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Analysis of the constituents of aqueous preparations of *Stachys recta* by HPLC–DAD and HPLC–ESI-MS

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ABSTRACT

In the present study a method based on liquid chromatography with diode array detection (HPLC/DAD) coupled to an electrospray ionization (ESI) interface for the simultaneous determination of phenolic constituents in three aqueous preparations of the herbal medicinal drug *Stachys recta*. The developed assay was simple and effective and permitted the quality control of *S. recta* decoctions and infusion. Overall, 30 constituents were detected and identified, belonging mainly to three classes of compounds: caffeoylquinic acids, phenylethanol glycosides and flavonoids. 15 of them were quantified having a lower limit not less than 0.02% of the lyophilized extracts. Only seven of them were previously reported in this species, while 23 were identified for the first time as constituents of *S. recta*. HPLC–DAD–ESI-MS analysis provided evidence for the certain identification of the main constituents and in some cases of their isomers. Eight constituents were isolated and their structure elucidated by HPLC–ESI-MS and 1D- and 2D-NMR spectroscopy. Among the investigated preparations, the infusion seems to be the best method to extract the native constituents of the plant, while decoction is a more aggressive treatment and causes partial degradation of some acylated flavonoids.

1. Introduction

Herbal teas are one of the most common forms of oral aqueous preparations, used also externally in traditional medicine and their wide use is mainly due to the convenience of their preparation. They are obtained from one or more herbal substances by means of decoction, infusion or maceration, usually prepared immediately before use [1]. In continuing our studies on traditional preparations of herbal drugs [2–4] we now report the investigation of infusions and decoctions of *Stachys recta* L. (yellow woundwort). This plant is largely diffused in Europe, well known for its anxiolytic properties, in Italy called "erba della paura", which means "herb that keeps away fear", which is used for oral administration and externally after preparation of different herbal teas [5]. Recent pharmacological studies concerning the hydroalcoholic extracts of plants of the genus *Stachys* have confirmed the antiinflammatory and anxiolytic properties of these plants and justified their ethnopharmacological uses. These properties are mostly attributed to the high phenolic content of *Stachys* sp. [6].

Aim of the study was to develop a simple and rapid HPLC method for the quality control of this herbal drug and for the analysis of its preparations, namely an infusion and two decoctions of *S. recta*, in order to establish which one could represent the best method of preparation, due to the highest concentration of active constituents. Generally, decoctions and infusions are preferable to cold maceration as microbial contaminations that sometimes occur during storage or processing of the initial herbal material are reduced with the employment of boiling water [7]. For this reason, in this study cold water maceration was not included among the methods of preparation.

2. Materials and methods

2.1. Plant material

Aerial parts of *S. recta* L. were collected in the Tuscan Apennines during the flowering period of the plant in May–June of 2008. The plant material was identified by Professor Laura Maleci Bini, Department of Vegetal Biology, University of Florence.

2.2. Chemicals

All solvents used were HPLC grade; CH_3CN and MeOH for HPLC were purchased from Merck (Darmstadt, Germany). Formic acid (85%, v/v) was provided by Carlo Erba (Milan, Italy). Water was purified by a Milli-Qplus system from Millipore (Milford, MA, USA).

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0.45 mm PTFE membrane filter was purchased from Waters Co. (Milford, MA). All laboratory chemicals used in this study were of reagent grade.

2.3. Standards

For the quantitative analysis the following standards were used: verbascoside reference standard (purity 98% by HPLC and NMR) was an isolated compound from IRB (Istituto di Ricerche Biotecnologiche) of Altavilla Vicentina. Chlorogenic acid was purchased from Fluka Chemie AG (Switzerland). Isoscutellarein-7-*O*-2″-*O*-[6‴-*O*-acetyl-β-D-allopyranosyl (1 → 2)]-β-D-glucopyranoside was isolated (purity more than 98% by HPLC and NMR) as described in Section 2.5. For the qualitative analysis, apart from the standards mentioned above, the following standards were used: caffeoylquinic esters (3-caffeoylquinic acid, 4-caffeoylquinic acid) are isolated products kindly supplied by Professor Corrado Trogolo Università la Sapienza, Roma [8]; forsythoside B was previously isolated from *Marrubium velutinum* [9].

2.4. Herbal preparations

To prepare the decoctions of *S. recta* aerial parts, two procedures were used: 10 g of dry aerial parts of *S. recta* was put in 500 ml of water and boiled for 20 min (SR-D1). After cooling it was filtered and finally lyophilized to give a solid residue of 1.7 g. The same procedure was repeated for another decoction (SR-D2) boiling for 5 min and affording a lyophilized solid residue of 1.9 g. The infusion (SR-I) was prepared as follows: 500 ml of boiling water was added to 10 g of the herbal drug and filtered after cooling. After lyophilization it gave a residue of 1.3 g.

The EtOH extract was obtained using 200 g of herbal drug (HD) macerated with 6 l of EtOH (3× 2 l). A total of 15.7 g of extract was obtained. For the HPLC–DAD–MS analysis samples were obtained by dissolving and filtrating the dried residues (about 3 mg exactly weighed) with 1 ml of MeOH:H_2O 2:1. Lyophilization and re-dilution were preferred to direct analysis of the prepared decoction/infusion in order to evaluate the yield of the preparata, and to assure stability.

2.5. Isolation of the characteristic constituents

Lyophilized ethanol extract from the leaves of *S. recta* (15.5 g) was redissolved in 200 ml of H_2O:MeOH 4:1, defatted with cyclohexane and then partitioned (in triplicate) with equal volumes of cyclohexane:diethylether 1:3 and with a mixture of ethyl acetate:cyclohexane 4:1 to obtain three major fractions: S_1–S_3. The first mixture was chosen in order to remove the terpenic content which is well documented in the literature [10], while the second system in an attempt to separate the more apolar acylated flavonoid glycosides characteristic of the genus [6]. Indeed, TLC and HPLC–DAD–MS analyses showed that fraction S_3 was richer in phenylethanol glycosides than S_2, while both extracts contained the representative acetylated isoscutellarein derivatives similar to the decoctions and infusion. Fraction S_2 (822 mg) was subjected to size exclusion chromatography over a Sephadex LH-20 (Sigma–Aldrich, Amersham, Sweden) column (22 cm × 3 cm), using a mixture of MeOH:H_2O 50:50 as mobile phase and afforded 10 fractions (A_1–A_{10}). Fraction A_2 (133.6 mg) was applied on column chromatography on silica gel using solvent mixtures of increasing polarity to yield 11.7 mg of 8-acetylharpagide (**6**) [11]. Fraction A_3 (24.6 mg) was subjected to CC over Sephadex LH-20 (MeOH:H_2O 50:50) and afforded pure 4′-methylisoscutellarein-7-*O*-2″-*O*-[6‴-*O*-acetyl-β-D-allopyranosyl (1 → 2)]-β-D-glucopyranoside (**24**) [12]. Fraction A_7 (29.6 mg) was further purified with column chromatography over Sephadex LH-20 (MeOH:H_2O 50:50) and yielded 9.3 mg of pure isoscutellarein-7-*O*-2″-*O*-[6‴-*O*-acetyl-β-D-allopyranosyl (1 → 2)]-β-D-glucopyranoside (**20**). Its purity was higher than 98% (measured by HPLC and ^{1}H NMR). ^{1}H NMR data were consistent with the previously published data [12]. Fraction A_8 (16.1 mg) was further purified with CC over Sephadex LH-20 (MeOH:H_2O 50:50) and afforded 10.1 mg pure isoscutellarein-7-*O*-6″-*O*-acetyl-2″-*O*-[6‴-*O*-acetyl-β-D-allopyranosyl (1 → 2)]-β-D-glucopyranoside (**25**) [13]. Fractions A_9 and A_{10} were combined and subjected to CC over Sephadex LH-20 (MeOH:H_2O 50:50) and afforded pure 3,5-di-caffeoylquinic acid (**18**) [14], a mixture of the latter with 4,5-di-caffeoylquinic acid (**19**) and impure apigenin-7-*O*-(6″-p-*E*-coumaroyl)-glucopyranoside (**27**) and apigenin-7-*O*-(3″-p-*E*-coumaroyl)-glucopyranoside (**29**).

