.45	14.41	0.63	1.45	*19.12*	0.48	12.12	14.48	*27.07*	*46.19*
SD	0.47	0.82	7.04	0.26	1.13	*7.02*	0.18	13.20	5.14	*17.36*	*16.24*
Median	1.245	1.135	12.135	0.57	0.845	*16.345*	0.435	5.725	13.58	*18.475*	*45.73*
Min	0.58	0.73	5.74	0.32	0.4	*9.53*	0.21	3.14	7.63	*13.67*	*29.09*
Max	1.94	3.25	24.94	1.42	3.7	*29.93*	0.87	42.85	24.45	*68.17*	*78.43*

TF, Total Flavonoids; *TC*, Total Caffeic acid derivatives; *TPP*, Total Polyphenols; Er, eriocitrin; Lr, luteolin-7-*O*-rutinoside; Lgr, luteolin-7-*O*-β-glucuronide; E, eriodictyol; L, luteolin; CA, caffeic acid; LA, lithospermic acid; RA, rosmarinic acid.

high (only 20% lower than that for 50% aq. methanol). A classical extraction method with hot methanol led to decomposition of polyphenols, especially caffeic acid esters and arbutin. The optimal concentrations of the main compounds (arbutin, luteolin-7-*O*-β-glucuronide, lithospermic acid, rosmarinic acid) were estimated for 50% aq. methanolic extracts (50MeOH-US) and hot water extracts (infuses). The 70% aq. methanolic extracts (70MeOH-US) were characterized by a lower concentration of arbutin (Fig. 4) and the insignificantly better extraction efficiency of luteolin-7-*O*-β-glucuronide. In addition, we did not observe any statistical differences between extraction times of 15 and 30 min. These results agreed with the observations of Wang et al. (2004) and Liu & colleagues (2006).

For the above experiment, the most suitable extraction method for polyphenolic compounds was ultrasonic extraction with 50% aq. methanol within 15 min.

3.3. Method validation

The external standard method was used to obtain the regression equations. The calculated results are given in Table 1. All of the standards showed good linearity ($r \geqslant 0.9991$) in a relatively wide concentration range. LOD and LOQ were expressed as ng per injection in the range from 2.09 ng for caffeic acid to 91.8 ng for arbutin and from 4.51 to 145 ng, respectively, for the same compounds (Table 1). In our experiment, comparable repeatability and intermediate precision, with a

Table 4
Contents of determined polyphenolic compounds (mg g^{-1}) in *Majorana hortensis* herb

