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Chromatographic performance of a new polar poly(ethylene glycol) bonded phase for the phytochemical analysis of *Hypericum perforatum* L.☆

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Abstract

The aim of this study was to evaluate the chromatographic performance of a poly(ethylene glycol) (PEG) stationary phase for the HPLC analysis of the secondary metabolites (chlorogenic acid, flavonoids, phloroglucinols and naphthodianthrones) in methanolic extracts of *Hypericum perforatum* L. (St. John's Wort) flowering tops, herbal medicinal products and dietary supplements. A fast and reliable method was developed. The analyses were carried out on a Supelco Discovery HS PEG column (150 mm × 4.6 mm i.d., 5 μm). A gradient mobile phase, composed of 0.1 M aqueous acetic acid solution (pH 2.8) and methanol–acetonitrile (5:4, v/v), was used. The flow rate was 1 mL/min. The photodiode array detector monitored the eluent at 270 (for chlorogenic acid, flavonoids and phloroglucinols) and 590 nm (for naphthodianthrones). The column was maintained at room temperature. The total running time was 40 min. The method was validated and showed good linearity, precision, accuracy, sensitivity and specificity. Through the above described phytochemical markers, this technique allowed the unequivocal identification and standardization of *H. perforatum* plant material and phytoproducts. The quantification data highlighted the fact that the products on sale, in particular those labeled as dietary supplements, varied widely in the quantitative composition of the active constituents. The developed method could be considered suitable for the quality control of *H. perforatum* herb and derivatives.
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Keywords: *Hypericum perforatum* L.; Flavonoids; Naphtodianthrones; Phloroglucinols; Poly(ethylene glycol) stationary phase; Phytochemical analysis; HPLC

1. Introduction

Hypericum perforatum L., also known as "St. John's Wort", is a herbaceous perennial plant belonging to the *Hypericaceae* family. It has been used as a medicinal plant since antiquity. Nowadays, the dried flowers or aerial parts are used for the preparation of herbal medicines employed in phytotherapy. A series of pharmacological properties, ranging from wound healing [1] and antiseptic [2] to antiviral [3], anti-inflammatory [4,5], anticancer [6], ethanol intake inhibition [7] and apoptosis-inducing activities [8,9] has been described. However, its preeminent and carefully validated application is the symptomatic treatment of mild and moderate depression and its efficacy has been demonstrated through a number of pharmacological and clinical trials [10–14].

In various studies the isolation and analysis of a very high number of substances from different structural types and their pharmacological properties have been described. These compounds (Fig. 1) include chlorogenic acid, a broad range of flavonoids (rutin, hyperoside, isoquercitrin, quercitrin and quercetin), phloroglucinols (hyperforin and adhyperforin), biflavonoids (I3′, II8-biapigenin or amentoflavone and I3, II8-biapigenin) and naphthodianthrones (protopseudohypericin, protohypericin, pseudohypericin and hypericin).

Several researches have concluded that the total pharmacological activity of *H. perforatum* preparations may depend not on a single compound, but on the combined activities of several plant constituents [15]. These compounds work "synergistically" and cannot be separated into active parts. Consequently, it is necessary to define all the phytochemical

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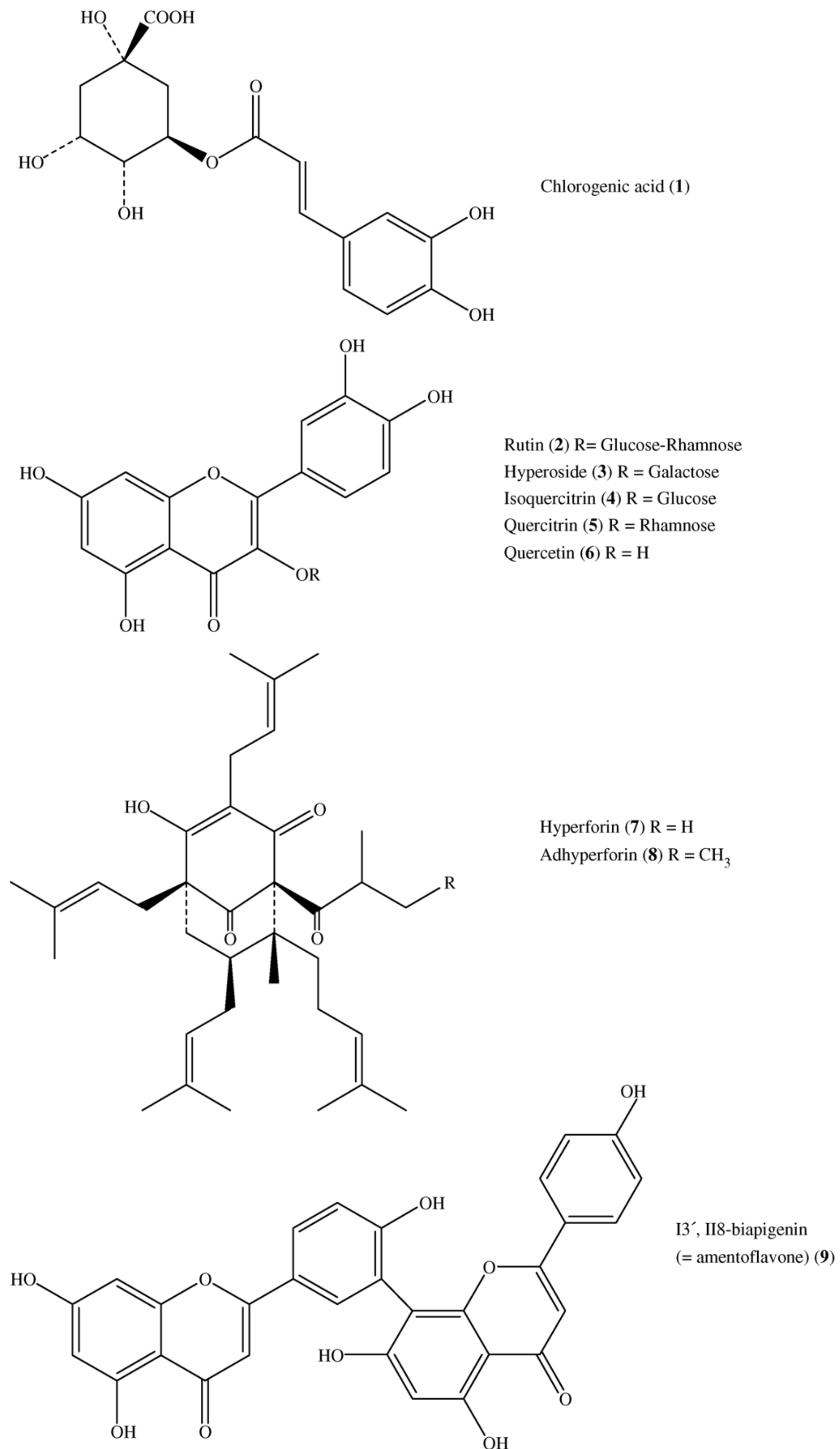

Fig. 1. Chemical structures of the active constituents of *Hypericum perforatum* L.