2.6. HPLC apparatus

2.6.1. HPLC–DAD analysis instrumentation

The HPLC system consisted of a HP 1100 L instrument with a Diode Array Detector and managed by a HP 9000 workstation (Agilent Techologies, Palo Alto, CA, USA). The column was a Hibars Prepacked column RT (250 mm × 4.6 mm) with a particle size of 5 mm (Merck, Darmstadt, Germany) maintained at 26 °C. The eluents were H_2O at pH 3.2 by formic acid (A) and acetonitrile (B). The following multi-step linear gradient was applied: from 13% B to 15% B in 10 min, then to 25% B in 10 min followed by a plateau for 5 min; 12 min to 60% B and a final step of 5 min to initial conditions. Total time of analysis was 38 min, equilibration time 10 min, and flow rate was 0.8 ml/min, oven temperature 26 °C. The UV–vis spectra were recorded between 220 and 500 nm and the chromatographic profiles were registered at 240, 280, 330 and 350 nm.

2.6.2. HPLC–MS analysis instrumentation

The HPLC system described above was interfaced with a HP 1100 MSD API-electrospray (Agilent Technologies, Palo Alto, CA, USA). The interface geometry, with an orthogonal position of the nebulizer with respect to the capillary inlet, allowed use of analytical conditions similar to those used for HPLC–DAD analysis in order to achieve the maximum sensitivity of ESI values. The same column, time period and flow rate were used during the HPLC–MS analyses. Mass spectrometry operating conditions were optimised in order to achieve maximum sensitivity values: negative and positive ionization mode, scan spectra from *m*/*z* 100 to 800, was used with a gas temperature of 350 °C, nitrogen flow rate of 10 l/min, nebulizer pressure 30 psi, quadrupole temperature 30 °C, capillary voltage 3500 V. The applied fragmentors were in the range 60–220 V.

2.7. Identification of peaks and peak purity

Identification of all constituents was performed by HPLC–DAD and MS analysis by comparing the retention time, the UV and MS spectra of the peaks in the samples with those of authentic reference samples or isolated compounds and in some cases data reported in the literature. The purity of peaks was checked by a Diode Array Detector coupled to the HPLC system, comparing the UV spectra of each peak with those of authentic references samples and/or by examination of the MS spectra.

2.8. Linearity and repeatability of the standards and samples

The linearity range of responses of the standards was determined on five concentration levels with three injections for each level. Calibration graphs for HPLC were recorded with sample amounts ranging from 10^{-3} μg to 20 μg: stock solutions of the standards were prepared at different concentrations ranging from 0.0005 to 0.8 mg/ml and injected into HPLC (injection volumes

varying from 2 to 25 μl). To evaluate the repeatability, six samples of each residue were analyzed by HPLC. The contents of each constituent were evaluated to calculate the relative standard deviation (RSD). To evaluate the repeatability of the injection integration, the standard solutions and each sample were injected six times and the relative standard deviation values were calculated.

Samples were stable for not more than 24 h, due to partial degradation of the acetylated flavonoid glycosides to their deacylated analogues. However, intra- and inter-day time variations were carried out. For the intra-day variability test a known sample (SD-2) was analyzed in six replicates within 1 day, while for inter-day variability test, the solutions were examined in triplicates for 3 consecutive days.

2.9. Quantitative determination of constituents

The method of external standard was applied to quantify each compound. Quantification of individual constituents was performed using a regression curve, each point in triplicate. Measurements were performed at 330 nm for the caffeoylquinic acids and phenylethanol glycosides, and for isoscutellarein-7-*O*-2″-*O*-[6‴-*O*-acetyl-β-D-allopyranosyl (1 → 2)]-β-D-glucopyranoside and its derivatives at 280 nm. LOD (limit of detection) and LOQ (limit of quantitation).

2.10. Other analytical methods

2.10.1. TLC analysis

TLC analysis of the fractionation was carried out on Merck plates pre-coated with silica gel 60 F_{254} (Art.5554) using CH_2Cl_2–MeOH–H_2O (85:15:1.5) as elution system. Detection was done under UV-light using Natturstoff spray reagent consisting of equal volumes of 1% (w/v) solution of diphenylboric acid 2-aminoethyl ester (Sigma Chemical) in methanol (A) and 5% (w/v) solution of polyethyleneglycol 400 (PEG) in ethanol (B) [15].

2.10.2. NMR analysis

Final checking of the purity of the isolated compounds which were used as standards was carried out by both LC–DAD–MS according to the method reported in Section 2.6 and by NMR spectroscopy. NMR spectroscopy was also used to elucidate the structure of all isolated constituents by means of 1D- and 2D-NMR (COSY, HSQC, HMBC, ROESY) experiments. NMR spectra were recorded in CD_3OD on a Bruker DRX-400 instrument at 295 K. Chemical shifts are given in ppm (δ) and were referenced to the solvent signals at 3.31 and 49.5 ppm for ^1H and ^{13}C NMR, respectively.

3. Results and discussion

The present paper reports on the qualitative profiles of different extracts of *S. recta* aerial parts and the quantification of the constituents of the lyophilized extracts. The herbal drug was submitted to different extraction procedures in accordance with the traditional preparations: two decoctions obtained with different boiling times and an infusion. An EtOH macerate was also prepared due to the very high capacity of this solvent to recover organic constituents and because it is a relatively gentle extraction procedure suitable to provide the chemical profile of the herbal drug avoiding degradation of labile constituents. As described in the experimental part the samples were lyophilized and submitted to HPLC/DAD/ESI-MS analysis to qualitatively and quantitatively evaluate the constituents.

The constituents of the three aqueous preparations were identified by UV and MS spectral data. The qualitative profiles of the decoctions and infusion were quite similar. In Fig. 1 the HPLC/DAD chromatograms of the investigated extracts of *S. recta* at 330 nm are presented. HPLC/DAD chromatogram of the EtOH extract was considered only for comparison reasons. Data concerning identification of the peaks are shown in Table 1, where the retention time, UV–vis absorptions and electrospray ionization mass spectrometry