No	Ab	Hq	*THQ*	Er	Lr	Lgr	L	*TF*	CA	RA	MeR	*TC*	*TPP*
1	18.5	0.69	*19.19*	0.4	0.73	2.93	nd	*4.06*	0.31	4.04	0.4	*4.75*	*28*
2	12.71	0.48	*13.19*	0.39	0.52	2.51	nd	*3.42*	0.22	4	0.35	*4.57*	*21.18*
3	13.18	0.35	*13.53*	0.31	0.7	2.7	nd	*3.71*	0.95	6.1	0.32	*7.37*	*24.61*
4	24.39	0.42	*24.81*	0.38	0.63	2.35	nd	*3.36*	0.61	4.94	0.4	*5.95*	*34.12*
5	28.98	0.28	*29.26*	0.64	2.17	8.74	0.96	*12.51*	1.35	11.36	0.95	*13.66*	*55.43*
6	23.77	0.26	*24.03*	0.5	1.84	7.6	0.96	*10.9*	1.19	12.88	0.73	*14.8*	*49.73*
7	26.5	0.28	*26.78*	0.62	1.94	8.16	0.51	*11.23*	1.26	12.9	0.8	*14.96*	*52.97*
8	27.13	0.27	*27.4*	0.61	2.06	7.21	0.49	*10.37*	0.97	14.69	0.84	*16.5*	*54.27*
9	24.08	0.28	*24.36*	0.58	1.97	7.02	0.36	*9.93*	1.29	14.14	0.8	*16.23*	*50.52*
10	25.25	0.3	*25.55*	0.46	1.55	6.69	0.3	*9*	1.25	14.82	0.75	*16.82*	*51.37*
11	25.47	0.28	*25.75*	0.56	1.63	6.95	0.25	*9.39*	0.86	12.5	0.55	*13.91*	*49.05*
12	25.45	0.28	*25.73*	0.58	1.61	6.68	0.24	*9.11*	0.87	12.51	0.26	*13.64*	*48.48*
13	33.82	0.25	*34.07*	0.3	2.7	9.68	0.21	*12.89*	1.22	19.48	0.55	*21.25*	*68.21*
14	32.56	0.27	*32.83*	0.34	2.85	9.89	0.22	*13.3*	1.28	19.44	0.61	*21.33*	*67.46*
15	28.61	0.12	*28.73*	0.57	1.38	5.79	0.38	*8.12*	0.78	8.57	0.49	*9.84*	*46.69*
16	21.01	0.14	*21.15*	0.52	1.3	5.61	0.39	*7.82*	0.72	7.99	0.42	*9.13*	*38.1*
17	26.29	0.3	*26.59*	0.54	1.24	5.28	0.42	*7.48*	0.79	8.66	0.35	*9.8*	*43.87*
18	17.53	0.27	*17.8*	0.55	1.81	7.54	0.4	*10.3*	0.94	14.86	0.18	*15.98*	*44.08*
19	17.01	0.28	*17.29*	0.55	1.76	7.5	0.44	*10.25*	0.92	14.43	0.19	*15.54*	*43.08*
20	16.89	0.27	*17.16*	0.5	1.5	6.57	0.37	*8.94*	0.92	13.02	0.35	*14.29*	*40.39*
21	17.97	0.3	*18.27*	0.59	2.01	7.24	0.29	*10.13*	1.17	11.81	0.78	*13.76*	*42.16*
22	16.83	0.32	*17.15*	0.59	1.91	7.16	0.3	*9.96*	1.16	11.88	0.73	*13.77*	*40.88*
23	16.99	0.28	*17.27*	0.58	1.8	7.06	0.28	*9.72*	1.18	11.92	0.78	*13.88*	*40.87*
24	16.98	0.12	*17.1*	0.58	1.86	7.35	0.23	*10.02*	0.92	12.48	0.71	*14.11*	*41.23*
25	17.98	0.12	*18.1*	0.54	1.77	6.98	0.24	*9.53*	0.92	12.36	0.56	*13.84*	*41.47*
26	16.56	0.15	*16.71*	0.49	1.74	6.75	0.24	*9.22*	0.7	12.41	0.45	*13.56*	*39.49*
27	18.87	0.44	*19.31*	0.5	0.66	3.3	0.7	*5.16*	0.61	5.91	0.34	*6.86*	*31.33*
28	18.63	0.45	*19.08*	0.46	0.5	3.8	0.67	*5.43*	0.64	6.4	0.36	*7.4*	*31.91*
29	18.59	0.41	*19*	0.53	0.59	3.71	0.69	*5.52*	0.62	5.5	0.41	*6.53*	*31.05*
30	57.69	0.49	*58.18*	0.8	1.74	5.74	0.97	*9.25*	1.11	10.02	0.96	*12.09*	*79.52*
31	60.45	0.45	*60.9*	0.76	1.83	6.06	0.99	*9.64*	1.02	11.75	0.95	*13.72*	*84.26*
32	59.26	0.5	*59.76*	0.71	1.9	6.3	1.1	*10.01*	0.98	9.25	0.83	*11.06*	*80.83*
33	35.57	0.61	*36.18*	0.39	1.92	6.11	0.33	*8.75*	1.08	12.49	1	*14.57*	*59.5*
34	37.02	0.59	*37.61*	0.45	1.96	6.48	0.35	*9.24*	1.03	12.29	1.12	*14.44*	*61.29*
35	36.5	0.68	*37.18*	0.42	1.89	6.01	0.3	*8.62*	0.9	12.1	0.99	*13.99*	*59.79*
Mean	26.14	0.34	*26.48*	0.53	1.6	6.21	0.47	*8.75*	0.93	11.14	0.61	*12.68*	*47.92*
SD	12.17	0.15	*12.23*	0.11	0.58	1.9	0.27	*2.56*	0.27	3.82	0.26	*4.12*	*15.3*
Median	24.08	0.28	*24.36*	0.54	1.77	6.68	0.37	*9.25*	0.94	12.1	0.56	*13.77*	*44.08*
Min	12.71	0.12	*13.19*	0.3	0.5	2.35	0.21	*3.36*	0.22	4	0.18	*4.57*	*21.18*
Max	60.45	0.69	*60.9*	0.8	2.85	9.89	1.1	*13.3*	1.35	19.48	1.12	*21.33*	*84.26*

nd, not detected; *THQ*, Total Hydroquinones; *TF*, Total Flavonoids; *TC*, Total Caffeic acid derivatives; *TPP*, Total Polyphenols; Ab, arbutin; Er, eriocitrin; Lr, luteolin-7-*O*-rutinoside; Lgr, luteolin-7-*O*-β-glucuronide; L, luteolin; CA, caffeic acid; RA, rosmarinic acid; MeR, methyl rosmarinate.

magnitude approximately 1.09–4.95%CV and 2.18–5.4%CV can be obtained in routine analysis. Recoveries were from 96.3% to 102%. As these methods could be used for the determination of polyphenols, to control quality, precision is the most important validation parameter. Therefore the repeatability and the intermediate precision of the suggested HPLC method for the determination of eighteen polyphenolic compounds in herbal drugs and spices from Lamiaceae are adequate.

