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Identification by high-performance liquid chromatography–diode array detection–mass spectrometry and quantification by high-performance liquid chromatography–UV absorbance detection of active constituents of *Hypericum perforatum*

M. Brolis, B. Gabetta, N. Fuzzati, R. Pace*, F. Panzeri, F. Peterlongo

Indena S.p.A. Laboratori Ricerca e Sviluppo, Via Don Minzoni 6, 20090 Settala (Milan), Italy

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Abstract

Hypericum perforatum is a medicinal plant which has been known in traditional medicine as an anti-inflammatory and healing agent. Nowadays, the alcoholic extract of its aerial parts finds wide application for its antidepressant activity. A high-performance liquid chromatography (HPLC) method for the identification of its constituents using a wide pore RP-18 column and a water–methanol–acetonitrile–phosphoric acid mobile phase system was developed. The identification of its flavonoid, naphthodianthrone and phloroglucinol constituents was performed using combined HPLC–diode array detection (DAD) analysis, HPLC–thermospray and HPLC–electrospray mass spectrometry. Chlorogenic acid, quercetin, quercitrin, isoquercitrin, rutin, hyperoside, I3,II8-biapigenin, pseudohypericin, hypericin, hyperforin and adhyperforin were separated by an aqueous phosphoric acid–acetonitrile–methanol gradient within 50 min. The quantification of the above constituents was performed using rutin as an external standard.

Keywords: *Hypericum perforatum*; Flavonoids; Naphthodianthrones; Phloroglucinols; Rutin; Chlorogenic acid

1. Introduction

Hypericum perforatum L. is a herbaceous perennial plant, belonging to Hypericaceae family, distributed in Europe, Asia, Northern Africa and naturalized in the USA. It is a well known medicinal plant since the antiquity and was recognized as ''Erba di S. Giovanni'' in Italy and ''St. John's Wort'' in Anglo-Saxon folk medicine. The extract of aerial part in blossom had since the Middle Ages a high reputation as an anti-inflammatory, antidepressive and healing agent.

Nowadays the use of *Hypericum* extracts is concerned mainly with antidepressive applications [1].

H. perforatum contains a number of constituents with documented biological activity [2,3] including chlorogenic acid, a broad range of flavonoids, naphthodianthrones and phloroglucinols (Fig. 1).

Generally, high-performance liquid chromatography (HPLC) analytical methods of *H. perforatum* preparations are concerned with the determination of

*Corresponding author.

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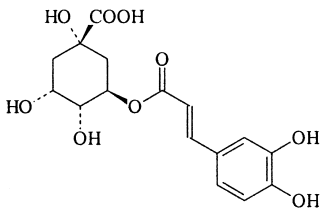
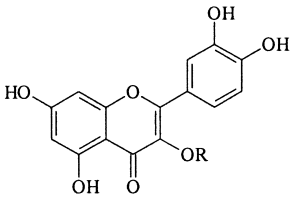


Chlorogenic acid (**3**)



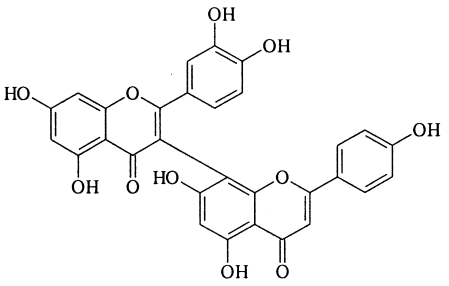
Rutin R = Glu-Rha (**4**)

Hyperoside R = Gal (**5**)

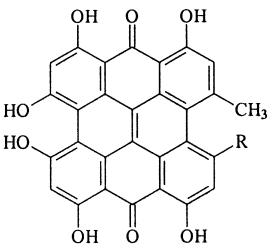
Isoquercitrin R = Glu (**6**)

Quercitrin R = Rha (**8**)

Quercetin R = H (**9**)

