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# Simultaneous determination of hypericin and hyperforin in human plasma and serum using liquid–liquid extraction, high-performance liquid chromatography and liquid chromatography–tandem mass spectrometry

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**Abstract**

A method for the simultaneous extraction of hypericin and hyperforin from a St. John's Wort extract, which is used in case of moderate depressions and skin injuries, from human plasma and serum by liquid–liquid extraction (LLE) with *n*-hexane–ethylacetate (70:30, w/w) was developed. A reversed-phase high-performance liquid chromatographic (RP-HPLC) method with UV, fluorescence (FLD) and mass spectrometric (MS) detection using electrospray ionization (ESI) was used to identify and quantify hypericin and hyperforin in the extracts from blood samples. Linearity was obtained in the ranges 8.4–28.7 ng/ml (hypericin) and 21.6–242.6 ng/ml (hyperforin). Recoveries were between 32.2 and 35.6% for hypericin and 100.1 and 89.9% for hyperforin. Intra-day accuracy and precision for this method ranged between 3.2 and 4.3% and 2.6 and 2.8%, respectively. After validation of the LLE, the method was tested on real plasma samples which were obtained by ingestion of St. John's Wort extract capsules. Blood samples were taken 2, 4, and 6 h after ingestion. Finally, this method proved to be highly suitable for clinical and pharmacologically relevant studies.


*Keywords:* Hypericin; Hyperforin

## 1. Introduction

St. John's Wort extract is used for the treatment of skin injuries, burns, neuralgia, for its antibacterial activity and as a treatment for mild to moderate depression [1–7]. Hypericin and hyperforin are discussed [8–13] as being the active components of the antidepressant activity in *Hypericum perforatum* L. extracts, although it is still unclear how and why St. John's Wort extract works as an antidepressant. The common notion is that the extract acts as a mild monoamine oxidase inhibitor and a strong serotonin reuptake inhibitor [14]. Some research has suggested that the antidepressant action of the extract is enhanced by the fact that it is also an immune-stimulator and thus relieve many of the physical symptoms associated with depression while strengthening the body's defense system overall. Nevertheless, hypericin and hyperforin act as standards of the extracts in the pharmaceutical industry. To date hyperforin and hypericin have been extracted separately from human serum and plasma. Liquid–

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liquid extraction (LLE) has been carried out using mixtures of *n*-hexane and ethylacetate for hyperforin and ethylacetate for hypericin [15–17]. On the other hand high-performance liquid chromatography (HPLC) with UV and fluorescence detection (FLD) is the most common technique used for the separation and detection of hypericin and hyperforin in plant and human plasma extracts. A known problem is the strong tailing of hypericin that can be caused by tautomerisation [18,19], association with other hypericin molecules [20,21] or strong interaction with silica [22–25]. Due to the steady progress of medicinal sciences more efficient extraction and analytical techniques are required. Therefore a method for the simultaneous extraction and RP-HPLC determination of hypericin and hyperforin from human plasma was developed. This method requires a shorter sample preparation time and less chemicals as well as a short analysis time.

## 2. Experimental

### 2.1. Materials and reagents

Dimethylsulfoxide (DMSO, puriss.) and acetonitrile (ACN), methanol, *n*-hexane and ethyl acetate (all analytical reagent grade) were purchased from Fluka (Buchs, Switzerland). Triethylamine (analytical reagent grade) was from Sigma–Aldrich (Vienna, Austria). Glacial acetic acid, orthophosphoric acid (85%) and potassium dihydrogenphosphate (all analytical reagent-grade) were from Merck (Darmstadt, Germany). Bidestilled water purified by a NanoPure-unit (Barnstead, Boston, MA, USA) was used. Hypericin and hyperforin were a gift from Herbextracts (Consell, Spain). The purities of hypericin and hyperforin were determined by HPLC–ESI-MS in the full scan mode (200–800 $m/z$). Hyperforin was found to be 94% pure; in the case of hypericin no impurities were found. Standard stock solutions of hypericin (68 μg/ml) and hyperforin (485 μg/ml) in methanol under nitrogen were stored at −18 °C for 2 months [25]. For the pharmacokinetic study St. John's Wort extract capsules purchased form Nature's Way Products (Springville, UT, USA) were used. Human plasma was obtained from the blood bank of the University Hospital (Innsbruck, Austria).