3.4. Isolation, chromatographic analysis and content assay

The hydrophilic extracts from the herbal drugs and spices, *T. herba*, *S. herba* and *M. herba,* were investigated by chromatographic techniques. We performed the isolation and identification of luteolin-7-*O*-β-glucuronide and lithospermic acid from the 50% aq. acetone extract of wild thyme, methyl rosmarinate, naringenin, eriodictyol, luteolin, luteolin-7-*O*-*β*-glucoside and luteolin-7-*O*-β-glucuronide from the 50% aq. acetone extract of thyme, and eriocitrin, luteolin-7-*O*-rutinoside, hesperidin and diosmin from the 50% aq. acetone extract of peppermint. Structures of isolated compounds were elucidated by comparison of their UV, ^{1}H NMR and ^{13}C NMR data with literature values (Dapkevicius et al., 2002; Harborne, 1994; Harborne & Mabry, 1982; Kelley, Hurruff, & Carmack, 1976; Kelley et al., 1975; Kobayashi et al., 2003; Lu & Foo, 2000; Parejo et al., 2004). Additionally, we recorded a ^{1}H–^{1}H COSY spectrum for LA and both ^{1}H–^{13}C HMQC and HRESI MS spectra for Lgr, LA, MeR. Lithospermic acid and luteolin-7-*O*-β-glucuronide were identified as new wild thyme constituents, and methyl rosmarinate and luteolin-7-*O*-β-glucuronide as new compounds in sweet marjoram. Methyl rosmarinate was isolated for the first time from thyme.

The presence of flavonoids, caffeic acid esters and hydroquinone derivatives in the commercial herbal drugs and spices was examined by planar chromatography using HPTLC Si60 and HPTLC NH_2 plates (Figs. 5 and 6) and by high-performance liquid chromatography, with UV

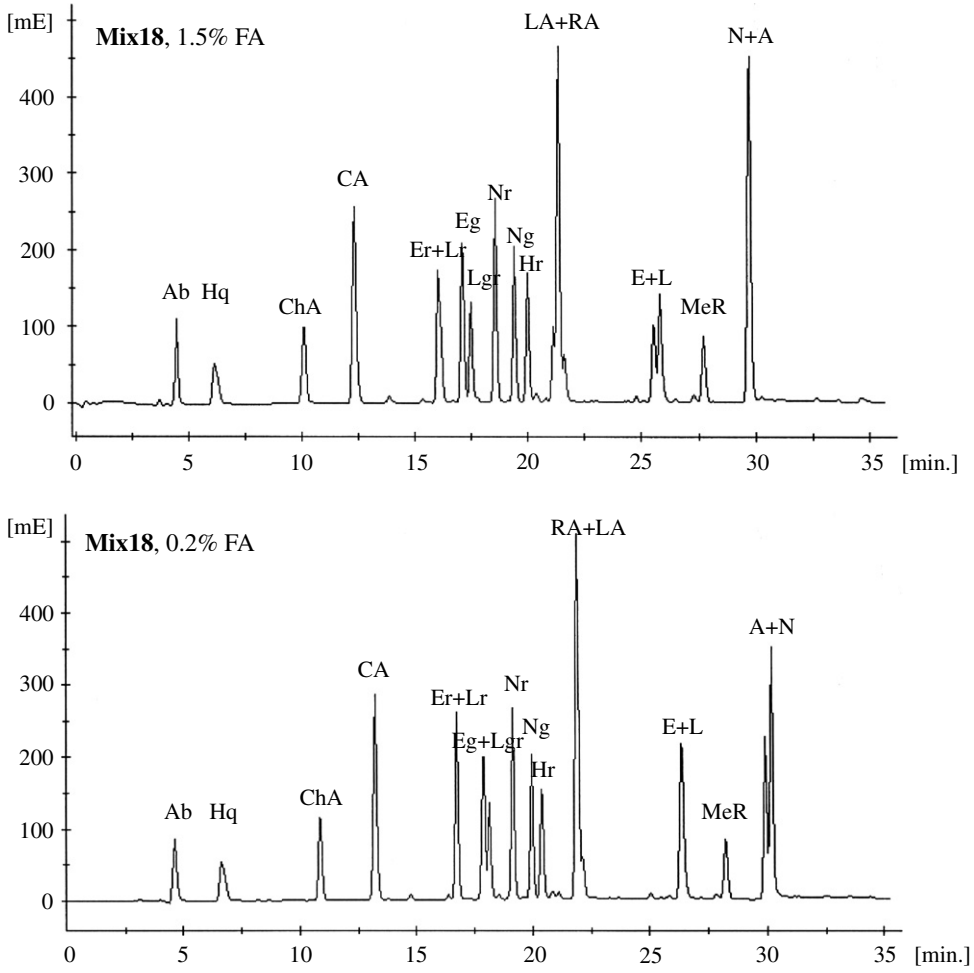


Fig. 2. HPLC RP18 chromatograms of standards Mix 18 (UV, $\lambda = 280$ nm) with various concentrations of FA in solvent systems. Ab, arbutin; Hq, hydroquinone; CA, caffeic acid; Er, eriocitrin; Lr, luteolin-7-*O*-rutinoside; Eg, eriodictyol-7-*O*-β-glucoside; Lgr, luteolin-7-*O*-β-glucuronide; Nr, narirutin; Ng, naringenin-7-*O*-β-glucoside; Hr, hesperidin; LA, lithospermic acid; RA, rosmarinic acid; E, eriodictyol; L, luteolin; MeR, methyl rosmarinate; N, naringenin, A, apigenin; (mE), milliextinction.