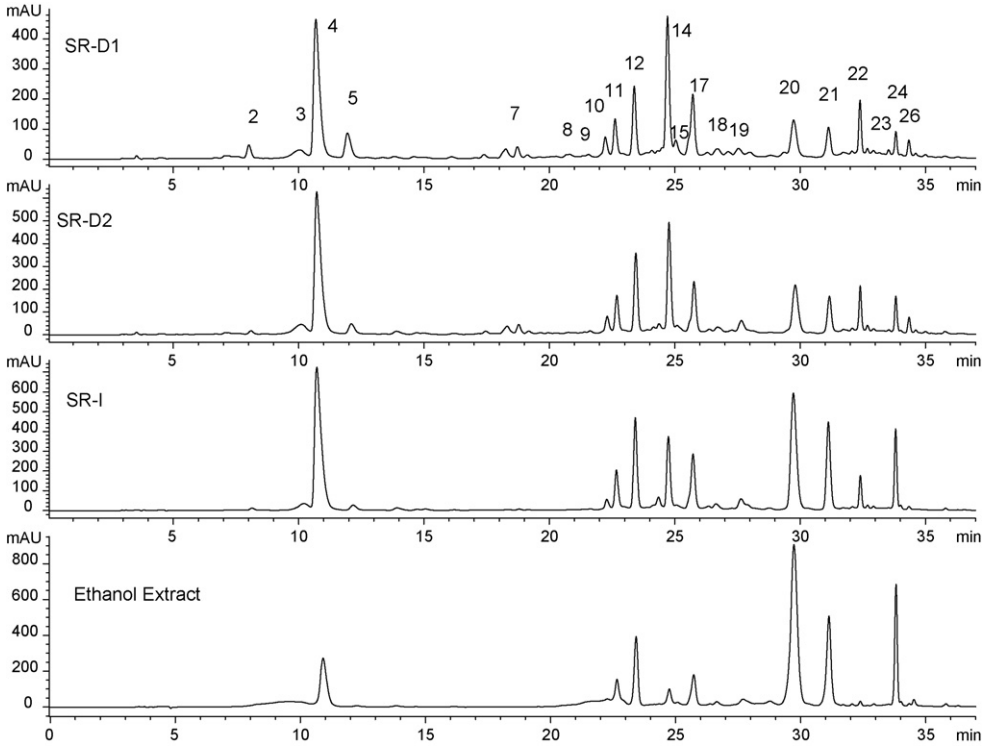


Fig. 1. Chromatograms of decoction 1 (SR-D1), decoction 2 (SR-D2) and infusion (SR-I) of *Stachys recta* recorded at 330 nm. Below, for comparison reasons the chromatogram of the ethanol extract is displayed.

Table 1
Positive and negative MS fragmentation and UV–vis absorption data of the compounds detected in the three extracts of *Stachys recta*.

No.		Rt	UV (nm)	Negative	Positive	Identification	Mode of identification
1	Ir1	4.8	257	**523** $[M-H]^-$	**547** $[M+Na]^+$, 563 $[M+K]^+$	Melittoside	UV/MS
2	1-CQA	7.1	297, 324	135, 179, 191, **353** $[M-H]^-$	117, 135, 163, **377** $[M+H]^+$	1-Caffeoylquinic acid (1-CQA)	UV/MS
3	3-CQA	9.3	298, 325	179, 191, **353** $[M-H]^-$	117, 135, 163, **377** $[M+H]^+$	3-Caffeoylquinic acid	UV/MS + std
4	5-CQA	10.5	298, 324	179, 191, **353** $[M-H]^-$	117, 135, 163, **377** $[M+H]^+$	Chlorogenic acid (5-caffeoylquinic acid, 5CQA)	UV/MS + std
5	4-CQA	10.9	297, 326	135 173, 179, 191, **353** $[M-H]^-$		4-Caffeoylquinic acid (4-CQA)	UV/MS + std
6	Ir2	11.3		**405** $[M-H]^-$	**407** $[M+H]^+$, 429 $[M+Na]^+$, 445 $[M+K]^+$	8-Acetylharpagide	UV/MS + NMR
7	Ph1	18.7	292, 328	591 $[M-\text{caffeoyl}]^-$, 639 $[M-\text{apiose}]^-$, 621 $[M-\text{apiose}-H_2O-H]^-$, 753 $[M-H_2O-H]^-$, **771** $[M-H]^-$	**795** $[M+Na]^+$	β-OH-Forsythoside B	UV/MS
	Ph1	19.2	291, 330	639 $[M-\text{Rha}]^-$, **771** $[M-H]^-$	**795** $[M+Na]^+$	β-OH-Forsythoside B isomer	UV/MS
8	Ph2	21.2	289, 327	135, 161, 179 $[\text{caffeoyl}]^-$, 487 $[M-\beta\text{-hydroxy tyrosol}]^-$, 459 $[M-\text{caffeoyl}]^-$, 621 $[M-H_2O-H]^-$, **639** $[M-H]^-$	**663** $[M+Na]^+$	β-OH-Acteoside	UV/MS
		21.7	289, 327	621 $[M-H_2O-H]^-$, **639** $[M-H]^-$	**663** $[M+Na]^+$	β-OH-Acteoside-distereomer	UV/MS
9	Ph3	21.9	285, 326	161,179, 193 $[\text{feruloyl}]^-$, 609 $[M-\text{feruloyl}]^-$, 621 $[M-\text{apiose-}H_2O]^-$, 639 $[M-\text{Rha}]^-$, 767 $[M-H_2O-H]^-$, 769 $[M-OH]^-$, **785** $[M-H]^-$	147, 163, **809** $[M+Na]^+$	Betonyoside E	UV/MS
10	F1	22.5	255, 277, 301, 337	301, 445 $[M-180]^-$, 463 $[M-162]^-$, **625** $[M-H]^-$	303, **627** $[M+H]^+$	Hypolaetin-7-*O*-(2-allosyl)-glucopyranoside	UV/MS
11	Ph4	22.9	291, 328	593 $[M-\text{caffeoyl}]^-$, 607 $[M-\text{apiose}]^-$, 623 $[M-\text{apiose residue}]^-$, **755** $[M-H]^-$	117, 135, 163, 609 $[M-\text{apiose}]^+$, **779** $[M+Na]^+$	Forsythoside B	std
12	Ph5	23.7	291, 330	**623** $[M-H]^-$	117, 135, 163, **647** $[M+Na]^+$	Acteoside	std
13	Ph6	24.8	290, 327	161, 347, 623 $[M-\text{caffeoyl}]^-$, 755 $[M-\text{OMe}]^-$, **785** $[M-H]^-$	163, 371, 647, 779 $[M-\text{OMe}+Na]^+$, **809** $[M+Na]^+$	β-OH-Forsythoside B methylether	UV/MS
14	F2	25.0	278,306, 326	285, 429 $[M-180]^-$, **609** $[M-H]^-$	287, **611** $[M+H]^+$	Isoscutellarein-7-*O*-[allosyl(1 → 2)]-glucopyranoside	UV/MS
15	Ph7	25.3	289, 327	**623** $[M-H]^-$	**625** $[M+H]^+$	Isoacteoside	UV/MS
16	3,4diCQA	25.9	297, 324	135, 161, 173, 191, 335, 353, **515** $[M-H]^-$	**517** $[M+H]^+$	3,4-Dicaffeoyl quinic acid	UV/MS + NMR
17	F3	26.1	255, 277, 299, 338	315, 459 $[M-180]^-$, **639** $[M-H]^-$	317, **641** $[M+H]^+$	3′-Hydroxy-4′-*O*-methylisoscutellarein-7-*O*-allosyl-(1 → 2)-glucopyranoside	UV/MS
18	3,5diCQA	26.6	297, 325	135, 179, 191, 353, **515** $[M-H]^-$	355, **517** $[M+H]^+$	3,5-Dicaffeoyl quinic acid	UV/MS + NMR
19	4,5diCQA	28.2	297, 324	161, 173, 179, 191, 353, **515** $[M-H]^-$	355, **517** $[M+H]^+$	4,5-Dicaffeoyl quinic acid	UV/MS + NMR
20	F4	30.5	276, 306, 326	285 [A−H], 429 $[M-180-\text{OAc}]^-$, **651** $[M-H]^-$	287 $[A+H]^+$, **653** $[M+H]^+$	Isoscutellarein-7-*O*-[6‴-acetyl-allosyl-(1 → 2)]-glucopyranoside	UV/MS + NMR
21	F5	31.5	254, 277, 300, 336	315 $[A-H]^-$, 459 $[M-180-\text{OAc}]^-$, **681** $[M-H]^-$	317 $[A+H]^+$, **683** $[M+H]^+$	3′-Hydroxy-4′-*O*-methylisoscutellarein-7-*O*-[6‴-acetyl-allosyl-(1 → 2)]-glucopyranoside	UV/MS
22	F6	32.5	278, 306, 324	**299**, 471 $[M-180]^-$, **623** $[M-H]^-$	**301**, 625 $[M+H]^+$	4′-*O*-Methylisoscutellarein-7-*O*-[allosyl-(1 → 2)]-glucopyranoside	UV/MS
23	F7	32.8	254, 277, 300, 336	315 $[A-H]^-$, **501** $[M-180]^-$, **681** $[M-H]^-$	317 $[A+H]^+$, **683** $[M+H]^+$	3′-Hydroxy-4′-*O*-methylisoscutellarein-7-*O*-(6″-acetyl-hexosyl)-hexoside Isomer of **21**	UV/MS
24	F8	34.0	279, 307, 325	299, **665** $[M-H]^-$	301 $[A+H]^+$, **667** $[M+H]^+$	4′-*O*-methylisoscutellarein-7-*O*-[6‴-acetyl-allosyl-(1 → 2)]-glucopyranoside	UV/MS + NMR
25	F9	34.1	277, 306, 325	285, **693** $[M-H]^-$	287 $[A+H]^+$, **695** $[M+H]^+$	Isoscutellarein-7-*O*-[6‴, 6‴-di-acetyl-allosyl(1 → 2)]-glucopyranoside	UV/MS + NMR
26	F10	34.2	255, 277, 301, 336	315, 501 $[M-180-\text{OAc}]^-$, 681 $[M-\text{OAc}]^-$, **723** $[M-H]^-$	**725** $[M+H]^+$	3′-Hydroxy-4′-*O*-methylisoscutellarein-7-*O*-[6″, 6‴-di-acetyl-allosyl (1 → 2)]-glucopyranoside	UV/MS