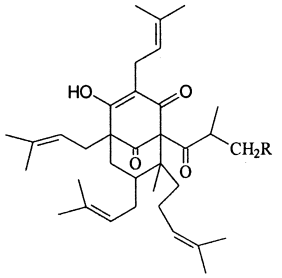


I 3 II 8 - biapigenin (**10**)



Pseudohypericin R = CH_2OH (**11**)

Hypericin R = CH_3 (**12**)



Hyperforin R = H (**15**)

Adhyperforin R = CH_3 (**16**)

Fig. 1. Constituents of *Hypericum perforatum*.

hypericin and pseudohypericin content [4–6]. The eluent system consists of acidic phosphate buffers with gradients of methanol and acetonitrile. Usually reversed-phase columns (RP-18) with a particle size of not more than 5 μm are recommended.

As the biological effects of *Hypericum* preparations are considered arising, rather than from presence of a single constituent, from the whole mixture of the above metabolites, the availability of a method allowing the analysis of the entire extract is desirable. In the present work we describe a HPLC analytical method which allows the identification and quantitation of all the metabolites listed in Fig. 1. In addition, since availability and chemical stability of several of the above constituents are critical, the use of the stable and easily available constituent rutin as

an external standard was considered for the assay of the constituents of the extract.

2. Experimental

2.1. Standards and samples

Hypericin was purchased from Analyticon (Berlin, Germany); isoquercitrin and chlorogenic acid from Extrasynthèse (Genay, France); rutin from Merck (Darmstadt, Germany); quercetin from Fluka Chemicals (Sigma–Aldrich Division, Milan, Italy).

Pseudohypericin, quercitrin, hyperoside, I3,II8-biapigenin, hyperforin were isolated and characterized at Indena Chemical Labs.

Hypericum perforatum extract was prepared by hot methanol extraction of dried flowering tops of plant material collected in July–August in Italy.

2.2. Solvents

Acetonitrile and methanol were HPLC grade from J.T. Baker (Deventer, Netherlands); 85% phosphoric acid reagent grade from Ashland (Milan, Italy). Water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA, USA).

2.3. Instrumentation

The HPLC system consisted of a 2690 Alliance Waters quaternary pump. The detection was performed with a Tunable UV–visible detector Waters 486 working at 270 nm and a diode array detector Waters 996 working in the range of 200–650 nm (Milford, MA, USA). The chromatographic data were recorded and processed by the Waters Millennium 2015 software.

For the liquid chromatography (LC)–thermospray (TSP) mass spectrometry (MS) analysis a Finnigan MAT (San Jose, CA, USA) TSQ-700 triple quadrupole mass spectrometer equipped with a 5100 DEC station, ICIS data system and a TSP-2 interface, was used. The HPLC system included a Waters 600-MS pump (Bedford, MA, USA) equipped with a gradient controller, a tunable UV–Vis Waters 486 detector and a Waters 717 automatic sample injection module.

For the LC–electrospray (ESI) MS analysis a Finnigan MAT LCQ ion trap mass spectrometer equipped with a Microsoft Windows NT data system and an ESI interface was used. The HPLC system included a Thermo Separation Product P4000 pump (San Jose, CA, USA) and a tunable Thermo Separation Products UV1000 detector.

Mass spectrometer conditions were optimized in order to achieve maximum sensitivity. TSP values: source block temperature 230°C, vaporizer temperature 90°C, discharge voltage 2000 V and repeller voltage 30 V. Electron multiplier voltage: 1800 V, dynode: 20 kV. Filament: off-mode. Full scan spectra from m/z 200 to 700 u in the positive ion mode were obtained (scan time 1 s). Concentration of the *Hypericum* extract: 30 mg/ml (methanol). Injection volume: 20 μl.

ESI values were: source voltage 3.6 kV, sheath gas flow-rate 62 p.s.i., auxiliary gas flow 9 p.s.i., capillary voltage −16 V and capillary temperature 200°C (1 p.s.i.=6894.76 Pa). Full scan spectra from m/z 200 to 700 u in the negative ion mode were obtained (scan time 1 s). Injected volume of *Hypericum* extract: 20 μl of a 1 mg/ml (methanol) solution.