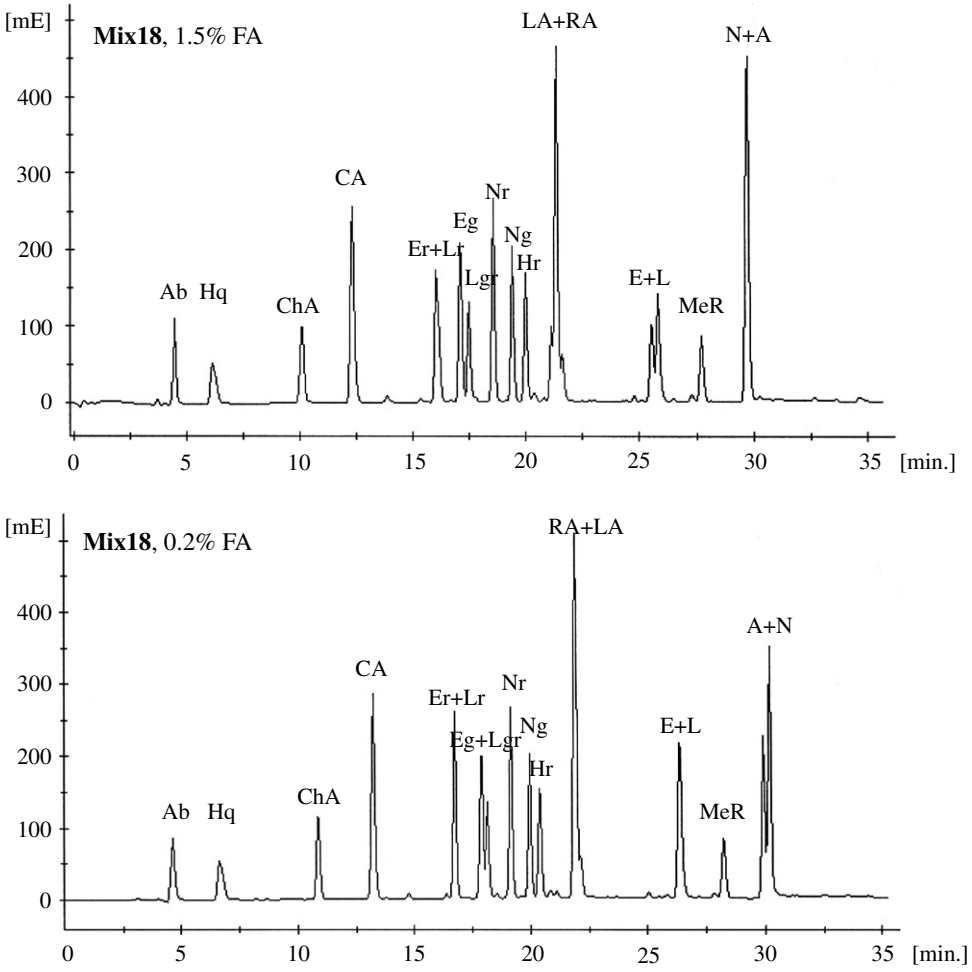


Fig. 2 (*continued*)

detection and the Beta Basic-18 column (Figs. 2 and 3). In this work we have also evaluated the contents of both glycosides and aglycones of flavones and flavanones, lithospermic acid, methyl rosmarinate, rosmarinic acid, caffeic acid, arbutin and hydroquinone in the dried herbs of thyme, wild thyme and sweet marjoram by the HPLC method. The contents of analyzed compounds have been expressed as mg per g of a dried herb and are presented in Tables 2–4.

The major of identified compounds in the hydrophilic extracts were caffeate oligomers, luteolin glycosides and hydroquinone glucoside. The predominant caffeic acid derivative was the caffeate dimer known as rosmarinic acid. Flavonoids included first of all flavones, both glycosides and aglycones, followed by minor amounts of flavanones. Seven compounds were common to all extracts: caffeic acid, rosmarinic acid, luteolin, luteolin-7-*O*-β-glucuronide, luteolin-7-*O*-β-glucoside, luteolin-7-*O*-rutinoside and eriocitrin. Two of them, rosmarinic acid and luteolin-7-*O*-β-glucuronide were present in the highest amounts, the mean concentration of RA was up to 2.19% and Lgr up to 1.44% (Fig. 7).

Herbal teas from thyme contained, first of all, rosmarinic acid at a level 1.1–3.53% of dried herb. The percentage concentrations of RA in wild thyme and sweet marjoram were in the range 0.76–2.44% and 0.4–1.95%, respectively. Contents of rosmarinic acid in examined lamiaceous species were comparable to the results of other researchers (Dorman et al., 2004; Kosar et al., 2005; Parejo et al., 2004; Wang et al., 2004). The wild thyme herb had a high amount of lithospermic acid, up to 4.28% (mean 1.21%) but, in thyme and sweet marjoram, the compound was absent. Methyl rosmarinate accrued only in thyme and sweet marjoram in amounts below 0.15% (mean 0.06%). Free caffeic acid was detected in all herbal drugs and spices at a low concentration (0.02–0.13%). Among analyzed polyphenols on recorded HPLC chromatograms, we observed two unknown compounds, named X_1 and X_2 (Fig. 3) which are probably caffeate oligomers. They were detected in hydrophilic extracts in variable quantities. Several unknown caffeate conjugates were also observed as blue or dark brown bands in the HPTLC chromatogram (Fig. 5). We did not record chlorogenic acid in any chromatograms (HPTLC and HPLC).