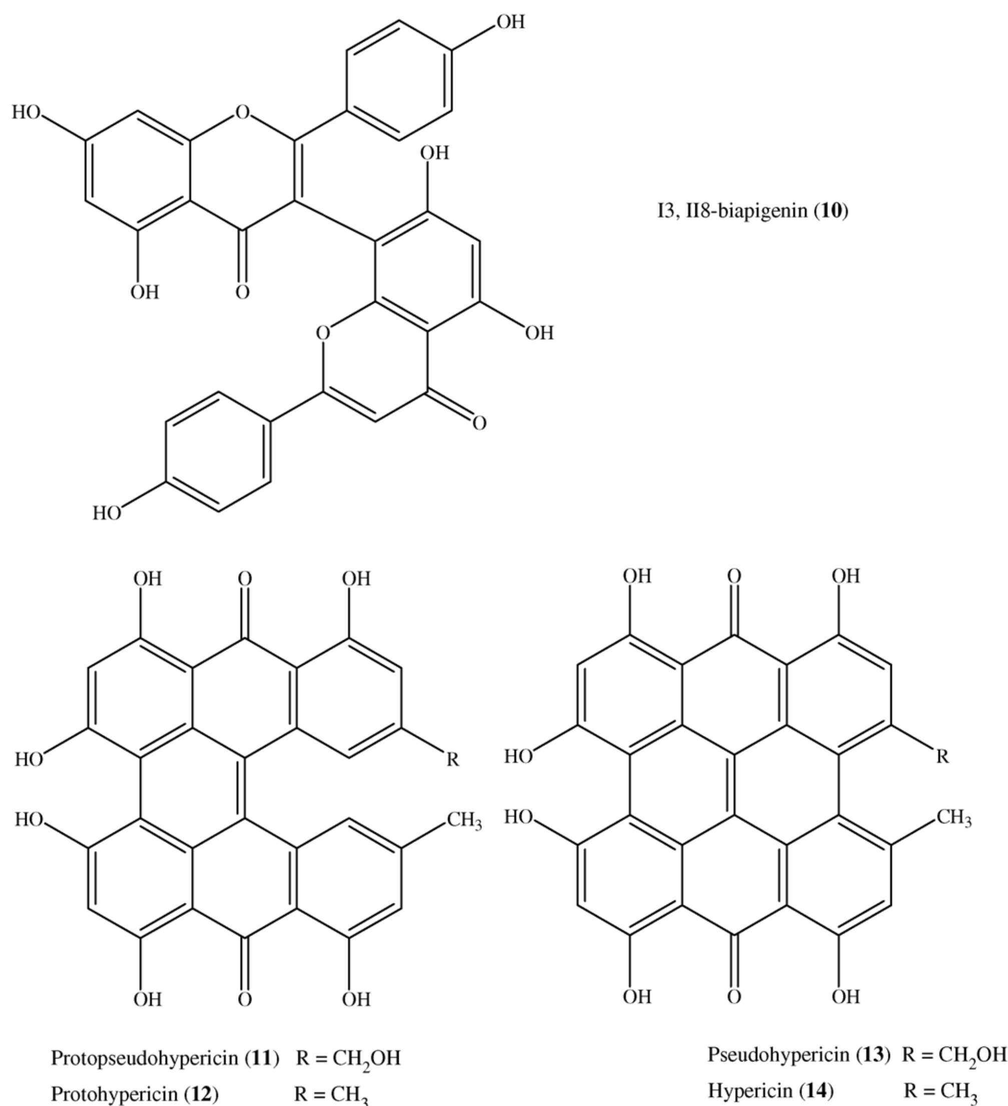

Fig. 1. (*Continued*).

constituents of botanical extracts to understand the bioactivity and possible adverse effects of the active compounds, and to enhance product quality control.

High-performance liquid chromatography (HPLC) has been widely applied for the analysis of the secondary metabolites of *H. perforatum*. So far, several HPLC methods deal with the analysis of all the active ingredients of *H. perforatum* extracts [15–28], while others are specific for the assay of naphthodianthrones [29–34] or phloroglucinols [35–39] or both [40–43]. Usually, HPLC analyses are carried out with C_{18} columns. However, most of the methods reported in the literature for the phytochemical analysis of all the active compounds *H. perforatum* are not economical in terms of time (60–90 min or more) and solvent usage.

In recent years, novel RP-HPLC stationary phases containing polar groups have been developed for efficient analysis of samples that contain a wide range of polarities, allowing a reduction in the analysis time. In particular, a poly(ethylene glycol) (PEG) packing is characterized by ether groups which can help to overcome traditional chromatographic problems related to the analysis of sample components with very different retention times. This improvement arises from a combination of increased retention for polar compounds and decreased retention for non-polar compounds, which reduces the total analysis time. Most plant materials comprise a complex mixture of different phytochemicals (plant secondary metabolites), with a wide range of polarities. One such case is the *H. perforatum* herb.

In this study, the performance of a new polar reversed-phase stationary phase was evaluated for the analysis of the active compounds of *H. perforatum* herb. To the best of our knowledge, no studies on the application of the PEG stationary phase in phytochemical analysis have been reported so far.

The validation procedure, according to ICH guidelines, proved that the method has good linearity, accuracy, precision

and sensitivity. The practical applicability of this procedure was tested by assaying the extracts of *H. perforatum* herbs and phytoproducts. Considering the results of this method, the PEG stationary phase could be further investigated and applied in the phytochemical analysis of various vegetable matrices and derivatives containing phenolic compounds.

2. Experimental

2.1. Chemicals and solvents

Rutin, hyperoside, quercitrin, quercetin, amentoflavone and hypericin were purchased from Extrasynthese (Genay, France). Isoquercitrin and hyperforin were from Roth (Karlsruhe, Germany). Methanol HPLC grade, acetonitrile HPLC grade, formic acid, phosphoric acid, trifluoroacetic acid and chlorogenic acid were from Sigma (Milan, Italy). Glacial acetic acid was from J.T. Baker (Milan, Italy). Water was purified using a Milli-Q PLUS 185 system from Millipore (Milford, MA, USA).

2.2. Plant material

Hypericum perforatum L. flowering tops were harvested in spring 2003 in an experimental field located in Ozzano (Bologna, Italy) from 3-year-old plants and were kindly donated by Dr. Maria Grazia Bellardi (Dipartimento di Scienze e Tecnologie Agroambientali (DiSTA), Patologia Vegetale, Università degli Studi di Bologna, Italy). The plant material was dried in airy rooms in the dark at ambient temperature; after drying, it was stored and protected from light and humidity until required for chemical analysis. A voucher specimen was deposited at the Herbarium of the Botanical Garden of the University of Modena and Reggio Emilia (Italy).

The dried plant material was ground on a IKA M20 grinder (Staufen, Germany) before extraction.

H. perforatum herbal medicinal products (tablets and capsules) were purchased in local pharmacies in summer 2004 and are representative of the Italian market. These products are classified as prescription drugs and are indicated in the text as *H. perforatum* HMP1-HMP4, respectively. The *H. perforatum* dietary supplements (tablets and capsules) under investigation were purchased in local shops in summer 2004. These products are indicated in the text as *H. perforatum* DS1-DS5, respectively. Of all the samples purchased, six were labeled as standardized for their content of hypericin (0.3%). Only single herb preparations of *H. perforatum* were purchased so that qualitative and quantitative determinations could be carried out without contamination from other plant extracts.