### 2.2. Instrumentation

#### 2.2.1. High-performance liquid chromatography

The reversed-phase HPLC system consisted of a low-pressure gradient pump (Model 510, Waters, Milford, MA, USA), a helium degassing system, an autosampler (Model 712 WISP, Waters) with a 200-μl loop, a fluorescence detector (Model 474, Waters) with a 16-μl flowcell, which was connected to a UV absorbance detector (Model 486, Waters) with a 8-μl flowcell. Data was recorded on a computer-based data system (MILLENIUM$^{32}$, Version 3.05.01, Waters). For RP chromatography a Bischoff Prontosil 120-5-$C_{18}$-AQ column (250×2 mm I.D., 5 μm, 120 Å, Bischoff, Leonberg, Germany) and a Shandon Hypersil BDS-$C_{18}$ HCT guard precolumn (10×4 mm I.D., 3 μm, 120 Å, Berger, Linz, Austria) were used. The mobile phase consisted of methanol–ethyl acetate–buffer (95:25:20, v/v/v). The buffer was composed of 0.5 g $KH_2PO_4$, 200 g bidestilled water and 0.4 ml orthophosphoric acid 85% which resulted in a pH value of 2.1. The flow-rate was 0.3 ml/min at a temperature of 45 °C.

#### 2.2.2. HPLC coupled to electrospray ionization (ESI) quadrupole ion trap mass spectrometry

For HPLC–ESI-MS experiments, a low-pressure gradient micropump (Model Rheos 2000, Flux, Karlskoga, Sweden), a degasser (Model DG-301, Phenomenex, Torrance, CA, USA), a microinjector (Model CC00030, Valco, Houston, TX, USA) with a 5-μl internal loop connected to a quadrupole ion trap mass spectrometer (Model LCQ, Finnigan, San Jose, CA, USA) were used. The following parameters were used in all experiments: negative ion mode; source voltage, 4.5 kV; source current, 80 μA; sheath gas flow-rate, 60 (nitrogen 4.6); capillary voltage, −47 V; temperature of the heated capillary, 200 °C; tube lens offset, −30 V; first octapole offset, 4.75/4.25 V (hyperforin/hypericin); second octapole offset, 6.5 V; inter-octapole lens, 16/14 V (hyperforin/hypericin). The mobile phase consisted of acetonitrile–buffer (170:30, v/v) and the buffer of 100 g bidestilled water and 0.2 ml glacial acetic acid

adjusted to pH 3.5 with triethylamine. The flow-rate was 0.3 ml/min at a temperature of 45 °C.

### 2.3. Sample preparation

LLE of all plasma samples was carried out as follows: to 1 ml plasma containing hypericin and hyperforin in a glass tube 0.4 ml DMSO and 0.15 ml ACN were added and vortex mixed for 30 s. Then 1 ml of ethyl acetate–*n*-hexane (70:30, w/w) was added and vortex mixed for 2 min. The tubes were centrifuged for 5 min at 5000 rpm and the organic layer was removed. The residue was extracted again using the same procedure, the combined organic layers were taken to dryness under a stream of nitrogen at room temperature and redissolved in 0.25 ml methanol.

### 2.4. Method validation

#### 2.4.1. Calibration curves

Weight least-squares (weighing factor = concentration$^{-2}$) was used to fit the response of the data versus the effective concentration to the equation

$$\text{height response} = \text{slope} \times \text{concentration} \pm \text{intercept}$$

Calibration curves were obtained by spiking human plasma with methanolic solutions of the analytes to achieve the following concentrations: hypericin 8.4, 12.6, 16.4, 21.0, 24.6 and 28.7 ng/ml; hyperforin: 21.6, 43.1, 145.6, 194.1 and 242.6 ng/ml.