The highest content of luteolin-7-*O*-β-glucuronide was estimated in *S. herba,* between 0.57% and 2.49%. *T. herba* and *M. herba* yielded 0.32–1.45% and 0.23–0.99% of this flavonoid, respectively. Among flavonoids, luteolin, eriodictyol and their rutinosides were present at a several-times lower concentrations (aglycones 0.003–0.37%, glycosides 0.03–0.32%). The presence of luteolin-7-*O*-β-glucoside was shown by the use of planar chromatography on HPTLC NH_2 plates with the mobile phase acetone – acetic acid (85:15, v/v) (Fig. 6) where luteolin-7-*O*-β-glucuronide remained at the start line and using the HPLC method with a solvent system containing 0.2% FA addition. The mean concentration of luteolin-7-*O*-β-glucoside was 0.22% (0.09–0.36%) in thyme herb, in sweet marjoram herb 0.05% (up to 0.1%) and in wild thyme herb 0.01% (up to 0.03%). Apigenin was recorded in Tv and Mh extracts at a concentration below its LOQ. Flavanones such as eriodictyol-7-*O*-β-glucoside, naringenin, naringenin-7-*O*-β-glucoside, naringenin-7-*O*-rutinoside (narirutin) and hesperidin were detected only in thyme (mean contents 0.06%, 0.04%, 0.06%, 0.03% and 0.1%, respectively) under the chromatographic conditions used. No traces of apigenin glycosides and diosmin were recorded in analyzed extracts.

Hydroquinone derivatives, represented mainly by arbutin (hydroquinone-*O*-β-glucoside), occurred in sweet marjoram and thyme which agreed with literature data. Arbutin is commonly used as urinary tract antiseptic. Therefore, it was interesting to evaluate the amount of this hydroquinone glucoside in the analyzed plants. The percentages of arbutin in sweet marjoram and thyme were in the ranges 1.27–6.04% and 0.06–0.11%, respectively. Free hydroquinone was recorded only in *M. herba* at a concentration below 0.07%. The total hydroquinone content in both species appeared at average levels, 2.65% for Mh and 0.08% for Tv.

The content of total polyphenols (TPP), represented by derivatives of hydroquinone (THQ), caffeic acid esters (TC) and flavonoids (TF), estimated with the HPLC method used, was 2.12–8.43% (mean 3.96–4.79%) of dried herbs and it was higher than the described essential oil contents. In thyme, the average TPP was insignificantly lower than those