2.3. Chromatographic apparatus

Chromatography was performed on an Agilent Technologies (Waldbronn, Germany) modular model 1100 system consisting of a vacuum degasser, a quaternary pump, an autosampler, a thermostatted column compartment and a photodiode array detector (PAD). The chromatograms were recorded with Agilent ChemStation for LC and LC–MS system (Rev. A.08.03) on a Pentium III personal computer.

2.4. HPLC method

The analyses were carried out on a Supelco Discovery HS PEG column (150 mm × 4.6 mm i.d., 5 μm) (Supelco, Bellefonte, PA, USA). To protect the integrity of the analytical column, all analyses were performed with a coupled Supelco Discovery HS PEG guard column (20 mm × 4.0 mm i.d., 5 μm). The mobile phase was (A) 0.1 M aqueous acetic acid solution (pH 2.8) and (B) methanol–acetonitrile (5:4, v/v). The gradient elution was modified as follows: initial 10% B; 0–18 min 10–30% B; 18–25 min 30–90% B; 25–40 min 90% B. The total running time was 40 min. The post-running time was 5 min.

The flow rate was 1 mL/min. The detector monitored the eluent at 270 (for chlorogenic acid, flavonoids and phloroglucinols) and 590 nm (for naphthodianthrones). The column was maintained at room temperature. The sample injection volume was 5 μL. Three injections were performed for each sample.

2.5. Identification of constituents and peak purity

Peaks were identified on the basis of their retention time (t_R) values and UV–vis spectra by comparison with those of the single compound in the standard solution. Peak identity was also confirmed by spiking the extracts with pure standards (standard addition method). Because of the lack of the other naphtodianthrones, pseudohypericin, protopseudohypericin, and protohypericin were identified by comparison of the UV–vis spectrum with literature data [41]. In particular, pseudohypericin has the same UV–vis spectrum as hypercin and it is reported in the literature as the major compound among the naphthodianthrones. Protopseudohypericin and protohypericin have a UV–vis spectrum similar but not identical to those of hypericin and pseudohypericin; protopseudohypericin and protohypericin peaks were identified in accordance with the polarity of these compounds.

Peak purity test was performed using a photodiode array detector coupled to the HPLC system, comparing the UV spectra of each peak with those of authentic reference samples.

2.6. Standard solutions and sample preparation for quantification

The stock standard solution of each standard compound (chlorogenic acid, rutin, hyperoside, isoquercitrin, quercitrin, quercetin, hyperforin, amentoflavone and hypericin) was prepared as follows: about 2.0 mg of each compound was accurately weighed and placed into a 5 mL volumetric flask.

Methanol–acetonitrile (5:4, v/v) was added and the solution diluted to volume with the same solvent.

External standard calibration curves were established on seven data points covering the concentration range of 4.40–440.40 μg/mL for chlorogenic acid, 4.19–419.20 μg/mL for rutin, 3.94–394.00 μg/mL for hyperoside, 4.58–458.20 μg/mL for isoquercitrin, 4.16–416.40 μg/mL for quercitrin, 4.22–421.60 μg/mL for quercetin, 5.29–264.60 μg/mL for hyperforin, 4.14–413.80 μg/mL for amentoflavone and 6.54–326.80 μg/mL for hypericin. Five microliters aliquots of each standard solution were used for HPLC analysis. Triplicate injections were performed for each standard solution. Each calibration curve was obtained by plotting the peak area of the compound at each level prepared versus the concentration of the sample.

Pseudohypericin has the same UV–vis spectrum as hypericin and was therefore quantified with the hypericin calibration curve [34,41]. The amounts of protopseudohypericin and protohypericin were evaluated using the calibration curve of hypericin [41].

Regarding the sample preparation, a weighed amount (0.5 g) of finely powdered plant material was extracted with 10 mL of methanol at room temperature using a magnetic stirrer (Multistirrer, Velp Scientifica, Milan, Italy) or an ultrasonic bath (Sonorex RK-100 H, Bandelin, Berlin, Germany) for 15 min. After centrifugation for 5 min at $2718 \times g$, the supernatant solution was filtered under vacuum into a volumetric flask. The residue was re-extracted in the same way. The filtrates of the two extractions were combined in a 25 mL volumetric flask and methanol was added to make the final volume. As far as possible, all extraction procedures were performed under protection from daylight, and amber glass utensils were used.

As for the herbal medicinal products and dietary supplements, a weighed amount (0.5 g) of finely powdered material (from 10 powdered tablets or from the contents of 10 capsules) was extracted according to the procedure previously described.

All the extracts were filtered through a 0.45 μm PTFE filter into a HPLC vial and capped. The use of PTFE filters enabled us to avoid the loss of naphthodianthrones due to filter absorption [25,31].

The extraction procedure was repeated twice for each sample.

3. Results and discussion

3.1. Selection of the extraction method

Different solvents and extraction methods are reported in the literature for the analysis of the active compounds of *H. perforatum* [15–43]. Methanol is one of the most commonly used solvents for an exhaustive extraction of *H. perforatum* [15,19,20,24–26,28,33,34,38,40,41] and was therefore chosen as the extraction solvent. In this study, two extraction methods were evaluated: magnetic stirring and sonication. Both these methods were carried out at room temperature to avoid the decomposition of the active constituents [28], such as naphthodianthrones and phloroglucinols. As shown in Fig. 2, magnetic stirring was more efficient than sonication for the extraction of the compounds of interest. Extraction using sonication is a very common practice in phytochemical research. However, the low ultrasonic frequency (35 kHz) applied by the equipment used in this study was not sufficient to obtain a satisfactory extraction of the compounds of interest.

In accordance with the above observations, in this work the extraction procedure was carried out at room temperature using a magnetic stirrer. It was found that two extraction procedures for each sample were sufficient to obtain a complete extraction of the secondary metabolites from *H. perforatum*; further extraction steps did not led to a more efficient extraction of the active compounds. The efficiency of the extraction method applied is this study was confirmed by the recovery data reported in Section 3.3.

Furthermore, since naphthodianthrones and phloroglucinols are sensitive to light [28], the extraction procedure was performed as far as possible under protection from daylight.

3.2. Method development and optimization

Several mobile phases have been described in the literature for the analysis of the secondary metabolites of *H. perforatum* [15–43]. Reversed-phase C_{18} columns are almost exclusively used [15–23,25–27,29,30–32,34–37,39–43]. Furthermore, a C_{12} column has been successfully used for the phytochemical analysis of *H. perforatum* [28]. Runs are generally an hour long or more, with equilibration between runs. Flow rate is usually 1 mL/min. Analyses are carried out at, or slightly above, room temperature. Injection volume is generally 20 μL. Photodiode array [15,16,19,21–25,27,28,30,31,34,35,37,38,41,43], mass spectrometric [15,17,19,22,28,29,32,37,38,43] and nuclear magnetic resonance [17] detection were employed to identify most of the components. Furthermore, fluorescence detection was employed for the analysis of hypericin and pseudohypericyn [32,40,42]. Recently, electrochemical detection was applied for the determination of hyperforin [39].