2.4. Analytical HPLC method

Analyses were carried out at 30°C on a 201 TP 54 RP-18 column (250×4.6 mm I.D., 5 μm, 300 Å, Vydac Separation Group Hesperia, CA, USA), protected with an Alltech direct-connect universal column prefilter of 2 μm porosity (Deerfield, IL, USA). Chromatographic separation was carried out using three solvents [A=water–85% phosphoric acid (99.7:0.3 v/v); B=acetonitrile; C=methanol] in a linear gradient programme shown in Table 1. The flow-rate was 1.0 ml/min, the injection volume was 10 μl and the dwell volume was 1.5 ml (the dwell volume given was based upon the use of Waters Alliance system: if another system were used, adjustments would be necessary). Peaks were detected at 270 nm. A typical chromatogram is reported in Fig. 2.

The t_R values for chlorogenic acid (**3**), rutin (**4**), hyperoside (**5**), isoquercitrin (**6**), quercitrin (**8**), quercetin (**9**), I3,II8-biapigenin (**10**), pseudohypericin (**11**), hypericin (**12**), hyperforin (**15**) and adhyperforin (**16**) were 11.27, 16.17, 16.52, 16.92,

Table 1
Linear gradient programme for HPLC analysis

Time (min)	Solvent A (%)[a]	Solvent B (%)[b]	Solvent C (%)[c]
Initial	100	0	0
10	85	15	0
30	70	20	10
40	10	75	15
55	5	80	15
56	100	0	0
65	100	0	0

[a] Solvent A = Water–85% phosphoric acid (99.7:0.3, v/v).
[b] Solvent B = Acetonitile.
[c] Solvent C = Methanol.

19.70, 28.20, 36.00, 40.93, 43.37, 46.07 and 46.65 min, respectively.

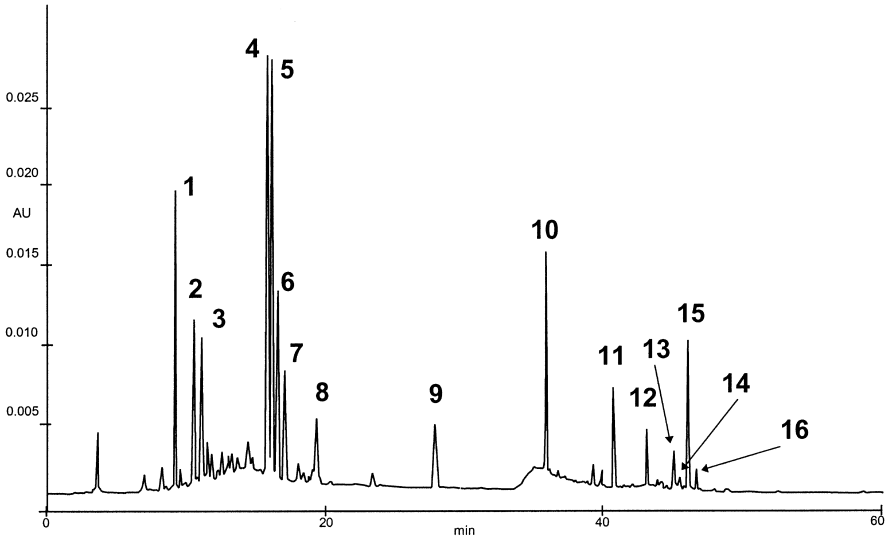
2.5. Identification and peak purity

Constituents **3–6**, **8–12**, **15** were identified by HPLC–MS analysis and by comparing the retention time of the peaks in the extracts with those of the authentic reference samples. The purity of the peaks was checked by photodiode array detection. The UV spectra of a peak at three different points (upslope, top and downslope) were compared with the UV spectrum of the authentic reference sample. Furthermore, the purity of peaks was confirmed by multivariate analysis using Waters Millennium 2015 software and by checking the MS spectra.