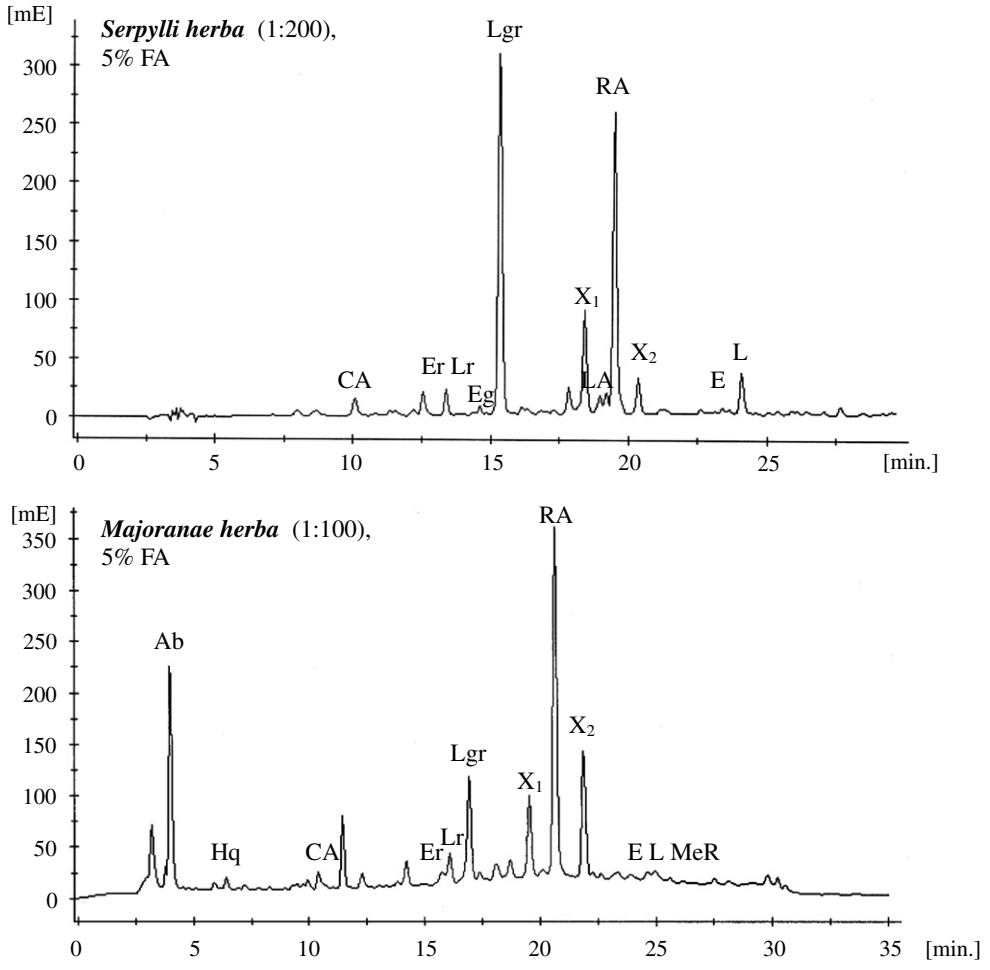


Fig. 3. HPLC RP18 chromatograms of typical 50% aq. methanolic extracts (UV, $\lambda = 280$ nm). Ab, arbutin; Hq, hydroquinone; CA, caffeic acid; Er, eriocitrin; Lr, luteolin-7-*O*-rutinoside; Eg, eriodictyol-7-*O*-β-glucoside; Lgr, luteolin-7-*O*-β-glucuronide; Nr, narirutin; Ng, naringenin-7-*O*-β-glucoside; Hr, hesperidin; LA, lithospermic acid; RA, rosmarinic acid; E, eriodictyol; L, luteolin; MeR, methyl rosmarinate; (mE), milliextinction.

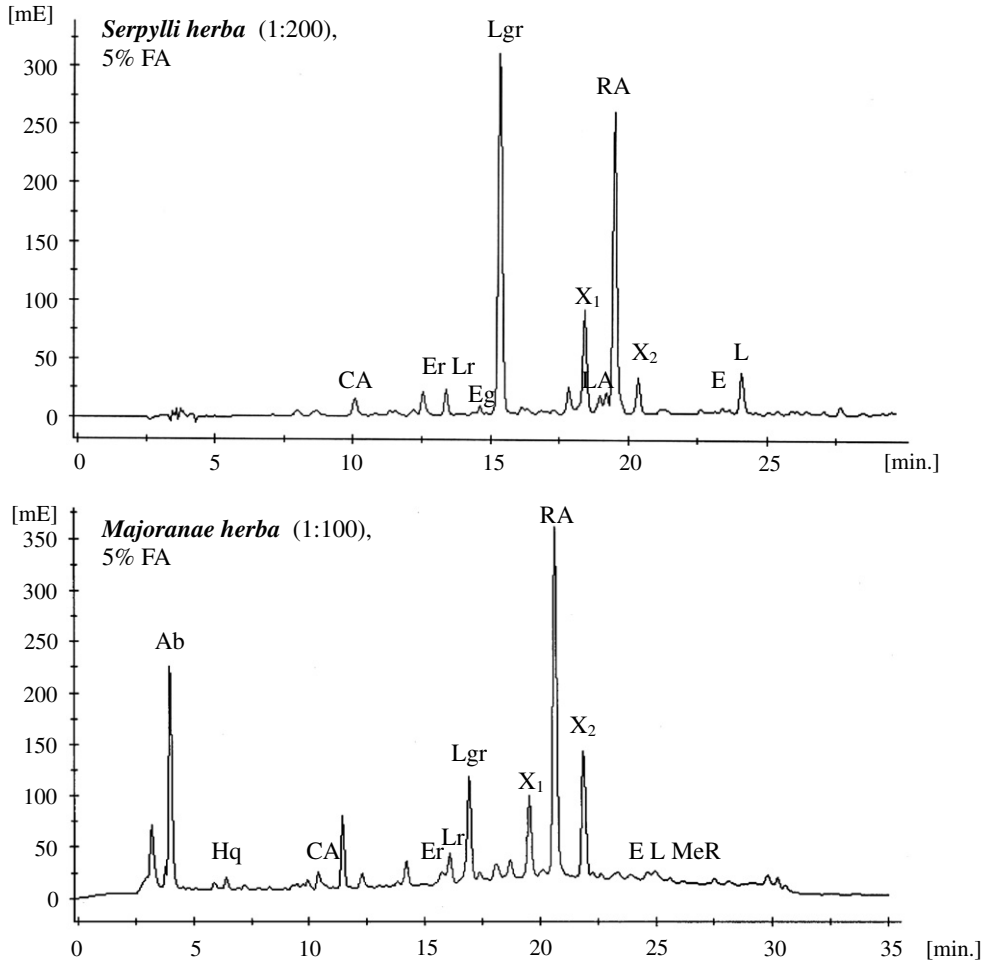


Fig. 3 (*continued*)

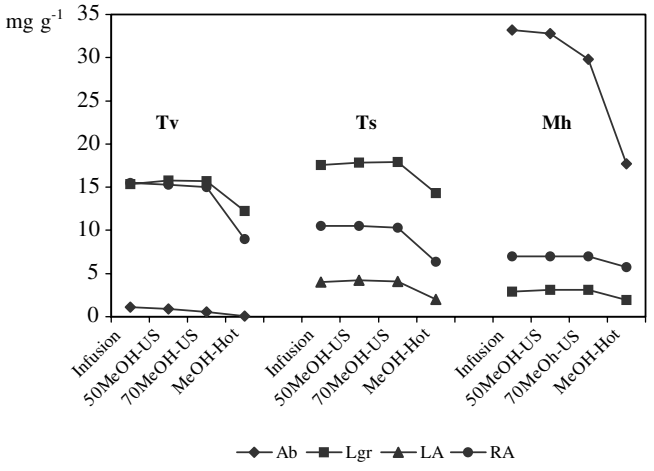


Fig. 4. Analysis of extraction efficiency. Concentration of main polyphenols (Ab, arbutin; Lgr, luteolin-7-*O*-β-glucuronide; LA, lithospermic acid; RA, rosmarinic acid) from thyme, wild thyme and sweet marjoram (Tv, Ts, Mh, respectively) is expressed as mg per g of dried herb achieved with various extraction procedure; extraction time is 15 min.