In this study, the chromatographic performance of a poly(ethylene glycol) bonded phase for the phytochemical analysis of *H. perforatum* secondary metabolites was evaluated. Preliminary results demonstrated that the application of this packing in normal phase mode did not allow a satisfactory separation of the compounds of interest. The reversed-phase chromatographic conditions were then optimized with the aim of obtaining chromatograms with a good resolution of adjacent peaks within a short analysis time. Two solvents were used as the mobile phase: (A) aqueous acidic solution and (B) methanol–acetonitrile (5:4, v/v). During the method development, water acidified with several acid additives, such as phosphoric, formic, acetic and trifluoroacetic acid was tested. Acetic acid is one of the most frequently chosen acid addi-

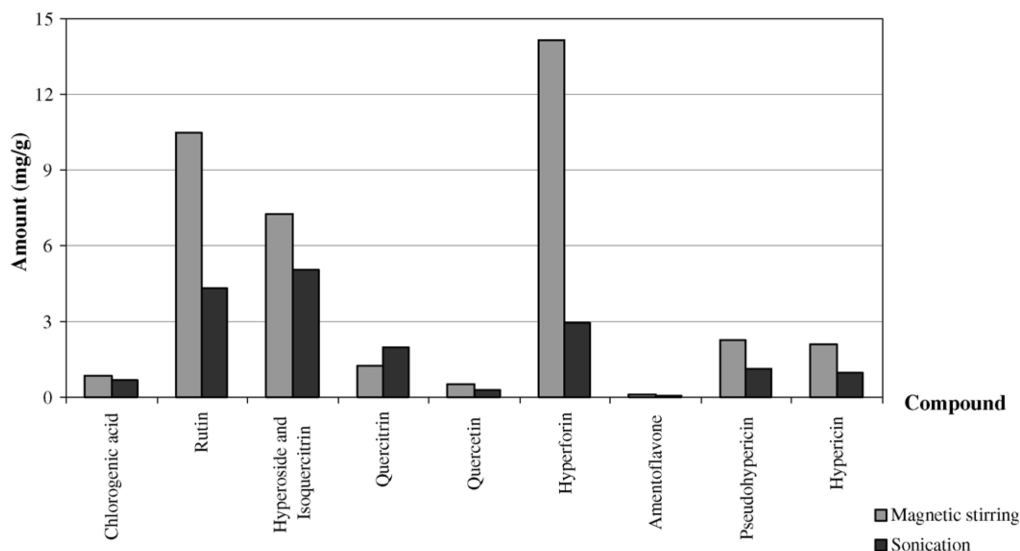

Fig. 2. Comparison between two methods of extraction of *H. perforatum* flowering tops.

tives, since it can protonate the phenolic hydroxyl groups, which makes it an effective additive for polyphenols. In this study, water containing 0.1 M acetic acid was finally selected as the solvent (A). Several pH values, from 2.5 to 8.0, of the aqueous solution were also evaluated. At more acidic pH values, phenol groups are protonated with a consequent reduction in peak tailing of phenolic compounds. The optimum pH value of the acetic acid solution was found to be 2.8.

Firstly, isocratic elution was tried, but it did not allow the elution of the non-polar components. Gradient elution was therefore carried out so as to ensure that the elution of all the compounds was completed within a short time. To optimize the mobile phase for a binary gradient profile, different compositions of methanol-acetonitrile (5:4, v/v) in water containing 0.1 M acetic acid were used. The gradient which gave the optimum separation was finally chosen. Under the gradient conditions reported in the previous section the peaks were well separated in a short time, with the exception of hyperoside and isoquercitrin. According to the literature [19,20,23], the resolution of hyperoside and isoquercitrin is problematic: these flavonoids have a very similar polarity and could not be separated under the applied chromatographic conditions.

Flow rates between 0.6 and 1 mL/min were studied. A flow rate of 1 mL/min gave an optimum signal-to-noise ratio with a reasonable separation time.

Data were collected at 270 nm for chlorogenic acid, flavonoids and phloroglucinols, and at 590 nm for hypericins since these wavelengths gave the best signal-to-noise response.

Fig. 3 shows the chromatogram of mixed standards at 270 nm.

When the solvent system and the chromatographic conditions reported in Section 2 were employed, the active components of *H. perforatum* were separated in a short time (40 min). The retention times of the compounds of interest are shown in Table 1.

Column performance results are presented in Table 1. As a measure of column performance, the number of theoretical plates (N) for each constituent was evaluated. The chromatographic parameters were satisfactory for these components: the a values were higher than 1.00 and the R_s values were higher than 1.50, indicating a good separation [44].

3.3. Method validation

For the validation of the analytical method, the guidelines of the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use and US Pharmacopeia 27 were followed [45,46].

Linear regression analysis for chlorogenic acid, flavonoids, hyperforin and hypericin was performed by the external standard method. The validating parameters of each calibration curve (slope (a), intercept (b), correlation coefficient (r^2), standard deviation of the slope and standard deviation of the intercept) are described in Table 2. Excellent linearity was observed for all these compounds between peak areas and concentrations over the range tested.

The accuracy of the analytical procedure was evaluated with the recovery test: this involved the addition of known quantities of reference standards, using each time the appropriate standard of the nine available in this study, to known amounts of *Hypericum* flowering tops. The fortified samples were then extracted and analyzed with the proposed HPLC method. Table 3 reports the recovery data, expressed as % recovery, that were obtained by comparing the results from samples and fortified samples.

Considering the results of the recovery test, the method is deemed to be accurate.