Table 1 (*Continued*)

No.		Rt	UV (nm)	Negative	Positive	Identification	Mode of identification
27	F11	34.5	271, 317	145, 269, **577** $[M-H]^-$	147, 271 $[A+H]^+$, **579** $[M+H]^+$	Apigenin-7-*O*-(6″-p-*E*-coumaroyl)-glucopyranoside	UV/MS + NMR
28	F12	34.7	271, 318	485 $[M-180]^-$, **665** $[M-H]^-$	301 $[A+H]^+$, **667** $[M+H]^+$	4′-*O*-Methylisoscutellarein-7-*O*-(hexosyl)-hexoside Isomer of **24**	UV/MS
29	F13	34.8	271, 318	269, 413 $[M-coumaroyl-H_2O]^-$, 431 $[M-coumaroyl]^-$, **577** $[M-H]^-$	147, 271 $[A+H]^+$, **579** $[M+H]^+$	Apigenin-7-*O*-(3″-p-*E*-coumaroyl)-glucopyranoside	UV/MS + NMR
30	F14	36.1	280, 306, 326	299 $[A-H]^-$, **707** $[M-H]^-$	301 $[A+H]^+$, **709** $[M+H]^+$, 731 $[M+Na]^+$	4′-*O*-Methylisoscutellarein-7-*O*-[6″, 6‴-di-acetyl-allosyl-(1 → 2)]-glucopyranoside	UV/MS

A: aglycon; Ir: iridoid; CQA: caffeoylquinic acid; Ph: phenylethanol glycoside; F: flavonoid; std: standard. The bold values indicate pseudomolecular ions.

in both positive and negative ion mode [16] of all the compounds detected in the infusions and decoctions of *S. recta* are reported. The developed analytical system led to the separation and identification of the majority of the constituents, overall, 30 compounds were identified, belonging to three representing classes of constituents: quinic acid derivatives, phenylethanol glycosides and flavonoids. Flavonoid glycosides of isoscutellarein and its derivatives were the main constituents of all extracts. Negative ionization mode at high voltage of 220 eV gave the best results, for the identification of both the flavonoid and phenylethanol glycosides, while positive ionization at 120 eV led to the formation of the $[M+Na]^+$ pseudomolecular ion in most of the cases. Instead, for smaller components such as chlorogenic acid derivatives, the pseudomolecular ions *m/z* $[M+H]^+$, $[2M+H]^+$ and $[2M+Na]^+$ were better observed in the lower voltage of 60 eV.

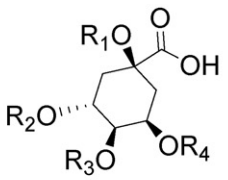
Main focus of our studies was the phenolic constituents, as they are associated with the biological properties of plants of this genus [6], however, terpenes, usually responsible for toxic activities [17] were absent in all the aqueous preparations.

3.1. Identification of caffeoylquinic acids

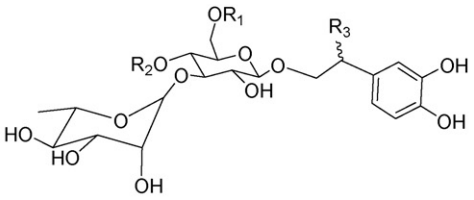
The first group of peaks of all the chromatograms consisted of four peaks at 7.1, 9.3, 10.5, 10.9 min which showed similar UV spectra with maximum absorbance at 325 nm and a shoulder around 298 nm, characteristic of caffeoylquinic acid derivatives. Quasi-molecular fragments at 353 $[M-H]^-$ and 377 $[M+Na]^+$ *m/z* and the characteristic ion at *m/z* 191 (Table 1), suggested the presence chlorogenic acid derivatives. Chlorogenic acid (5-caffeoylquinic acid, 5-CQA) was identified as the predominant phenolic acid (compound **4**). Its complete identification was further proved by comparing its UV spectra and retention times with those of the reference substance. Two further peaks at 9.3 and 10.9 min showing a quasi-molecular ion $[M-H]^-$ at *m/z* 353 (compounds **3** and **5**) were assigned to 3-caffeoylquinic acid (3-CQA) and 4-caffeoylquinic acid (4-CQA), respectively. Their fragmentation patterns were of diagnostic importance [18]. Apart from typical fragments at *m/z* 353, 191 and 179, 4-CQA gave a characteristic signal at *m/z* 173 [quinic acid–H–H_2O]$^-$ which is not observed for the other isomers [19]. The peak assignment was supported by taking into account the elution order which is reported in previous studies [18–20] and further proved with co-chromatography with pure isolates that were available in our laboratory [8]. Similarly, three more minor peaks at 25.9, 26.3 and 28.2 min were attributed to di-caffeoylquinic acid derivatives. All of them gave common quasi-molecular ions at *m/z* 515 $[M-H]^-$, 353 $[M-163]^-$ and 191 $[M-162-162-H]^-$, suggesting the presence of two caffeoyl units and a quinic acid. Co-elution with cynarin (1,3-di-caffeoylquinic acid, Rt = 13.8 min) indicated a less polar nature of these quinic acid derivatives. The peak at 26.3 min was identified as 3,5-di-caffeoylquinic acid after co-elution with the pure constituent isolated from the plant (compound **18**) (Section 2.5). A careful examination of the other two peaks revealed the presence of characteristic ions at *m/z* 173, suggesting a substitution at position 4 of the quinic acid. Considering the elution order [20], the peaks were tentatively assigned as 3,4-di-caffeoylquinic acid and 4,5-di-caffeoylquinic acid, respectively (compounds **16** and **19**). Examining the same peaks under different fragmentations (120 eV) the peak at 25.9, but not that at 28.2 min showed a very small quasi-molecular ion at *m/z* 335 which is present only in the case of the 1,3-, 1,4-, 1,5- and 3,4-di-caffeoylquinic acid derivatives, confirming the given assignment. All the above compounds are identified for the first time in *S. recta*, while there are only a few reports in the genus *Stachys* [21,22].