2.6. Determination of the relative response factors

In order to evaluate the content (%) of constituents **3–6**, **8–12**, **15**, **16** in the extract using rutin as an external standard, the respective response factors relative to rutin were calculated.

Relative response factor (RRF) was determined calculating the ratio between the average response



1	Chlorogenic acid isomer	**9**	Quercetin
2	3-*O*-p-Coumaroylquinic acid	**10**	I3, II8 Biapigenin
3	Chlorogenic acid	**11**	Pseudohypericin
4	Rutin	**12**	Hypericin
5	Hyperoside	**13**	Hyperforin analogue
6	Isoquercitrin	**14**	Hyperforin analogue
7	3,3', 4', 5, 7-Pentahydroxyflavanone 7-*O*-Rhamnopyranoside	**15**	Hyperforin
		16	Adhyperforin
8	Quercitrin		

Fig. 2. Profile of *Hypericum perforatum* extract with the HPLC–MS attributions of the components detected.

factor of each compound and the average response factor of rutin at 270 nm.

The purity value of the reference standard of compounds **3–6**, **8–12**, **15** was calculated considering residual solvents, moisture and amount of impurities. The results are summarized in Table 2. For each compound three different preparations and three different injections were used.

2.7. Linearity

Linearity was determined for rutin reference standard and for the constituents **3–6**, **8–12**, **15**, **16** in the *Hypericum perforatum* extract.

Linearity of responses was determined on five levels of concentration with three injections for each level. Rutin was linear from 4.5 to 100 μg/ml and all the curves had coefficient of linear correlation ≥0.999 with *y*-intercepts which were close to zero.

2.8. Stability

Rutin and *Hypericum perforatum* extract were solubilized in methanol and in methanol–water (9:1, v/v), respectively, before the analysis. The stability at room temperature of these solutions was evaluated.

The analyses were performed by injecting the stability solutions every 4 h up to 48 h and the regression lines of the stability studies were calculated using the area responses vs. time intervals.

Table 2
Relative response factors of constituents **3–6**, **8–12**, **15**, **16**

Constituents	Reference standard purity[a]	RRF
Chlorogenic acid (**3**)	96.23	0.506
Rutin (**4**)	88.17	1.000
Hyperoside (**5**)	89.79	1.283
Isoquercitirn (**6**)	82.56	1.275
Quercitrin (**8**)	85.63	1.368
Quercetin (**9**)	87.16	1.704
I3,II8-Biapigenin (**10**)	91.94	2.469
Pseudohypericin (**11**)	84.86	1.550
Hypericin (**12**)	83.97	1.608
Hyperforin (**15**)	94.46	0.570
Adhyperforin (**16**)	–	0.570[b]

[a] Calculated considering the content of residual solvents, moisture and amount of impurities.
[b] Considered as hyperforin.

The slope of stability lines was used to estimate the stability of constituents up to 48 h.

2.9. Reproducibility of the injection integration and repeatability

The reproducibility of the injection integration procedure was determined for rutin and for the constituents **3–6**, **8–12**, **15**, **16** in *Hypericum perforatum* extract. The solutions were injected ten times and the relative standard deviation (R.S.D.) values were calculated (chlorogenic acid 1.26%, rutin 0.17%, hyperoside 0.18%, isoquercitrin 0.22%, quercitrin 0.38%, quercetin 0.40%, I3,II8-biapigenin 0.17%, pseudohypericin 2.32%, hypericin 0.84%, hyperforin 1.27%, adhyperforin 0.66%).