in wild thyme and sweet marjoram (Fig. 7). High concentrations of caffeate oligomers up to 6.82%, and flavonoids, up to 2.99%, were observed in wild thyme herb (mean TC – 2.71%, TF – 1.91%) Similarly, the thyme herb was a source of caffeate oligomers, up to 3.61%, and flavonoids, up to 2.16% (mean TC – 2.3%, TF – 1.58%). Sweet marjoram herb delivered, first of all, hydroquinone derivatives, up to 6.09% (mean THQ – 2.65%), next caffeate oligomers, up to 2.13% and flavonoids, up to 1.33% (mean TC – 1.27% and TF – 0.87%).

Polyphenolic compounds are plant secondary metabolites of biological and pharmacological significance. Luteolin-7-*O*-β-glucuronide was confirmed, among others, in *Thymus* (Dapkevicius et al., 2002; Kobayashi et al., 2003), *Salvia* (Lu & Foo, 2001, 2002) and *Lycopus* (Gumbinger, Winterhoff, Wylde, & Sosa, 1992). It reveals antioxidant (Lu & Foo, 2001; Parejo et al., 2004), antiallergic (Kobayashi et al., 2003) and antigonadotropic effects (Gumbinger et al., 1992). Caffeic acid derivatives exhibit various properties, such as antioxidative, antimutagenic, antiinflamatory, hepatoprotective and antimicrobial (Findley, Hollstein, & Besch, 1985; Liam, Fernandes-Ferreira, & Pereira-Wilson, 2006; Lu & Foo, 2001; Tewtrakul et al., 2003). In addition, rosmarinic acid shows antithrombotic, antiallergic, as well as antiviral, activity against herpes simplex virus and human immunodeficiency virus (Findley et al., 1985; Lu & Foo, 2001; Parejo

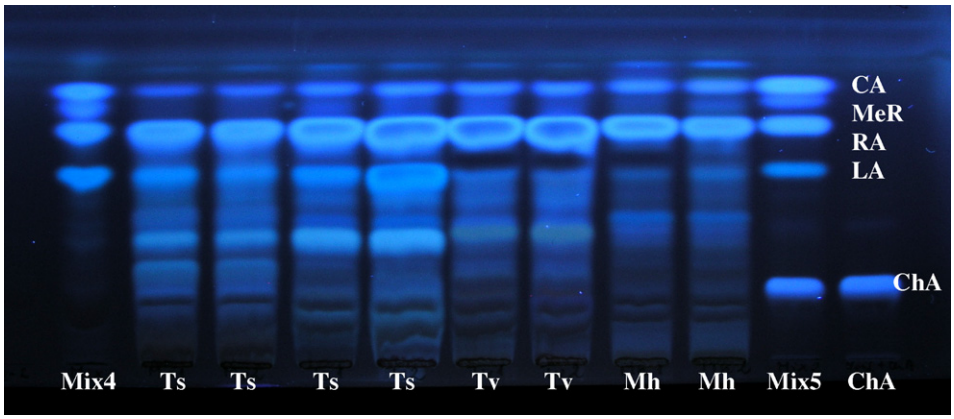


Fig. 5. HPTLC Si60 chromatogram of analyzed 50% aq. methanolic extracts and standards developed with diisopropyl ether–acetone–water–formic acid (50:30:10:10). Compounds were detected under UV light at 365 nm. Tracks: CA, caffeic acid; ChA, chlorogenic acid; LA, lithospermic acid; MeR, methyl rosmarinate; RA, rosmarinic acid; Mix4, LA + RA + MeR + CA; Mix5, ChA + LA + RA + MeR + CA; Tv, *Thymus vulgaris* harb; Ts, *Thymus serpyllum* herb; Mh, *Majorana hortensis* herb.