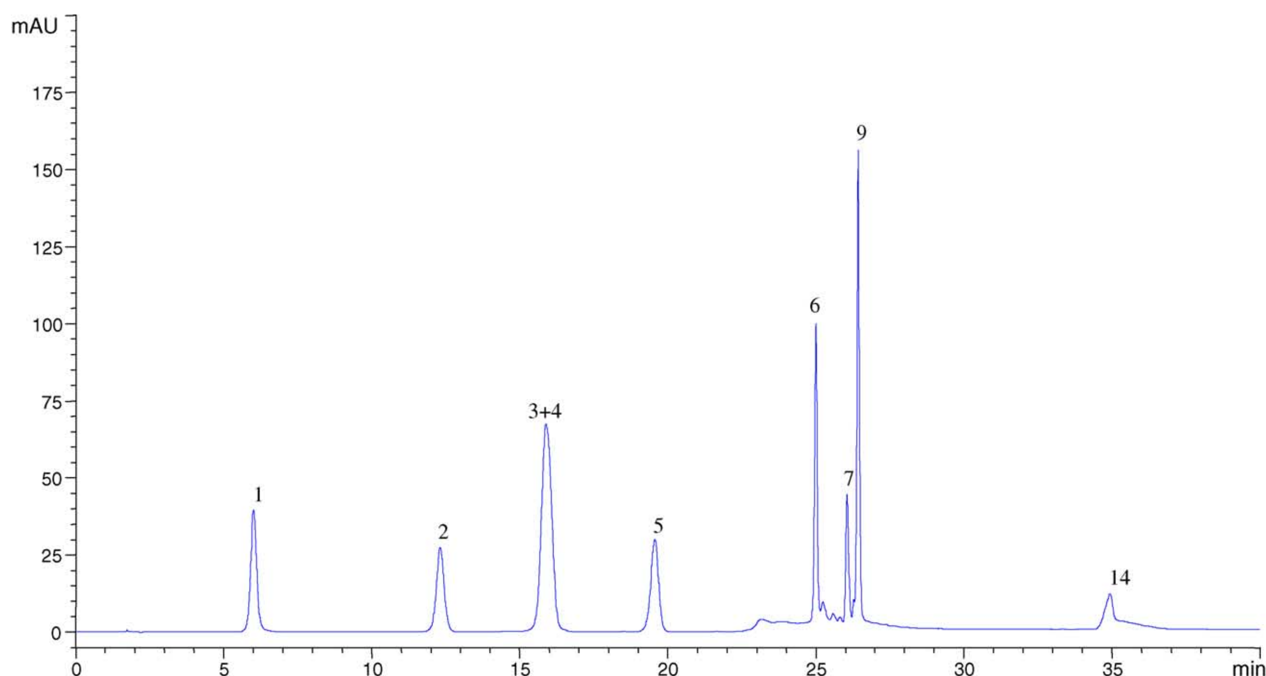

Fig. 3. Chromatogram of a standard mixture of the secondary metabolites of *H. perforatum*. For peak identification, see Fig. 1. Concentrations of compounds were: 122.5 μg/mL for chlorogenic acid, 68.9 μg/mL for rutin, 69.8 μg/mL for hyperoside, 93.6 μg/mL for isoquercitrin, 59.6 μg/mL for quercitrin, 62.0 μg/mL for quercetin, 155.9 μg/mL for hyperforin, 47.6 μg/mL for amentoflavone, 176.8 μg/mL for hypericin. Experimental conditions as in Section 2.4. Detection: UV at 270 nm.

Table 1
System-suitability report for the separation of the major compounds of *H. perforatum*

Compound	t_R (min)	Theoretical plates (*N*)	Resolution (R_s)	Selectivity (α)	Peak symmetry
Chlorogenic acid	6.01	4492	–	–	0.89
Rutin	12.31	10785	15.11	2.05	0.96
Hyperoside and isoquercitrin	15.90	9522	6.35	1.29	0.86
Quercitrin	19.56	26302	6.46	1.23	1.09
Quercetin	25.00	407270	16.99	1.28	1.02
Hyperforin	26.05	323846	6.16	1.04	0.70
Amentoflavone	26.43	488906	2.29	1.01	1.01
Hypericin	34.94	74680	25.63	1.32	0.80

Experimental conditions: Supelco Discovery HS PEG column (150 mm × 4.6 mm i.d., 5 μm) coupled to a Supelco Discovery HS PEG guard column (20 mm × 4.0 mm i.d., 5 μm). Mobile phase: 0.1 M aqueous acetic acid solution (pH 2.8) and methanol-acetonitrile (5:4, v/v), gradient. Flow rate: 1 mL/min. Injection volume: 5 μL. Temperature: ambient. Detection: 270 and 590 nm.

Table 2
Statistical analysis for the calibration curves of the active compounds of *H. perforatum*[a]

Compound	Wavelength (nm)	Linearity range (μg/mL)	Slope (*a*)	Intercept (*b*)	r^2
Chlorogenic acid	270	4.40–440.40	4.808 (±0.014)	−8.355 (±2.652)	0.9998
Rutin	270	4.19–419.20	7.368 (±0.014)	−0.946 (±2.544)	0.9999
Hyperoside	270	3.94–394.00	10.350 (±0.016)	−12.033 (±2.821)	0.9999
Isoquercitrin	270	4.58–458.20	10.234 (±0.020)	−2.354 (±3.977)	0.9999
Quercitrin	270	4.16–416.40	9.656 (±0.023)	−12.158 (±4.211)	0.9999
Quercetin	270	4.22–421.60	11.883 (±0.030)	−23.510 (±5.492)	0.9999
Hyperforin	270	5.29–264.60	2.027 (±0.004)	9.233 (±0.478)	0.9999
Amentoflavone	270	4.14–413.80	20.773 (±0.026)	−8.362 (±4.653)	1.0000
Hypericin	590	6.54–326.80	3.011 (±0.006)	0.727 (±0.852)	0.9999

[a] For each curve the equation is $y = ax + b$, where *y* is the peak area, *x* the concentration of the analyte (μg/mL), *a* the slope, *b* the intercept and r^2 is the correlation coefficient. SD values are given in parenthesis. The *P* value was <0.0001 for all calibration curves. Experimental conditions as in Table 1.

Table 3
Recovery data of the active constituents of *H. perforatum*

Compound	Spiked amount (mg)	Recovery (%)	Mean ($n = 3$)	RSD (%)
Chlorogenic acid	1.072	97.8–99.3	98.6	0.8
Rutin	1.288	102.7–104.1	103.2	0.7
Hyperoside	0.966	101.0–103.9	102.7	1.4
Isoquercitrin	1.209	97.5–98.4	97.9	0.4
Quercitrin	1.143	95.4–96.0	95.6	0.3
Quercetin	0.942	97.3–101.8	100.0	2.3
Hyperforin	1.198	100.2–103.8	101.6	1.9
Amentoflavone	1.328	101.3–101.7	101.5	0.2
Hypericin	1.392	101.1–103.9	102.1	1.5

RSD (%) = (standard deviation/mean) × 100. Experimental conditions as in Table 1.

Table 4
Intra- and inter-day precision data for retention time (t_R) of the major constituents of *H. perforatum*

Compound	Intra-day precision ($n = 10$, mean)						Inter-day precision ($n = 30$, mean)	
	Day 1		Day 2		Day 3		t_R (min)	RSD (%)
	t_R (min)	RSD (%)	t_R (min)	RSD (%)	t_R (min)	RSD (%)		
Chlorogenic acid	5.96	0.80	5.95	1.05	5.94	1.11	5.95	0.97
Rutin	11.84	0.95	11.83	1.04	11.77	1.36	11.81	1.12
Hyperoside and isoquercitrin	15.50	0.95	15.50	1.16	15.39	1.24	15.46	1.13
Quercitrin	19.24	0.02	19.27	0.87	19.11	1.01	19.20	0.94
Quercetin	25.03	0.14	25.03	0.19	24.99	0.20	25.02	0.19
Hyperforin	26.05	0.01	26.07	0.03	26.06	0.01	26.06	0.03
Amentoflavone	26.45	0.09	26.45	0.12	26.42	0.13	26.44	0.12
Hypericin	32.84	0.15	33.64	2.01	35.19	0.43	33.86	3.19

Experimental conditions as in Table 1.

The precision of the chromatographic system was tested by performing intra- and inter-day multiple injections of a solution of all the compounds of interest and then checking the percent relative standard deviation (%RSD) of the retention times and peak areas. Ten injections were performed each day and this was repeated for 3 consecutive days. Tables 4 and 5 describe the %RSD values of retention times and peak areas.

It was concluded that there was no significant difference for the analyses tested within and between days.