In order to evaluate the repeatability of the method, three solutions at different concentrations (0.8 mg/ml, 1.0 mg/ml, 1.2 mg/ml of dry extract in methanol) of *Hypericum perforatum* extract were prepared. Each solution was injected three times. The contents of constituents **3–6**, **8–12**, **15**, **16** were calculated in order to estimate the R.S.D. (chlorogenic acid 1.59%, rutin 1.92%, hyperoside 1.92%, isoquercitrin 1.90%, quercitrin 2.70%, quercetin 2.15%, I3,II8-biapigenin 1.93%, pseudohypericin 2.62%, hypericin 3.96%, hyperforin 2.00%, adhyperforin 1.95%).

2.10. Robustness

To evaluate the reliability of the analytical method a full factorial design study was applied.

The effects of the chromatographic parameters (factors) as wavelength of the detector and column temperature on the assay of the constituents **3–6**, **8–12**, **15**, **16** were evaluated.

Each factor is used at a high level (+1, detector wavelength 272 nm, column temperature 35°C) and a low level (−1, detector wavelength 268 nm, column temperature 25°C) with respect to the value specified in the analytical method (central level 0, detector wavelength 270 nm, column temperature 30°C).

The number of experiments required for a full factorial design of two factors at two levels is $2^2=4$; one central level combination is included in the design so that five experiments were performed.

2.11. Quantitation

The dried flowering tops of *Hypericum perforatum* (1.0 g) were extracted with 100 ml of methanol for 6 h with a Soxhlet apparatus. A portion (2 ml) of the cold supernatant was diluted to 10 ml in a volumetric flask with methanol–water (9:1, v/v) and the obtained samples were filtered through a cartridge-type sample filtration unit with a polytetrafluoroethylene (PTFE) membrane before HPLC analysis.

Rutin (10 mg) was dissolved in 400 ml of methanol (0.025 mg/ml). After verification of the suitability of the system, all standards and samples (two preparation for each of them) were injected alternatively. Injections of methanol–water (9:1, v/v) were performed at the beginning and at the end of the analysis sequence.

The contents of constituents **3–6**, **8–12**, **15**, **16** can be calculated by the following equation:

$$\text{Contents (\%)} = \frac{A_{\text{sample}}}{\text{RF}_{\text{std}} C_{\text{sample}}} \cdot \frac{1}{\text{RRF}} \cdot 100$$

where A_{sample} is the peak area of the considered constituent in the test solution (area counts), RF_{std} the mean response factor of rutin in the reference solutions [response factor = area/(conc.$_{(\text{mg/ml})}$ · purity/100)], C_{sample} the concentration of the test solution (mg/ml) and the RRF the response factor of the considered constituent relative to rutin (Table 2).

3. Results and discussion

The analytical method was designed to fulfil the following requirements: to give accurate quantitative data on the most important constituents; to employ widely available HPLC columns; to provide good specificity and reproducibility; to employ an easily available reference substance.

Several organic aqueous-based mobile phases were tested, including water–acetonitrile in combination with ammonium acetate buffer, phosphate buffer, phosphoric acid, trifluoroacetic acid, tetrahydrofuran–trifluoroacetic acid, tetrahydrofuran–phosphoric acid, methanol–trifluoroacetic acid, methanol–phosphoric acid, acetonitrile–methanol–trifluoroacetic acid and acetonitrile–methanol–phosphoric acid. These organic aqueous-based mobile phases were tested with RP-8, RP-18 and CN stationary phases with granulometry of 80, 100, 120 and 300 Å. Eventually, it was found that a water–acetonitrile–methanol–phosphoric acid solvent system (Table 1) with RP-18 300 Å column successfully separated the chemicals within 50 min (Fig. 2). Furthermore, it became evident that the temperature of the column oven affected the separation. For convenience, 30°C was selected as the oven temperature for the following analyses, since satisfactory separation of all constituents was achieved at this temperature. UV detection was fixed at 270 nm, as all the constituents of the extract showed appreciable absorbance at this wavelength.

HPLC–MS analyses were performed in order to identify the constituents and to verify the specificity of the analytical method. Since phosphoric acid was not suitable for HPLC–MS operations, separation was performed using aqueous 0.3% (v/v) formic acid, without appreciable variations in the chromatographic profile.