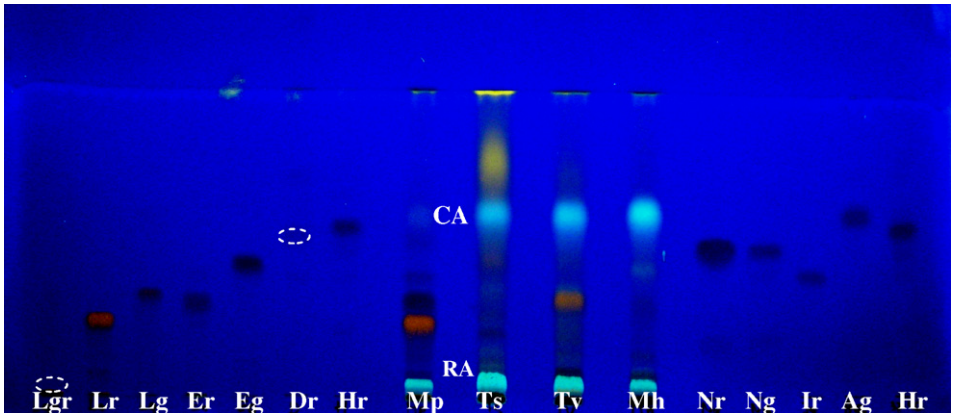


Fig. 6. HPTLC NH_2 chromatogram of analyzed 50% aq. methanolic extracts and standards developed with acetone-acetic acid (85:15). Compounds were detected under UV light at 365 nm. Tracks: Lgr, luteolin-7-*O*-β-glucuronide; Lr, luteolin-7-*O*-rutinoside; Lg, luteolin-7-*O*-β-glucoside; Er, eriocitrin; Eg, eriodictyol-7-*O*-β-glucoside; Dr, diosmin; Hr, hesperidin; Nr, narirutin; Ng, naringenin-7-*O*-β-glucoside; Ir, isorhoifolin; Ag, apigenin-7-*O*-β-glucoside; Tv, *Thymus vulgaris* herb; Ts, *Thymus serpyllum* herb; Mh, *Majorana hortensis* herb; Mp, *Mentha* × *piperita* leaf.

et al., 2004; Tewtrakul et al., 2003). Lithospermic acid was previously found in several species of *Lithospermum, Lycopus* and *Salvia* (Findley et al., 1985; Kelley et al., 1975, 1976; Lu & Foo, 2002). It was proved that lithospermic acid, just like rosmarinic acid, also had radical-scavenging and antiviral properties. Moreover, rosmarinic and lithospermic acids inhibit gonadotrophin release, xanthine oxidase and adenylate cyclase activities (Findley et al., 1985; Kohda et al., 1989; Lu & Foo, 2001, 2002). They prevent lipid peroxidation and platelet aggregation (Lin, Chang, Kuo, & Shiao, 2002). Arbutin possesses urinary anti-infective and skin bleaching properties (inhibitor of melanin synthesis) (Bruneton, 1999).

4. Conclusions

The investigated herbal drugs and spices provide arbutin, luteolin-7-*O*-β-glucuronide, rosmarinic acid, lithospermic acid and other polyphenols in high amounts, even up to 84.3 mg g^{-1} of a dried herb. In conclusion, the detected polyphenolic compounds, together with acidic polysaccharide, acetophenones, hydroxyjasmones, biphenyls, terpenoids and essential oil constituents, might be considered as potential active ingredients of aqueous, aq. alcoholic and alcoholic extracts from examined aromatic plants.

The presented chromatographic methods (HPLC, HPTLC) are simple and therefore easy to use in routine tests. The reversed-phase HPLC method allows successful separation of all analyzed compounds and could be used for rapid quality-control analysis of various plants and commercially available herbal drugs. By testing this method, using validation parameters, we were able to show that the presented chromatographic conditions were suitable for estimating these eighteen polyphenols in herbal products.

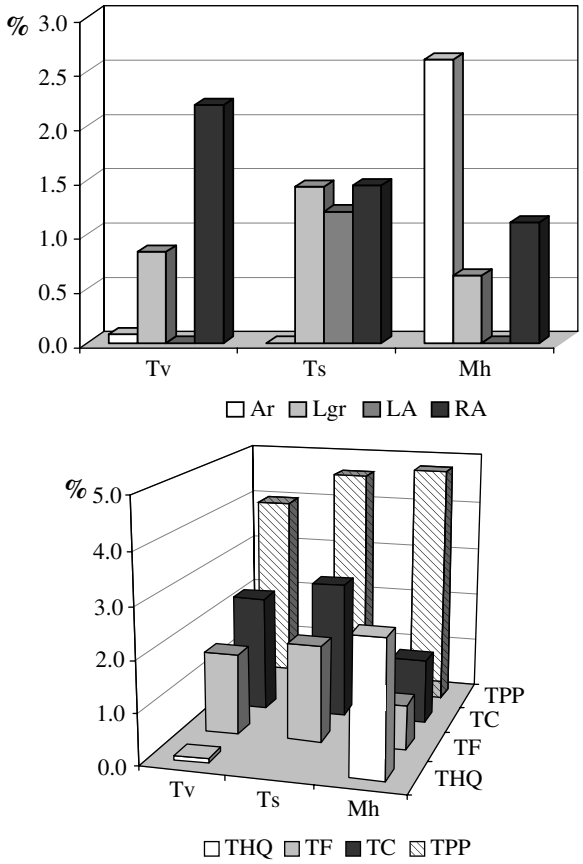


Fig. 7. Percentage distribution of individual polyphenols (average content of Ar, arbutin; Lgr, luteolin-7-*O*-β-glucuronide; LA, lithospermic acid; RA, rosmarinic acid) and total polyphenolic compounds (THQ, Total Hydroquinones; TF, Total Flavonoids; TC, Total Caffeic acid derivatives; TPP, Total Polyphenols) present in 50% aq. methanolic extracts of thyme, wild thyme and sweet marjoram (Tv, Ts, Mh, respectively).

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