#### 2.4.2. Recovery

Absolute recoveries for hypericin and hyperforin from spiked human plasma were determined at two concentrations (8.2, 20.5 ng/ml and 97.1, 291.2 ng/ml, respectively) for each and compared with the height ratios of reference samples. Reference samples were prepared by LLE of blank plasma and the extracts were redissolved in methanolic solutions of the appropriate drug to obtain the same concentrations as the spiked plasma extracts; this was done six times for each concentration.

### 2.5. Study on real plasma samples

To get access to real plasma samples, three St. John's Wort extract capsules standardized to 0.3% hypericin were ingested and 10 ml blood was withdrawn 2, 4 and 6 h afterwards from a volunteer. The blood samples were centrifuged for 10 min at 5000 rpm and the plasma was frozen for 24 h at −18 °C prior to LLE.

## 3. Results and discussion

### 3.1. Optimization and validation of LLE

Optimization of the LLE was done by varying the amounts of DMSO and ACN added to the plasma and changing the ratio of *n*-hexane to ethylacetate for the extraction. It was found that with less than 0.4 ml DMSO or 0.15 ml ACN, hypericin could not be extracted. On the other hand, using more DMSO or ACN caused protein precipitation which caused a loss in recoveries. Finally the plasma with DMSO, ACN and *n*-hexane–ethylacetate was incubated at 37 °C for 10 min but no increase of hypericin and hyperforin recoveries could be obtained. The optimized RP-HPLC using UV and fluorescence detection was applied for the validation of the LLE.

#### 3.1.1. Precision and accuracy

Precision (repeatability) of the LLE was checked by calculating the intra-assay variation at three concentrations for each compound (hypericin 21.0, 16.4 and 8.4 ng/ml; hyperforin 291.2, 242.6 and 145.6 ng/ml). Accuracy was defined as the percentage difference between the effective concentration and the mean calculated concentration of hyperforin and hypericin in the extracts. In order to ensure highest possible reliability of the results each concentration was extracted six times and finally determined by HPLC using fluorescence and UV detection. The results in Table 1 indicate that the extraction method was reliable within the reported concentration ranges for hypericin and hyperforin in human blood after taking St. John's Wort extracts. Maximum relative standard deviation (RSD) values

Table 1
Intra-assay precision (repeatability) and accuracy for hypericin and hyperforin, $n = 6$

| Analyte | Effective concentration (ng/ml) | Mean calculated concentration (ng/ml) | SD (ng/ml) | RSD (%) | Accuracy (%) |
|---|---|---|---|---|---|
| Hypericin | 21.0 | 20.92 | 0.68 | 3.24 | 0.38 |
| | 16.4 | 16.60 | 0.52 | 3.16 | 1.22 |
| | 8.4 | 9.18 | 0.39 | 4.30 | 9.29 |
| Hyperforin | 291.2 | 287.76 | 7.42 | 2.58 | 1.81 |
| | 242.6 | 233.91 | 4.90 | 2.10 | 3.58 |
| | 145.6 | 146.36 | 2.82 | 2.82 | 0.52 |

for hypericin and hyperforin were found to be 4.3 and 2.8%, respectively.

### 3.1.2. Linearity

Calibration plots of peak height versus concentration for hypericin and hyperforin were obtained by linear regression analysis of the average of at least three data points per concentration in a concentration range of 8.4–28.7 ng/ml and 21.6–242.6 ng/ml for hypericin and hyperforin, respectively. The regression equations were as follows: $y = 42.94x - 144.16$ for hypericin and $y = 7.6811x + 8.0832$ for hyperforin. Values for correlation coefficients were found at 0.9948 and 0.9991.

### 3.1.3. Recovery

The mean absolute recoveries for hypericin at the concentrations of 8.2 and 20.5 ng/ml were 32.2 and 35.6%, respectively, and for hyperforin were 100.1 and 89.9% for the concentrations of 97.1 and 291.2 ng/ml. The low recovery for hypericin is presumably due to a stronger plasma protein binding than hyperforin [13]. Nevertheless, the recovery of hypericin is not a drawback for the simultaneous analysis of both compounds because this method covers a wide concentration range of hypericin and within this range it is highly reproducible.