The obtained TSP-MS spectra exhibited $[M+H]^+$ and, frequently, $[M+Na]^+$ and $[M+K]^+$ ions.

Peaks **1** and **3**, which displayed identical UV spectra with maxima at 236 and 327 nm, showed $[M+H]^+$ ions at m/z 355 and $[M+H-H_20]^+$ ions at m/z 337. Peak **3** was identified as chlorogenic acid by comparison with an authentic sample. Peak **1** was therefore considered as an isomer of chlorogenic acid. The TSP-MS spectrum of peak **2** exhibited a $[M+H]^+$ ion at m/z 339 and a $[M+H-H_20]^+$ ions at m/z 321. These data, together with an ipsochromic shift of 16 nm in the UV maximum (331 nm) compared to that of chlorogenic acid, allowed the identification of **2** as 3-*O*-*p*-coumaroylquinic acid. Constituents **4–6** and **8** displayed identical UV absorptions with maxima at 259 and 355 nm, which is typical of flavonols. Moreover, the presence in all TSP-MS spectra of an ion at m/z 303 indicated that constituents **4–6** and **8** are quercetin derivates. Peak **4**, which exhibited $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ ions at m/z 611, 633 and 649, respectively, and fragment ions at m/z 465 and 303 due to the successive loss of a deoxy-hexose unit and a hexose unit, respectively, was identified as rutin by comparison with an authentic sample. Peak **5**, which exhibited $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ ions,

at m/z 465, 487 and 503, respectively, and a fragment ion at m/z 303 due to the loss of a hexose unit, was identified as hyperoside by injection of an authentic sample. Peak **6**, which exhibited the same TSP-MS spectrum as **5**, was identified as isoquercitrin by injection of an authentic sample. Peak **8** exhibited a TSP-MS spectrum with an $[M+H]^+$ ion at m/z 449, an $[M+Na]^+$ ion at m/z 471 and a fragment ion at m/z 303 due to the loss of a deoxy-hexose. Peak **8** was identified as quercitrin by injection of an authentic sample.

Peak **7** displayed an UV spectrum with a maximum at 292 nm and a shoulder at 342 indicating that it was due to a flavanone [11]. The TSP-MS exhibited a $[M+H]^+$ ion at m/z 451, a $[M+Na]^+$ ion at m/z 473 and a fragment ion at m/z 305 due to the loss of deoxy-hexose, allowing the tentative identification of **7** as 3,3′,4′,5,7-pentahydroxyflavanone 7-*O*-rhamnopyranoside, previously isolated from *Hypericum japonicum* [7].

Peak **9** exhibited UV maxima at 257 and 371 nm together with a shoulder at 310 nm and the TSP-MS spectrum displayed a $[M+H]^+$ ion at m/z 303. It was therefore identified as quercetin, and the assignment was confirmed by injection of an authentic sample.

Peak **10** exhibited a UV spectrum with maxima at 270 and 334 nm together with a $[M+H]^+$ ion at m/z 539 in the TSP-MS spectrum. Compound **10** was therefore identified as I3,II8-biapigenin, which is a known constituent of *Hypericum perforatum* [4]. Injection in comparison with an authentic sample confirmed the attribution.

Peaks **13-16** displayed identical UV absorptions with a maximum at 274 nm. The TSP-MS spectrum of peak **15** exhibited a $[M+H]^+$ ion at m/z 537 suggesting that compound **15** was hyperforin. The presence of fragment ions at m/z 481, 469, 411 and 277, arising from the loss of the side chains [8,9], and the co-injection with an authentic sample confirmed the identification. The TSP-MS spectrum of peak **16** exhibited a $[M+H]^+$ ion at m/z 551 together with a fragment ions at m/z 495, 483, 427 and 291, thus allowing the identification of **16** as adhyperforin [8,9]. Peaks **13**, **14** exhibited the same TSP-MS spectrum with a fragmentation pattern similar to that of hyperforin. The $[M+H]^+$ ion at m/z 553, together with the fragment ions at m/z 497, 485, 427 and 293, indicated that **13** and **14** contained an additional oxygen compared to hyperforin. These *Hypericum* metabolites have not been described in literature so far. Their isolation and identification is presently in progress.