3.2. Identification of phenylethanol glycosides

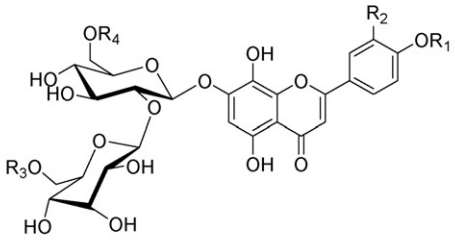
Overall seven phenylethanol glycosides were detected in *S. recta* extracts for the first time (Fig. 2), whereas these are reported in other *Stachys* species. The main phenylethanol glycosides present in all the extracts of *S. recta* were verbascoside (**12**) and forsythoside B (**11**) at 23.7 and 22.9 min, respectively. Both compounds are reported several times in the genus *Stachys* but for the first time they are identified in *S. recta*. Their identification was based on their UV absorbance and the fragmentation pattern and finally co-elution with reference standard or with previously isolated compounds available in our laboratory [9]. Especially for forsythoside B at the high voltage of 180 and 220 eV the ion *m/z* 593 [M−caffeoyl]$^-$ was observed [23] and ions at *m/z* 623 and 607 corresponding to the loss of apiose without or with the anomeric oxygen, respectively. Two peaks at earlier retention times at 18.7 and 19.2 min were assigned as two isomers of β-OH-forsythoside B (**7**). Both of them gave characteristic fragmentation patterns (Table 1) which enabled their unambiguous identification. In particular the quasi-molecular ions $[M-H_2O-H]^-$ at *m/z* 753 and $[M-H_2O-H-apiose]^-$ at *m/z* 621 correspond to intermediate – dioxane type – products which are diagnostic for this type of compounds [24]. At 21.2 and 21.7 min (compound **8**), again according with the elution order, another pair of diastereomeric forms of similar constituents were identified. Their pseudomolecular peaks at *m/z* 639 $[M-H]^-$ and the ion $[M-H_2O-H]^-$ at 621 *m/z* suggested the presence of campneoside I or campneoside II diastereomers previously reported in other *Stachys* sp. [25,26]. A peak at 21.9 min was tentatively assigned as Betonyoside E (compound **9**) previously isolated from *Stachys officinalis* [25]. Its identification was based again on the presence of ions $[M-H_2O-H]^-$ at *m/z* 767, $[M-Rha]^-$ at *m/z* 639, and $[M-Rha-H_2O]^-$ at *m/z* 621. A series of other ions at *m/z* 193, 179, 161 were typical of the presence of ferulic acid moiety [27]. The peak at 24.8 min was tentatively assigned as a methoxy derivative



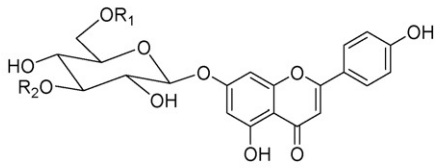
		R_1	R_2	R_3	R_4
2	**1-CQA**	Caf	H	H	H
3	**3-CQA**	H	Caf	H	H
4	**5-CQA**	H	H	H	Caf
5	**4-CQA**	H	H	Caf	H
16	**3,4-di-CQA**	H	Caf	Caf	H
18	**3,5-di-CQA**	H	Caf	H	Caf
19	**4,5-di-CQA**	H	H	Caf	Caf



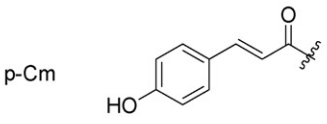
		R_1	R_2	R_3
7	**Ph1**	apiose	Caf	OH
8	**Ph2**	H	Caf	OH
9	**Ph3**	apiose	Fer	OH
11	**Ph4**	apiose	Caf	H
12	**Ph5**	H	Caf	H
13	**Ph6**	apiose	Caf	OCH_3
15	**Ph7**	Caf	H	H

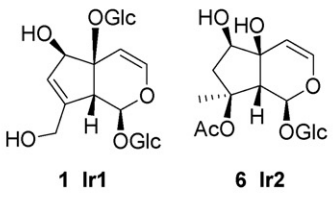


		R_1	R_2	R_3	R_4
10	**F1**	H	OH	H	H
14	**F2**	H	H	H	H
17	**F3**	CH_3	OH	H	H
20	**F4**	H	H	OAc	H
21	**F5**	CH_3	OH	OAc	H
22	**F6**	CH_3	H	H	H
23	**F7**	CH_3	OH	H	OAc
24	**F8**	CH_3	H	H	OAc
25	**F9**	H	H	OAc	OAc
26	**F10**	CH_3	OH	OAc	OAc
28	**F12**	CH_3	H	OAc	H
30	**F14**	CH_3	H	OAc	OAc



		R_1	R_2
27	**F11**	p-Cm	H
29	**F13**	H	p-Cm





1 Ir1 **6 Ir2**

Fig. 2. Structures of the identified constituents.

of β-OH-forsythoside B (compound 13) with the methoxy group preferably on the tyrosol unit. This hypothesis was based on the fact that the above described dioxan-type intermediates were not observed whereas the fragmentation pattern suggested the presence of a caffeoyl unit instead of ferulic one. Finally, a peak at 25.3 min (compound **15**) having pseudomolecular ions at *m*/*z* 623 $[M-H]^-$ and at *m*/*z* 625 $[M+H]^+$ was assigned to isoacteoside. This is the first report of the presence of this class of constituents in *S. recta*.

3.3. Identification of flavonoid glycosides

Fourteen flavonoid glycosides were detected and identified in the extracts (Fig. 2). The majority of the constituents were represented by isoscutellarein derivatives whereas only four apigenin-p-coumaroyl derivatives were detected. Both of these flavonoid groups are considered as important chemotaxonomic markers [28,29] of the genus *Stachys*. The presence of a hydroxyl group at position 8 of the flavonoid moiety causes a strong bathochromic shift of the band II to 278–280 nm [12]. For this reason the wavelength of 280 nm was selected for the quantitative analysis of the flavonoids in all tested extracts. Hydroxyl substitution on position 3′ of the isoscutellarein causes a further absorption maximum at 255 nm. Results are shown in Table 1. Negative ionization at 180 and 220 eV gave the best results, while positive ionization mode confirmed the results. In general, apart from the pseudomolecular ion $[M-H]^-/[M+H]^+$ in all of the cases a fragment belonging to the aglycon $[A-H]^-/[A+H]^+$ was also observed. The presence of allose in the molecules is suggested for biosynthetic reasons, since the main constituents according to detailed NMR analysis contained allose, whereas the presence of this particular flavonoid glycosides characterises

a large number of plants belonging to genus *Stachys* subgenus *Stachys*. In most cases of constituents acetylated on the external allose unit, in the higher voltage of 180 or 220 eV the intermediate ions $[M-180-OAc]^-$ were observed. In contrast, a peak at 32.8 min (compound **23**) was detected and tentatively identified as the isomer of 3′-hydroxy-4′-*O*-methylisoscutellarein-7-*O*-[6‴-acetyl-allosyl-(1 → 2)]-glucopyranoside (compound **21**), based on the product ion $[M-180]^-$ which indicated that the acetyl group was attached on the internal sugar moiety, without however being able to identify the identity of the hexoses. Similarly, a peak at 34.7 min (compound **28**) was detected and tentatively identified as 4′-*O*-methylisoscutellarein-7-*O*-[6″-acetyl-hexosyl-(1 → 2)]-hexoside, as it produced the same ion $[M-180]^-$, instead of ion $[M-180-OAc]^-$.