### 3.1.4. Limit of detection

The limits of detection were obtained by successively decreasing the concentrations of hypericin and hyperforin in the plasma samples and using 1 ml of spiked plasma for the LLE. For hypericin the limit of detection was found to be 1.8 ng/ml (signal-to-noise ratio, 3:1). Due to a intense peak eluting after the hyperforin peak the limit of detection with a signal-to-noise ratio of 3:1 could not be determined but at a concentration of 9.5 ng/ml hyperforin could be still definitely identified.

## 3.2. HPLC using coupled UV-absorbance and fluorescence detection

For the analysis of hypericin and hyperforin (Fig. 1) in human plasma a RP-HPLC system was assembled, that requires the shortest possible analysis time with satisfactory selectivity and sensitivity. Therefore, the highest efficiency was achieved by using an eluent consisting of methanol–ethyl acetate–$KH_2PO_4$ buffer (95:25:20, v/v/v). This system was used for the validation of the established LLE using

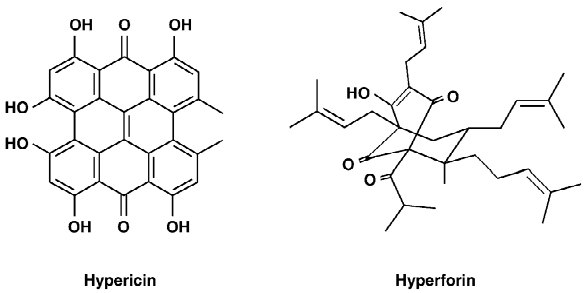


Fig. 1. Chemical structures of hypericin and hyperforin.

UV and fluorescence detection. As depicted in Fig. 2 hyperforin and hypericin show peaks at 10.81 min using UV detection ($\lambda = 276$ nm) and at 9.46 min using fluorescence detection ($\lambda_{ex} = 591$ nm; $\lambda_{em} = 601$ nm).