Peaks **11** and **12** exhibited UV spectra with maxima in the red-visible region suggesting that these constituents were hypericin derivates. Injection with authentic samples allowed the identification of **11** as pseudohypericin and **12** as hypericin. The TSP-MS conditions were not suitable to obtain ionization of these constituents. It was found that electrospray ionization process is a useful technique for the detection of naphthodianthrones derivates [10]. Therefore, a HPLC–ESI-MS analysis was also performed.

The ESI-MS spectra, in the negative ion mode, of all the detected constituents exhibited an $[M-H]^-$ ion confirming the results obtained with the TSP-MS analysis. In particular, the ESI-MS spectra of peaks **11** and **12** displayed $[M-H]^-$ ion at m/z 519 and m/z 503, thus confirming their identification as pseudohypericin and hypericin, respectively.

The HPLC–MS and purity peaks analysis demonstrate the specificity of the analytical method.

The easily available rutin (peak **4** in the chromatogram) was chosen as a reference substance for the determination of the assay of the individual metabolites.

Rutin was found to be stable in methanol solution at room temperature for at least 48 h.

Constituents **3–6**, **8–12**, **15**, **16** in the extract were stable at room temperature when kept in the dark in methanol–water (9:1, v/v) solution for at least 48 h.

The content of each constituent **3–6**, **8–12**, **15**, **16** was calculated using the response factor, relative to rutin (RRF), calculated as described in Section 2.6. Table 2 reports the RRF values.

Repeatability was investigated on two different instrumentations, in two different days and by two different operators.

The method was found to be linear and precise when a concentration of 0.8–1.2 mg/ml of *Hypericum perforatum* extract was injected.

The results of the robustness study, applying a full factorial design, show that the method is also robust. Small variations in the most important chromatographic parameters do not drastically affect the

analysis. However the experimental parameters examined, both column temperature and wavelength accuracy, should be strictly controlled in this analytical procedure.

4. Conclusions

A new HPLC method has been developed for the analysis of *Hypericum perforatum* extracts allowing the identification and quantitation of 16 constituents using rutin as external standard.

The method was found to be specific and suitable for routine analysis because of its simplicity, specificity, accuracy and reproducibility.

References

[1] E. Bombardelli, P. Morazzoni, Fitoterapia 66 (1995) 43.
[2] G. Lavie, Y. Marzur, D. Lawie, D. Merelo, Med. Res. Rev. 15 (1995) 111.
[3] J. Hölzl, S. Sattler, H. Shütt, Pharm. Ztg. 139 (1994) 3959.
[4] J. Holz, E. Ostrowski, Dtsch. Apoth. Ztg. 23 (1987) 1227.
[5] H. Häberlein, K.P. Tshiersch, S. Stock, J. Hölzl, Pharm. Ztg. Wiss. 137 (1992) 169.
[6] W. Krämer, R. Wiartalla, Pharm. Zrg. Wiss. 137 (1992) 202.
[7] Dictionary of Natural Products on CD-ROM, Chapman and Hall, London, 1997, release 5:2.
[8] P. Maisenbacher, K.A. Kovar, Planta Med. 58 (1992) 291.
[9] J. Bruneton, Pharmacognosy, Phytochemistry, Medicinal Plants, Lavoisier, Paris, 1993.
[10] G. Piperopoulos, R. Lotz, A. Wixforth, T. Schmierer, K.-P. Zeller, J. Chromatogr. B 695 (1997) 309.
[11] T.J. Mabry, K.R. Markham, M.B. Thomas, The Systematic Identification of Flavonoids, Springer-Verlag, Berlin, 1970.