The precision of the extraction procedure was validated by repeating the extraction procedure on seven samples of *H. perforatum* flowering tops. An aliquot of each extract was then injected and quantified. This parameter was evaluated by repeating the experiment on a different day with newly prepared mobile phase and samples. The SD data of the repeated analysis of *H. perforatum* methanolic extracts were found to be: 0.01 for chlorogenic acid, 0.48 for rutin, 0.30 for hyperoside and isoquercitrin, 0.11 for quercitrin, 0.04 for quercetin, 0.74 for hyperforin, 0.02 for amentoflavone, 0.14 for pseudohypericin and 0.23 for hypericin. The low values of SD indicate the high level of precision of the method.

The instrument detection limit (IDL) was calculated from the results of the replicate analyses of the lowest concentration calibration standard used, by using the formula $\mathrm{IDL} = t_{n-1} \times \mathrm{SD}$, where t_{n-1} is the t is the Student's value at 95% confidence level for $n-1$ degrees of freedom, n

Table 5
Intra- and inter-day precision data for peak area of the major constituents of *H. perforatum*

Compound	Intra-day precision ($n = 10$, mean)						Inter-day precision ($n = 30$, mean)	
	Day 1		Day 2		Day 3		Area (mAU × s)	RSD (%)
	Area (mAU × s)	RSD (%)	Area (mAU × s)	RSD (%)	Area (mAU × s)	RSD (%)		
Chlorogenic acid	869.94	0.69	874.40	0.42	884.58	0.47	876.31	0.88
Rutin	733.08	0.63	745.29	0.49	755.41	0.41	744.59	1.34
Hyperoside and isoquercitrin	2401.46	0.61	2441.11	0.28	2465.66	0.12	2436.08	1.17
Quercitrin	803.51	0.67	816.96	0.34	824.98	0.12	815.15	1.18
Quercetin	954.92	1.77	951.58	1.17	966.46	0.46	957.65	1.38
Hyperforin	291.82	0.49	292.56	0.41	293.43	0.96	292.61	0.63
Amentoflavone	1407.82	1.32	1418.90	0.67	1447.40	0.89	1424.71	1.53
Hypericin	542.44	0.72	525.11	1.08	523.67	1.22	530.41	1.91

Experimental conditions as in Table 1.

is the number of replicates and SD the standard deviation of replicate analyses. The IDL values were 0.06 μg/mL for chlorogenic acid, 0.02 μg/mL for rutin, 0.05 μg/mL for hyperoside, 0.04 μg/mL for isoquercitrin, 0.06 μg/mL for quercitrin, 0.02 μg/mL for quercetin, 0.04 μg/mL for hyperforin, 0.02 μg/mL for amentoflavone, 0.56 μg/mL for hypericin. The limit of detection (LOD) of the method was evaluated in the light of the analyte concentration that would yield a signal-to-noise ratio (S/N) of 3; the limit of quantification (LOQ) represents the analyte concentration that would yield a signal-to-noise ratio (S/N) of 10. The LOD and LOQ values were experimentally verified by injections of standard solutions of the compounds at the LOD and LOQ concentrations. The LOD values were found to be 0.44 μg/mL for chlorogenic acid, 0.21 μg/mL for rutin, 0.12 μg/mL for hyperoside, 0.14 μg/mL for isoquercitrin, 0.12 μg/mL for quercitrin, 0.06 μg/mL for quercetin, 0.16 μg/mL for hyperforin, 0.06 μg/mL for

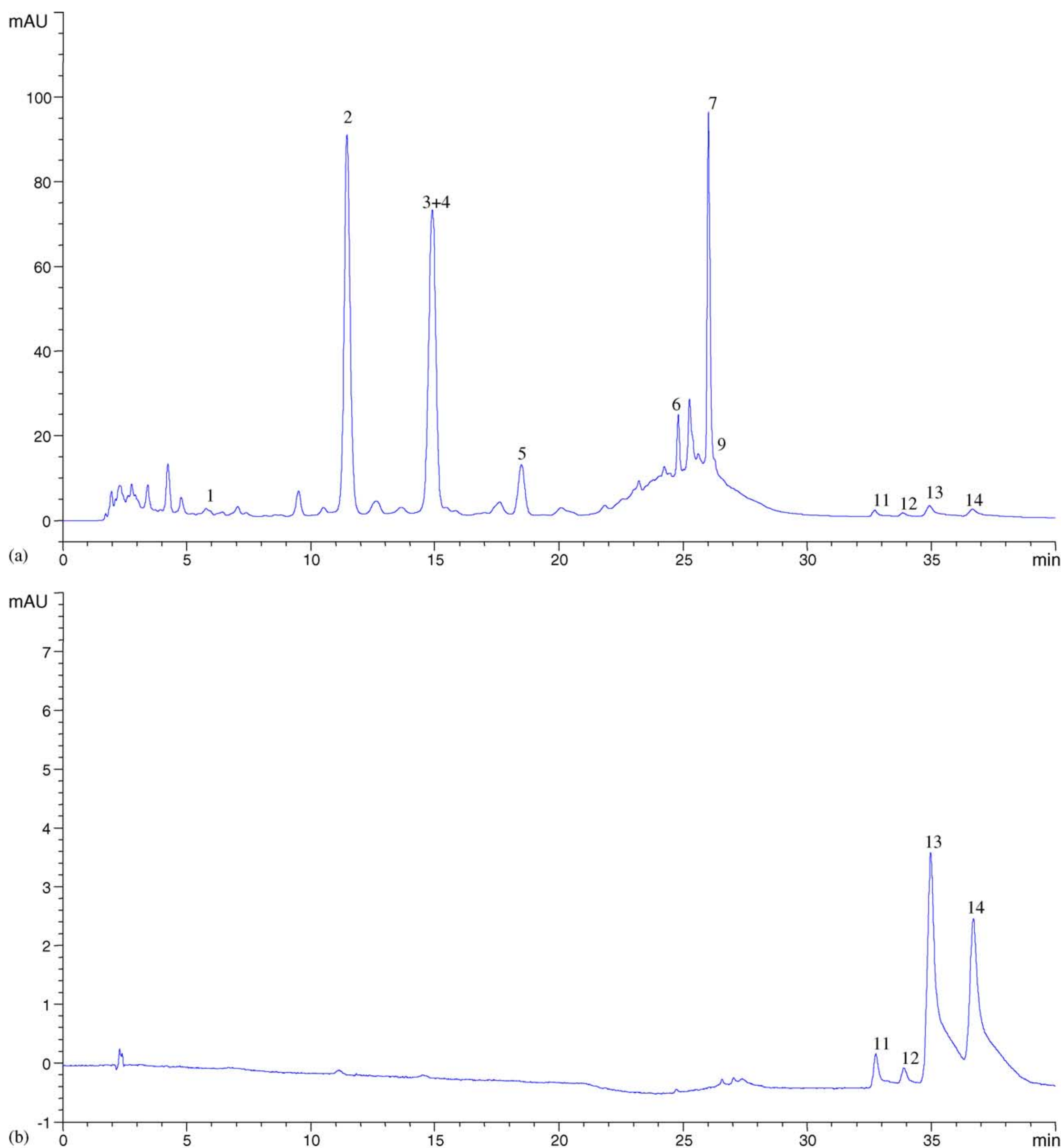

Fig. 4. Chromatogram of a methanolic extract of *H. perforatum* flowering tops. For peak identification, see Fig. 1. Experimental conditions as in Section 2.4. Detection at 270 nm (Fig. 4a) and 590 nm (Fig. 4b).