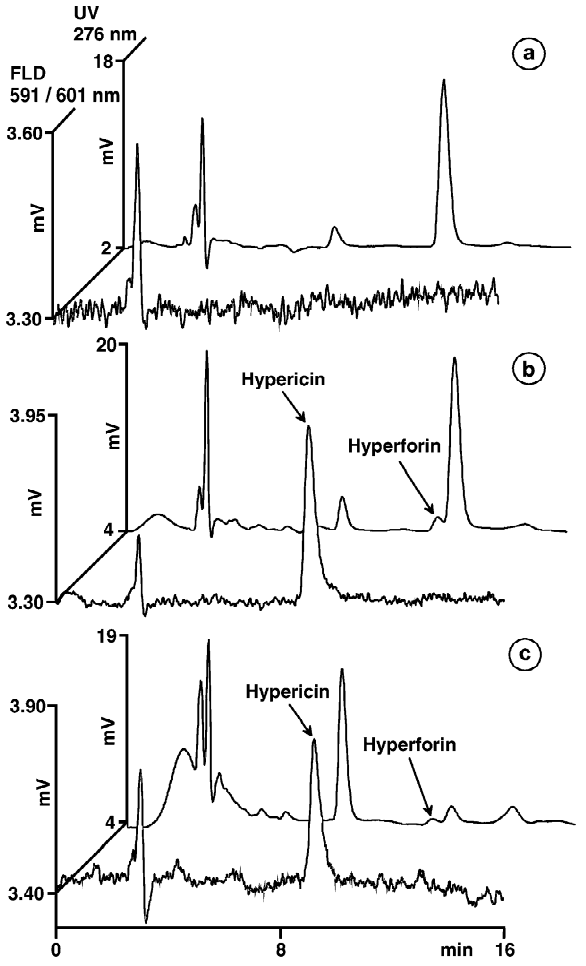


Fig. 2. Reversed-phase HPLC of spiked human plasma using fluorescence detection (hypericin) and UV-absorbance detection (hyperforin). (a) Blank plasma; (b) hypericin and hyperforin spiked human plasma; injected amount of hypericin 0.14 ng of hyperforin 5.8 ng; (c) 6 h after oral administration of St. John's Wort extract capsules. Stationary phase, Prontosil 120-5-$C_{18}$-AQ (250×2 mm I.D., 5 μm, 120 Å); mobile phase, methanol–ethyl acetate–buffer (95:25:20; v/v/v); buffer (pH 2.1), 0.5 g $KH_2PO_4$, 200 g bidestilled water and 0.4 ml orthophosphoric acid 85%; flow-rate, 0.3 ml/min; detection, FLD, 591/601 nm; temperature, 45 °C; sample volume, 20 μl.

### 3.3. RP–HPLC–ESI -MS–MS

The usefulness of RP-HPLC separation for a more specific and selective identification of hypericin and hyperforin was greatly enhanced by MS detection, especially in the plasma extract. The RP-HPLC system was coupled to a mass spectrometer via an ESI interface. Changing methods from UV absorbance and fluorescence detection to ESI-MS detection is rarely straightforward, because conventional eluents usually contain buffers and other additives to improve chromatographic performance, which generally interfere with the ESI procedure. On the other hand, the selective choice of an appropriate additive can influence the ionization of analytes positively. Therefore, phosphoric acid was replaced by acetic acid, which guarantees optimal assay performance balancing the highly individual requirements for both LC performance and ESI efficiency. Both hypericin and hyperforin could be efficiently transformed into deprotonated molecules $[M-H]^-$. Hypericin and hyperforin were tracked in the total ion current (Fig. 3a) from selected ion traces at $m/z = 503$ (Fig. 3c) and $m/z = 535$ (Fig. 3b), respectively, in the negative ion mode. RP-HPLC–ESI-MS–MS studies were carried out to prove that after LLE the detected analytes were no artefacts and facilitated further specific identification even in the case of coeluting peaks. Tandem mass spectra of the parent ions of the methanolic standards hypericin and hyperforin and in the plasma extract are depicted in Figs. 4 and 5. It can be deduced that the fragmentation products are the same in the methanolic solutions and in the spiked plasma extracts, confirming that the detected ions in the extracts result from hypericin and hyperforin.

### 3.4. Study on real plasma samples

To demonstrate the effectivness of the developed methodology, real blood samples were investigated. The results of this study showed increasing amounts of hyperforin and hypericin in human plasma (Fig. 6), although no hypericin was detected in the blood sample taken 2 h after ingestion of the St. John's Wort extract capsules. On the other side, the hyperforin concentration–time curve is linear in the observed span. Finally the results showed that the