Two peaks at 34.5 and 34.8 min were identified as apigenin-7-*O*-(6″-p-*E*-coumaroyl)-glucopyranoside (**27**) and apigenin-7-*O*-(3″-p-*E*-coumaroyl)-glucopyranoside (**29**), respectively. Their presence and complete characterization was further confirmed during the isolation process, by means of 1D- and 2D-NMR spectra. Their complete NMR (^{1}H and ^{13}C NMR) assignments in MeOD are reported in Sections 3.4.1 and 3.4.2. In the case of the constituent **29** a fragment at *m*/*z* 413 [M-coumaroyl-H_2O]$^-$ was detected.

3.4. Structure elucidation of flavonoid glycosides by NMR

3.4.1. Apigenin-7-O-(6″-E-p-coumaroyl)-glucopyranoside (27)

^{1}H NMR (ppm, MeOD): *δ*: aglycone: 6.51 (1H, d, *J* 2.0 Hz, H-6), 6.52 (1H, s, H-3), 6.75 (1H, d, *J* 2.2 Hz, H-8), 6.85 (2H, d, *J* 8.8 Hz, H-3′, 5′), 7.79 (2H, d, *J* 8.8 Hz, H-2′, 6′); β-glucopyranoside: 3.40 (1H, dd, *J* 9.1, 9.6 Hz, H-4″), 3.55-3.53 (1H, m, H-2″, H-3″), 3.86 (1H, m, H-5″), 4.28 (1H, dd, *J* 11.8, 8.2 Hz, H-6″b), 4.63 (1H, ddd, *J* 11.7, 2.8, 2.2 Hz, H-6″a), 5.10 (1H, d, *J* 7.4 Hz, H-1″); p-coumaroyl group: 6.27 (1H, d, *J* 15.9 Hz, H-8‴), 6.61 (2H, d, *J* 8.6 Hz, H-3‴, 5‴), 7.19 (2H, d, *J* 8.6 Hz, H-2‴, 6‴), 7.52 (1H, d, *J* 16.0 Hz, H-7‴). ^{13}C NMR (ppm, MeOD): *δ*: aglycone: 96.0 (C-8), 101.2 (C-6), 103.5 (C-3), 106.12 (C-10), 118.2 (C-3′, 5′), 122.8 (C-1′), 129.6 (C-2′, C-6′), 158.7 (C-9), 163.2 (C-5), 165.5 (C-4′), 164.7 (C-7), 167.2 (C-2), 184.1 (C-4); β-glucopyranoside: 64.9 (C-6″), 72.3 (C-4″), 74.7 (C-2″), 75.7 (C-5″), 78.0 (C-3″), 101.2 (C-1); *E*-p-coumaroyl group: 112.4 (C-8‴), 117.0 (C-3‴, 5‴), 127.0 (C-1‴), 131.2 (C-2‴, 6‴), 147.3 (C-7‴), 161.7 (C-4‴), 169.3 (C-9‴).

3.4.2. Apigenin-7-O-(3″-E-p coumaroyl)-glucopyranoside (29)

^{1}H NMR (ppm, MeOD): *δ*: aglycone: 6.51 (1H, d, *J* 2.2 Hz, H-6), 6.64 (1H, s, H-3), 6.85 (1H, d, *J* 2.2 Hz, H-8), 6.88 (2H, d, *J* 8.8 Hz, H-3′, 5′), 7.87 (2H, d, *J* 8.8 Hz, H-2′, 6′); β-glucopyranoside: 3.63-3.69 (1H, m, H-5″), 3.67-3.71 (1H, m, H-4″), 3.71 (1H, dd, *J* 7.8, 9.8 Hz, H-2″), 3.78 (1H, dd, *J* 5.4, 12.5 Hz, H-6″b), 3.96 (1H, brd, *J* 12.3 Hz, H-6″a), 5.18 (1H, dd, *J* 10.2, 9.4 Hz, H-3″), 5.22 (1H, d, *J* 7.8 Hz, H-1″); p-coumaroyl group: 6.44 (1H, d, *J* 15.9 Hz, H-8‴), 6.81 (2H, d, *J* 8.6 Hz, H-3‴, 5‴), 7.48 (2H, d, *J* 8.7 Hz, H-2‴, 6‴), 7.69 (1H, d, *J* 15.9 Hz, H-7‴). ^{13}C NMR (ppm, MeOD): *δ*: aglycone: 96.2 (C-8), 101.2 (C-6), 103.6 (C-3), 106.2 (C-10), 118.1 (C-3′, 5′), 122.9 (C-1′), 129.8 (C-2′, C-6′), 159.2 (C-9), 163.2 (C-5), 165.4 (C-4′), 165.0 (C-7), 167.2 (C-2), 184.2 (C-4); β-glucopyranoside: 62.3 (C-6″), 69.7 (C-4″), 73.7 (C-2″), 78.6 (C-5″), 78.7 (C-3″), 101.6 (C-1); *E*-p-coumaroyl group: 114.5 (C-8‴), 117.1 (C-3‴, 5‴), 127.8 (C-1‴), 131.3 (C-2‴, 6‴), 147.0 (C-7‴), 162.0 (C-4‴), 170.1 (C-9‴).

3.5. Validation data

3.5.1. Linearity, repeatability of the standards and samples, time precision of the samples

All compounds showed good linearity. The following r^2 values were obtained: 5-caffeoylquinic acid (chlorogenic acid) $r^2 = 0.9999$; verbascoside $r^2 = 0.9999$; isoscutellarein-7-*O*-2″-*O*-[6‴-*O*-acetyl-β-D-allopyranosyl (1 → 2)]-β-D-glucopyrano-side $r^2 = 0.9996$. The data of LOD (limit of detection) and LOQ (limit of quantitation) for each investigated compound were found to be between 0.4 and 12 ng (corresponding to 1.5 nmol), indicating that the method is sensitive for the quantitative evaluation of the major constituents of the preparata of *S. recta*. LOD for chlorogenic acid was 4×10^{-4} μg (0.000206 μg/ml, 2 μl of injection) and LOQ was 6×10^{-4} μg (0.000206 μg/ml, 3 μl of injection). For verbacoside LOD was calculated as 1.2×10^{-3} μg (0.0006 μg/ml, 2 μl of injection) and LOQ was 2×10^{-3} μg (0.0005 μg/ml, 2 μl of injection), whereas for isoscutellarein-7-*O*-2″-*O*-[6‴-*O*-acetyl-β-D-allopyranosyl (1 → 2)]-β-D-glucopyranoside LOD was 1×10^{-3} μg (0.0005 μg/ml, 2 μl of injection) and LOQ was 2×10^{-3} μg (0.0005 μg/ml, 8 μl of injection).

The repeatability, based on six samples of each residue was analyzed by HPLC and the relative standard deviation (RSD) of the contents of each constituent ranged between 1.04% and 3.58%. The repeatability of the injection integration, was good, as a range 0.51–2.02% was obtained (RSD of the standard solutions and each sample). The overall intra- and inter-day time variations (time precision) of the eleven major analytes of *S. recta* extracts were less than 1.36% and 0.84%, respectively.

3.6. Quantitative analysis

Caffeoyl quinic acids are expressed as chlorogenic acid equivalents phenylethanol glycosides as verbascoside and flavonoid glycosides as isoscutellarein-7-*O*-[6‴-acetyl-allosyl-(1 → 2)]-glucopyranoside. Results are presented in detail in Table 2. The main class of compounds found in the aqueous preparations of *S. recta* are flavonoid glycosides of isoscutellarein, followed by caffeoyl quinic acids and phenylethanol glycosides.