Table 6
Content of flavonoids of *H. perforatum* flowering tops and derivatives by means of the HPLC method

Sample	Content dry weight (mg/g[a])					
	Chlorogenic acid	Rutin	Hyperoside and isoquercitrin	Quercitrin	Quercetin	Amentoflavone
H. perforatum herb (flowering tops)	0.83 ± 0.01	10.47 ± 0.48	7.27 ± 0.30	1.26 ± 0.11	0.51 ± 0.04	0.10 ± 0.02
H. perforatum herbal medicinal products						
HMP1[b]	3.62 ± 0.10	7.99 ± 0.12	11.03 ± 0.11	2.08 ± 0.03	4.14 ± 0.38	0.23 ± 0.03
HMP2[c]	6.07 ± 0.15	14.77 ± 0.58	20.37 ± 0.73	3.54 ± 0.08	2.70 ± 0.08	0.13 ± 0.02
HMP3[b]	1.69 ± 0.17	14.41 ± 0.31	14.30 ± 0.19	2.41 ± 0.08	2.09 ± 0.07	0.08 ± 0.01
HMP4[c]	3.15 ± 0.21	22.47 ± 0.52	23.26 ± 0.29	3.58 ± 0.25	4.53 ± 0.15	0.17 ± 0.01
H. perforatum dietary supplements						
DS1[c]	2.73 ± 0.23	19.89 ± 0.36	22.54 ± 0.44	2.22 ± 0.09	3.00 ± 0.07	0.10 ± 0.02
DS2[c]	<LOD	0.07[d]	0.07[d]	0.09[d]	0.26 ± 0.03	0.03[d]
DS3[b]	2.27 ± 0.04	12.63 ± 0.03	15.03 ± 0.22	1.94 ± 0.04	1.01 ± 0.02	0.07 ± 0.01
DS4[c]	1.49 ± 0.03	29.37 ± 0.13	16.49 ± 0.11	2.97 ± 0.04	2.63 ± 0.03	0.33 ± 0.01
DS5[c]	0.54 ± 0.03	5.68 ± 0.04	5.20 ± 0.04	0.76 ± 0.01	0.72 ± 0.05	0.08 ± 0.01

Experimental conditions as in Table 1.
[a] Data are expressed as mean ± SD (standard deviation). For each sample $n = 6$.
[b] Non-standardized.
[c] Standardized for the content of "total hypericins" (0.3%). The terms non-standardized and standardized refer to label claims.
[d] SD < 0.01.

amentoflavone and 1.34 μg/mL for hypericin. The LOQ values were 1.47 μg/mL for chlorogenic acid, 0.70 μg/mL for rutin, 0.39 μg/mL for hyperoside, 0.46 μg/mL isoquercitrin, 0.42 μg/mL for quercitrin, 0.21 μg/mL for quercetin, 0.53 μg/mL for hyperforin, 0.20 μg/mL for amentoflavone and 4.48 μg/mL for hypericin. These results indicate that the proposed HPLC method was sufficiently sensitive for the determination of the secondary metabolites in *H. perforatum* samples.

Specificity was tested by applying the HPLC method to herbal formulations containing extracts of *H. perforatum* and excipients. By comparison with the assay results of *H. perforatum* flowering tops, the chromatograms obtained from the herbal products showed that the HPLC method is able to discriminate the active constituents of *H. perforatum* from the excipients, and the results are unaffected by the presence of these materials. Furthermore, peak purity test was performed using the photodiode array detector to demonstrate that the analyte chromatographic peak is pure, i.e. not attributable to more than one component, with the exception of hyperoside and isoquercitrin.

Stability was tested with *H. perforatum* methanolic extracts that were stored in amber glass flasks at 4 °C and at room temperature (about 25 °C) and analyzed every 12 h. The analytes in solution did not show any appreciable change in chromatographic profile for 72 h. No degradation products were detected. According to the literature [25], hyperforin in the methanolic extracts of *H. perforatum* is more stable than in its pure form, characterized by a very high sensitivity to oxidation.

Table 7
Content of phloroglucinols and naphthodianthrones of *H. perforatum* flowering tops and derivatives by means of the HPLC method

Sample	Content dry weight (mg/g[a])				
	Hyperforin	Protopseudohypericin	Protohypericin	Pseudohypericin	Hypericin
H. perforatum herb (flowering tops)	14.17 ± 0.74	<LOQ	<LOQ	2.26 ± 0.14	2.09 ± 0.23
H. perforatum herbal medicinal products					
HMP1[b]	67.89 ± 0.66	0.21[d]	<LOQ	1.82 ± 0.07	1.35 ± 0.08
HMP2[c]	17.04 ± 0.65	<LOQ	<LOD	1.57 ± 0.02	1.03 ± 0.06
HMP3[b]	9.13 ± 0.25	0.22 ± 0.02	<LOD	1.13 ± 0.02	0.62 ± 0.04
HMP4[c]	63.91 ± 0.97	0.45 ± 0.02	0.25 ± 0.03	4.31 ± 0.09	1.69 ± 0.02
H. perforatum dietary supplements					
DS1[c]	26.69 ± 0.87	0.56 ± 0.02	0.24 ± 0.02	3.80 ± 0.13	1.26 ± 0.07
DS2[c]	0.70 ± 0.04	<LOD	<LOD	<LOQ	<LOQ
DS3[b]	8.07 ± 0.21	0.23 ± 0.02	<LOQ	2.27 ± 0.07	1.62 ± 0.04
DS4[c]	49.15 ± 0.62	1.08 ± 0.05	0.43 ± 0.02	5.11 ± 0.05	3.62 ± 0.03
DS5[c]	8.16 ± 0.08	0.46 ± 0.01	0.24[d]	3.22 ± 0.04	1.97 ± 0.03

[a] Data are expressed as mean ± SD (standard deviation). For each sample $n = 6$.
[b] Non-standardized.
[c] Standardized for the content of "total hypericins" (0.3%). The terms non-standardized and standardized refer to label claims.
[d] SD < 0.01.

The validation data highlighted the suitability of the proposed HPLC method for the analysis of *H. perforatum* samples.

3.4. Applications to H. perforatum extracts

The HPLC method previously described was used to identify and quantify the amount of the secondary metabolites in *H. perforatum* plant material and commercially available herbal medicinal products and dietary supplements.

Fig. 4 shows the chromatograms of the HPLC analysis of the methanolic extract of *H. perforatum* flowering tops.