In general, the infusion (SR-I) had the highest content in all the above classes of constituents followed by the decoction 2 (SR-D2) and then by decoction 1 (SR-D1). In all of the extracts the main caffeoylquinic acid was chlorogenic acid, while phenylethanol glycosides are represented mainly by verbascoside and forsythoside B. Concerning the flavonoid content a closer observation (Fig. 3) of the results showed significant quantitative differences among the main constituents of the tested samples. In both the decoctions the polar flavonoids are more abundant, opposite to the infusion, where the acetylated derivatives of isoscutellarein allosyl-glucosides predominate. This is better shown in Fig. 4 which depicts the acylated versus the deacylated flavonoid contents expressed as percentage of the total flavonoid content in all the preparata. In the infusion the acylated derivatives are almost double than the deacylated. This could be explained in terms of the preparation procedure. In order to understand better the relation between quantitative differences among the extracts and mode of preparation, a comparison with the initial ethanol extract was necessary (Fig. 1). Indeed, the

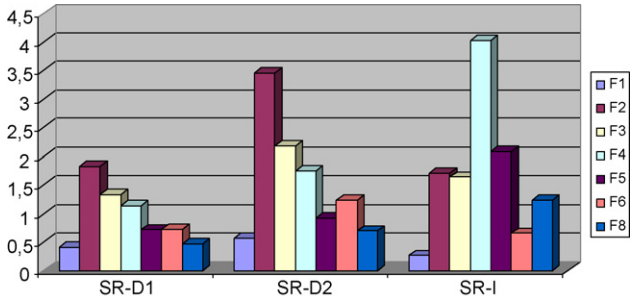


Fig. 3. Content (mg/g) of flavonoids F1–F8 in the decoctions and the infusion of *Stachys recta*. During boiling, flavonoid F4 is deacylated partially to F2, F5 is deacylated partially to F3 and F8 is deacylated partially to F6.

Table 2
Contents (mg/g) of the main constituents in the lyophilized preparations of *S. recta*, as evaluated by HPLC–DAD analysis.

Peak no.		Decoction I Mean + SD	RSD[a]	Decoction II Mean + SD	RSD[a]	Infusion Mean + SD	RSD[a]
2	1-CQA	0.02 ± 0.001	2.27	tr	–	tr	–
3	3-CQA	tr	–	0.85 ± 0.02	2.33	tr	–
4	5-CQA	2.60 ± 0.01	0.19	2.84 ± 0.03	1.02	3.49 ± 0.06	1.75
5	4-CQA	0.05 ± 0.00	1.92	0.04 ± 0.00	0.27	0.02 ± 0.00	1.77
Total CQA		2.67 ± 0.01		3.73 ± 0.01		3.51 ± 0.06	
7	Ph1	0.51 ± 0.00	0.58	0.62 ± 0.01	2.40	tr	–
11	Ph4	0.72 ± 0.01	0.83	0.70 ± 0.02	2.41	0.72 ± 0.01	1.66
12	Ph5	1.31 ± 0.03	2.29	1.56 ± 0.02	1.32	1.83 ± 0.02	1.04
15	Ph7	0.44 ± 0.01	1.36	0.33 ± 0.01	3.02	tr	–
Total phenylethanol glycosides		2.98 ± 0.04		3.21 ± 0.15		2.55 ± 0.03	
10	F1	0.42 ± 0.01	2.61	0.56 ± 0.01	2.13	0.26 ± 0.01	2.53
14	F2	1.83 ± 0.05	2.68	3.46 ± 0.04	1.30	1.70 ± 0.00	2.93
17	F3	1.34 ± 0.04	3.07	2.17 ± 0.03	1.38	1.64 ± 0.06	3.42
20	F4	1.14 ± 0.03	3.06	1.75 ± 0.02	1.03	4.02 ± 0.09	2.23
21	F5	0.72 ± 0.01	1.12	0.92 ± 0.01	1.52	2.08 ± 0.07	3.27
22	F6	0.73 ± 0.03	3.58	1.24 ± 0.02	1.86	0.66 ± 0.02	3.19
24	F8	0.48 ± 0.01	2.10	0.70 ± 0.01	1.30	1.24 ± 0.02	2.02
Total flavonoids		6.66 ± 0.13		10.80 ± 0.12		11.60 ± 0.32	

tr: traces.
[a] Each sample was injected six times.

Table 3
Amounts (mg) of total flavonoids, total phenylethanol glycosides and total caffeoylquinic acids in the three lyophilized preparations corresponding to their minimum intake in two cups of each herbal tea.

Sample	Herbal drug	Lyophilized residue	Total flavonoids in lyophilized extract	Total phenylethanol glycosides in lyophilized extract	Total CQA in lyophilized extract
SR-D1	10 g	1.7 g	113.2 ± 2.2	50.7 ± 0.7	45.4 ± 0.1
SR-D2	10 g	1.9 g	205.2 ± 2.4	60.7 ± 2.9	70.9 ± 0.1
SR-I	10 g	1.3 g	150.8 ± 4.1	33.2 ± 0.4	45.6 ± 0.8

ethanol extract is characterised by the presence of acetylated isoscutellarein allosyl-glucosides, while their deacetylated analogues are comparingly at very lower ratio. Infusion therefore, seems to be closer to the real chemical profile of the plant. Instead, in both the decoctions the deacetylated forms are at higher content. This is not understandable in terms of solubility, as in all cases the same solvent was used (water 100%). However, it reflects the differences in the preparation mode. It seems that during the preparation of the decoctions a limited deacetylation of the flavonoids can take place. This is better depicted in the more analytical Fig. 3, where the percentages of the main flavonoids of all the extracts are reported. As it can be observed, especially in the case of SR-D2 and SR-I, during boiling, flavonoid F4 is hydrolyzed partially to F2, F5 to F3 and F8 to F6. Finally, the more sensitive iridoids were detected in the preparations only in trace amounts (mainly by MS). This was probably due to degradation caused by high temperatures.

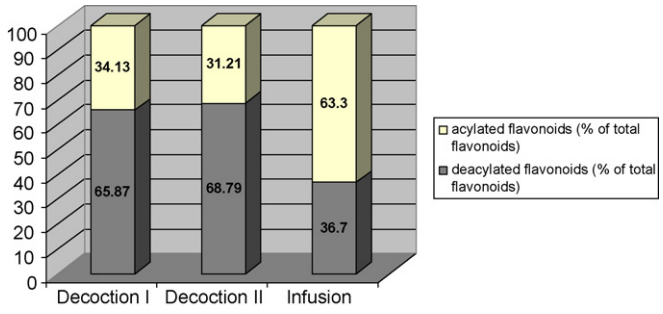


Fig. 4. Contents of acylated and deacylated flavonoids in the decoctions and the infusion expressed as percentages of total flavonoids. In the infusion prevail the acylated flavonoids, whereas in the decoctions the deacylated derivatives.

Based on the quantitative results of the total content of each group of components in the preparations we calculated the amounts (Table 3) of each characteristic group in the lyophilized extracts which correspond to 10 g of herbal drug, corresponding to two cups of herbal tea prepared with each method.

4. Conclusions

An effective and simple HPLC assay was developed and validated for *S. recta* aqueous preparations. The developed analytical method provided a good separation of the different classes of constituents present in such extracts, such as caffeoylquinic acids, phenylethanol glycosides, flavonoids and in traces iridoids. Overall 30 constituents were detected and identified and 15 of them were quantified. The rest of the constituents were detected in trace amounts. Only seven of them were previously reported in this species, while 23 were identified for the first time as constituents of *S. recta*. HPLC–DAD and HPLC–ESI-MS analysis provided evidence for the certain identification of the main constituents and in some cases of their isomers. Phytochemical isolations as well as NMR spectroscopy further confirmed these findings. Eight constituents were isolated and their structure elucidated by HPLC–ESI-MS and 1D- and 2D-NMR spectroscopy. For compounds **27** and **29** the complete NMR (^{1}H and ^{13}C NMR) data in MeOD are given for the first time. This method could be applied for the quality control not only of *S. recta* but for other *Stachys* species as well. A detailed literature survey showed the lack of an HPLC–DAD or HPLC–ESI-MS method for the quality control of *Stachys* sp. preparations which would

include different classes of constituents. A previous HPLC–DAD and HPLC–APICI-MS study by Marin et al. [28] is focussed solely on the detection of flavonoids. No quantitative data are provided, as the study was carried out for chemotaxonomic purposes. Among the investigated preparations infusion seems to be the best method to extract the native constituents of the plant, while decoction is a more aggressive treatment and causes a partial degradation of some acylated flavonoids.

The developed analytical method provided a satisfactory specificity and reproducibility. Good linearity of the calibration curves was achieved, while the repeatability and time precision (intra- and inter-day) of the method were satisfactory. To the best of our knowledge this is the first extensive HPLC–DAD and HPLC–ESI-MS analysis of *S. recta* preparations and of *Stachys* sp. reporting four different classes of constituents of the phytocomplex. The method of preparation of the herbal tea influenced considerably the quantitative profile of the active polyphenols and these differences in quality may reflect changes in the efficacy exerting different potencies.

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References

[1] European Pharmacopoeia, vol. 1, 6th ed., Council of Europe, Strasbourg, 2008, p. 685.
[2] A.R. Bilia, F. Eterno, M.C. Bergonzi, G. Mazzi, F.F. Vincieri, Evaluation of the content and stability of the constituents of mother tinctures and tinctures: the case of *Crataegus oxyacantha* L. and *Hieracium pilosella* L., J. Pharm. Biomed. Anal. 44 (2007) 70–78.
[3] A.R. Bilia, D. Salvini, G. Mazzi, F.F. Vincieri, Characterization of Calendula, Milk-thistle and passionflower tinctures by HPLC-DAD and HPLC–MS, Chromatographia 53 (2001) 210–215.
[4] A.R. Bilia, M.C. Bergonzi, G. Mazzi, F.F. Vincieri, HPLC–DAD and HPLC–MS analysis and stability of the constituents of Artichoke and St. John's wort tinctures, Drug Dev. Ind. Pharm. 25 (2002) 611–622.
[5] F. Camangi, A. Stefani, Le piante nella magia e nella superstizione: alcuni esempi di pratiche popolari in Toscana, Rivista di Preistoria, Etnografia e Storia naturale, Istituto Storico Lucchese 1 (2003) 1–5.
[6] A. Meremeti, A. Karioti, H. Skaltsa, J. Heilmann, O. Sticher, Secondary metabolites from *Stachys ionica*, Biochem. Syst. Ecol. 32 (2004) 139–151.
[7] V. Schulz, R. Hänsel, M. Blumenthal, V.E. Tyler, Rational Phytotherapy—A Reference Guide for Physicians and Pharmacists, 5th ed., Springer, Berlin, Heidelberg, New York, 2004.
[8] N. Mulinacci, D. Prucher, M. Peruzzi, A. Romani, P. Pinelli, C. Giaccherini, F.F. Vincieri, Commercial and laboratory extracts from artichoke leaves: estimation of caffeoyl esters and flavonoidic compounds content, J. Pharm. Biomed. Anal. 34 (2004) 349–357.
[9] A. Karioti, H. Skaltsa, J. Heilmann, O. Sticher, Acylated flavonoid and phenylethanoid glycosides from *Marrubium velutinum*, Phytochemistry 64 (2003) 655–660.
[10] M. Adinolfi, G. Barone, R. Lanzetta, G. Laonigro, L. Mangoni, M. Parrilli, Diterpenes from *Stachys recta*, J. Nat. Prod. 47 (1984) 541–543.
[11] A. Lenherr, B. Meier, O. Sticher, Iridoid glucosides and acetylated flavonoids in the group of *Stachys recta*, Planta Med. (1984) 403–409.
[12] A. Lenherr, M.F. Lahloub, O. Sticher, Three flavonoid glycosides containing acetylated allose from *Stachys recta*, Phytochemistry 23 (1984) 2343–2345.
[13] A. Lenherr, T.J. Mabry, Acetylated allose-containing flavonoid glucosides from *Stachys anisochila*, Phytochemistry 26 (1987) 1185–1188.
[14] X. Zhu, H. Zhang, R.J. Lo, Phenolic compounds from the leaf extract of Artichoke (*Cynara scolymus* L.) and their antimicrobial activities, J. Agric. Food Chem. 52 (2004) 7272–7278.
[15] H. Wagner, S. Bladt, E.M. Zgainski, Plant Drug Analysis, Springer-Verlag, Berlin, 1984.
[16] F. Cuyckens, M. Claeys, Mass spectrometry in the structural analysis of flavonoids, J. Mass Spectrom. 39 (2004) 1–15.
[17] F. Stickel, E. Patsenker, D. Schuppan, Herbal hepatotoxicity, J. Hepatol. 43 (2005) 901–910.
[18] M.N. Clifford, K.L. Johnston, S. Knight, N. Kuhnert, Hierarchical scheme for LC–MS^n identification of chlorogenic acids, J Agric. Food Chem. 51 (2003) 2900–2911.
[19] G.M. Weisz, D.R. Kammerer, R. Carle, Identification and quantification of phenolic compounds from sunflower (*Helianthus annuus* L.) kernels and shells by HPLC–DAD/ESI-MS^n, Food Chem. 115 (2009) 758–765.
[20] M.N. Clifford, S. Knight, N. Kuhnert, Discriminating between the six isomers of dicaffeoylquinic acid by LC–MS^n, J. Agric. Food Chem. 53 (2005) 3821–3832.
[21] V.B. Vundac, Z. Males, M. Plazibat, P. Golja, B. Cetina-Cizmek, HPTLC determination of flavonoids and phenolic acids in some Croatian *stachys taxa*, J. Planar Chromatogr. Mod. TLC 18 (2005) 269–273.
[22] A.I. Derkach, Biologically active substances of some species of the genus *Stachys* L. of the flora of the Ukraine, Rastit. Resur. 34 (1998) 57, CAN 129: 173065.
[23] H. Kırmızıbekmez, P. Montoro, S. Piacente, C. Pizza, A. Dönmez, I. Çalış, Identification by HPLC–PAD-MS and quantification by HPLC–PAD of phenylethanoid glycosides of Five *Phlomis* species, Phytochem. Anal. 16 (2005) 1–6.
[24] H. Guo, A.H. Liu, M. Yang, D.A. Guo, Characterization of phenolic compounds in the fruits of *Forsythia suspensa* by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry, Rapid Commun. Mass Spectrom. 21 (2007) 715–729.
[25] T. Miyase, R. Yamamoto, A. Ueno, Phenylethanoid glycosides from *Stachys officinalis*, Phytochemistry 43 (1996) 475–479.
[26] T. Murata, Y. Endo, T. Miyase, F. Yoshizaki, Iridoid glycoside constituents of *Stachys lanata*, J. Nat. Prod. 71 (2008) 1768–1770.
[27] J. Gruz, O. Novák, M. Strnad, Rapid analysis of phenolic acids in beverages by UPLC–MS/MS, Food Chem. 111 (2008) 789–794.
[28] P.D. Marin, R.J. Grayer, S. Grujic-Jovanovic, G.C. Kite, N.G. Veitch, Glycosides of tricetin methyl ethers as chemosystematic markers in *Stachys* subgenus *Betonica*, Phytochemistry 65 (2004) 1247–1253.
[29] F.A. Tomas-Barberan, M.I. Gil, F. Ferreres, F. Tomas-Lorente, Flavonoid *p*-coumaroylglucosides and 8-hydroxyflavone allosylglucosides in some Labiatae, Phytochemistry 31 (1992) 3097–3102.