With respect to traditional C_{18} columns [15,17,19,22,24, 26,28,35,41], the stationary phase employed in this study provided a different elution order of the phloroglucinols, which eluted before the naphthodianthrones. Furthermore, the order

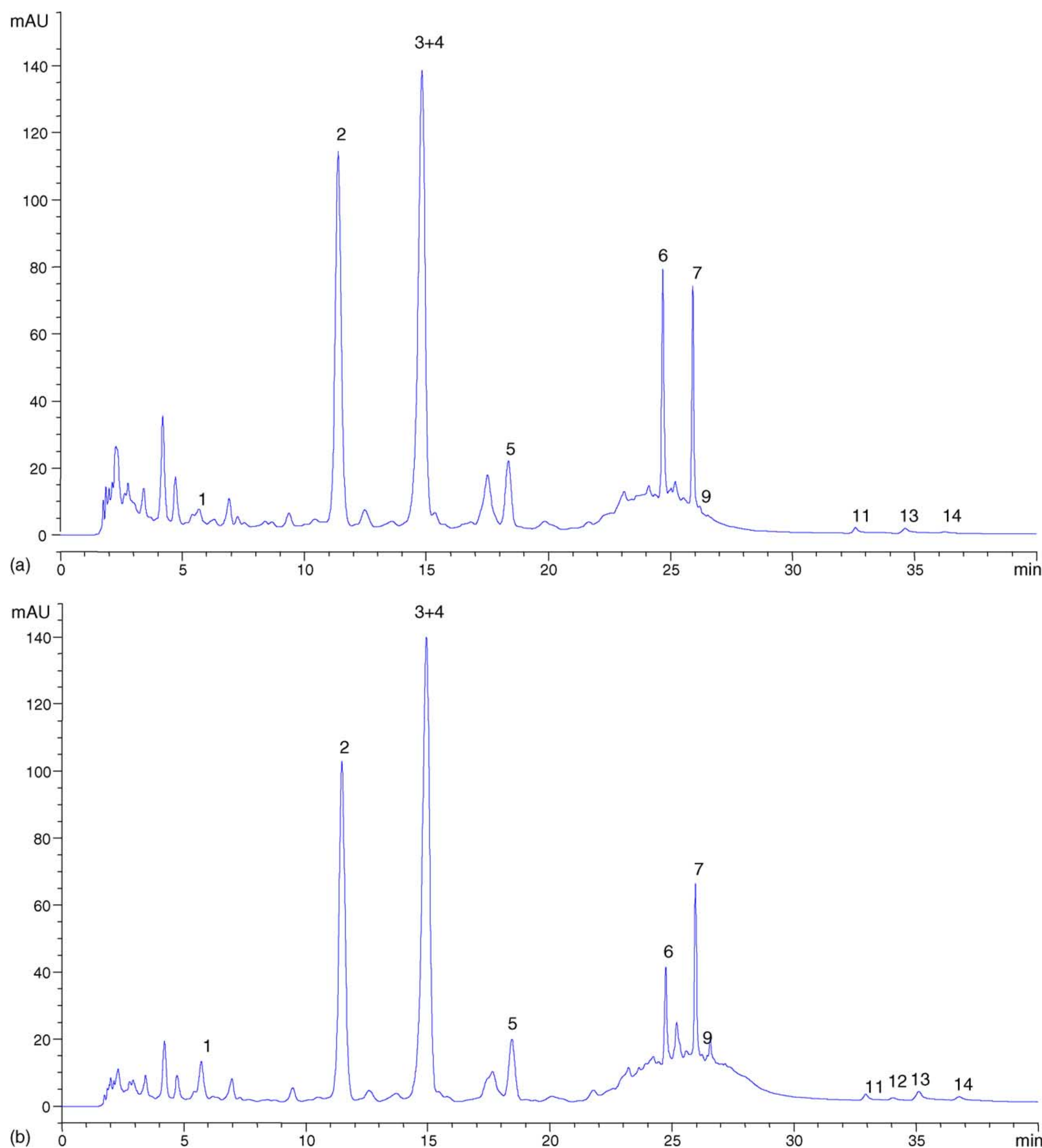

Fig. 5. Chromatogram of: (a) *H. perforatum* herbal medicinal product (HMP3); (b) *H. perforatum* dietary supplement (DS3). For peak identification, see Fig. 1. Experimental conditions as in Section 2.4. Detection UV at 270 nm.

of elution of the naphthodianthrones is different than those reported in the literature [17,29,31,34,41].

Tables 6 and 7 report the amounts of these compounds in *H. perforatum* herb (flowering tops).

Data are expressed as mg/g of dry weight. These quantification data are within the range of those of previous reports [20,23,27,33,34,38,40]. Rutin, hyperoside and isoquercitrin were the main flavonoid compounds of *H. perforatum* flowering tops. The content of hyperforin was high. Protopseudohypericin and protohypericin were detected but not quantified because their level was below the LOQ value.

Tables 6 and 7 also describe the results of the HPLC analysis of *H. perforatum* herbal medicinal products and dietary supplements. The chromatogaphic profiles of these products (Fig. 5) were the same as those reported for *H. perforatum* plant material (Fig. 4).

However, there is a great variability in the concentrations of the active constituents among the commercial samples on sale on the Italian market. This observation is in accordance with the literature [22,24–26,28,40]. Some of these products are categorized as "herbal medicinal products" and are subject to prescription-only control. The others, categorized as "dietary supplements", are not regulated as drugs. Most of the products are standardized for the content of total hypericin. The phloroglucinol hyperforin is unstable in the presence of light and oxygen; for this reason, the naphthodianthrones, expressed as total hypericin, are usually considered the marker compounds and are applied to standardize extracts and phytopharmaceuticals. However, label claims of a minimum of 0.3% hypericin in the extracts were not confirmed in this study. This means that standardization did not guarantee that the sample contained as much compound as was declared on the label. The product labels do not specify by which method the extract was standardized, i.e. by rather unspecific spectrophotometry or by the much more specific HPLC.

This study on *H. perforatum* commercial products showed that the main differences in the quantitative composition of secondary metabolites relate in particular to the products labeled as dietary supplements. Some preparations contained a very high amount of constituents (DS1, DS4), while others had very low levels (DS2). All these natural remedies require a pharmaceutical level of assurance for efficacy and safety in their use. The chemical composition of these natural products can differ significantly. Even the same plant material may vary depending on genetic variation and environmental factors, such as light, temperature, agronomic practices and so on. Furthermore, drying temperature, extraction methods, formulations and storage conditions may have occasioned this variability. In addition to the necessity of appropriate labeling of *H. perforatum* products for sale, the quality of the plant material and its derivatives used for clinical studies should be clarified to ensure better validity of the results. Futhermore, considering the several cases of drug-herb interactions reported in the literature, the possible role of hyperforin in these adverse effects [35,38,43] and the variability in the content of this compound reported in this study, its quantification seem to be very important to ensure the safety of *H. perforatum* natural products.

Modern HPLC analytical methods allow the rapid and reliable analysis of complex mixtures such as plant extracts: the correlation of chromatograms, used as fingerprints, between authentic as against unknown samples, allows the identification of plant material and facilitates the search for any adulteration. In particular, the proposed HPLC method could be applied to monitor the quality of *H. perforatum* crude drugs, extracts and commercial products.

4. Conclusion

The HPLC technique reported in this study, using a poly(ethylene glycol) stationary phase, is suitable for the analysis of the active compounds in *H. perforatum* extracts and natural products. The method is simple, precise and economical in terms of time and solvent usage. Through these phytochemical markers, this method allows the unequivocal identification and standardization of this plant material. The validation procedure confirms that this technique affords reliable analysis of these components and is appropriate for the quality control of complex matrices such as *H. perforatum* crude drugs, extracts and herbal medicines.

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