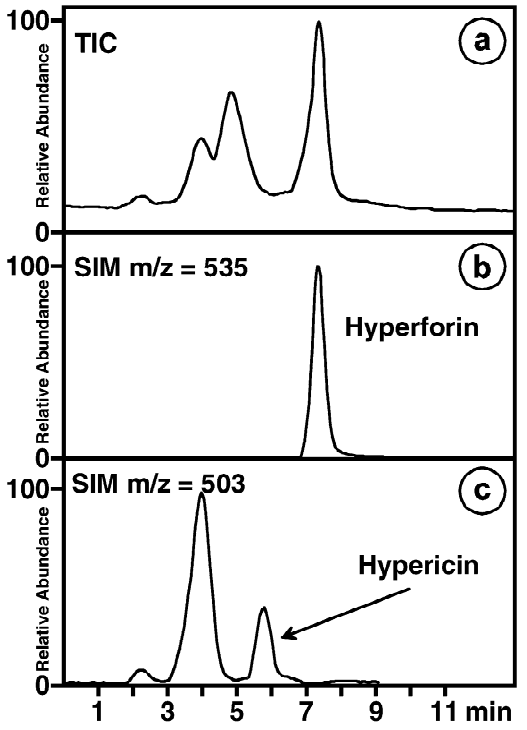


Fig. 3. RP HPLC–ESI-MS of spiked human plasma. (a) ESI, full scan 200–800; (b) SIM, $m/z = 534–536$; (c) SIM, $m/z = 502–504$. Stationary phase, Prontosil 120-5-$C_{18}$-AQ (250×2 mm I.D., 5 μm, 120 Å); mobile phase, acetonitrile–buffer (170:30, v/v); buffer, 100 g bidestilled water and 0.2 ml glacial acetic acid adjusted to pH 3.5 with triethylamine; flow-rate, 0.3 ml/min; temperature, 45 °C; sample volume, 5 μl; injected amount of hypericin 0.10 ng of hyperforin 0.97 ng.

established LLE method is highly suitable for further clinical studies.

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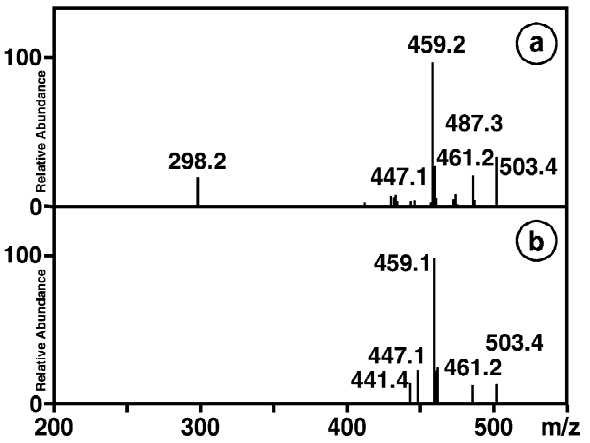


Fig. 4. Tandem mass spectra of the peak with the retention time 5.81 min of (a) methanolic solution of hypericin; injected amount of hypericin 2.49 ng (b) spiked human plasma; injected amount of hypericin 0.10 ng. Stationary phase, Prontosil 120-5-$C_{18}$-AQ (250×2 mm I.D., 5 μm, 120 Å); mobile phase, acetonitrile–buffer; buffer, 100 g bidestilled water and 0.2 ml glacial acetic acid adjusted to pH 3.5 with triethylamine; flow-rate, 0.3 ml/min; detection, ESI, scan 200 –550; relative collision energy, 59%; temperature, 45 °C; sample volume, 5 μl.

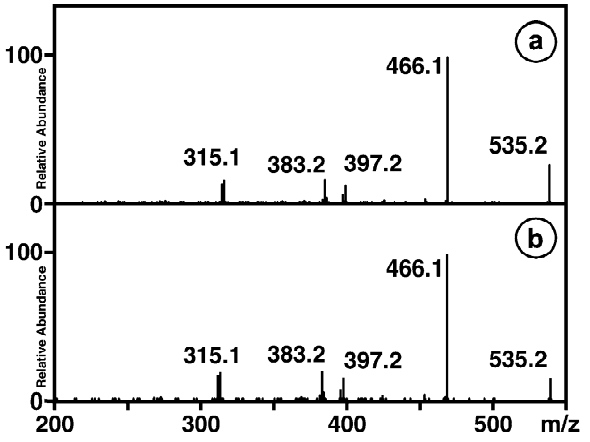


Fig. 5. Tandem mass spectra of the peak with the retention time 7.11 min of (a) methanolic solution of hyperforin; injected amount of hyperforin 2.4 ng (b) spiked human plasma; injected amount of hyperforin 0.97 ng. Stationary phase, Prontosil 120-5-$C_{18}$-AQ (5 μm, 120 Å, 250×2 mm I.D); mobile phase, acetonitrile–buffer; buffer, 100 g bidestilled water and 0.2 ml glacial acetic acid adjusted to pH 3.5 with triethylamine; flow-rate, 0.3 ml/min; detection, ESI, scan 200 –550; relative collision energy, 40%; temperature, 45 °C; sample volume, 5 μl.

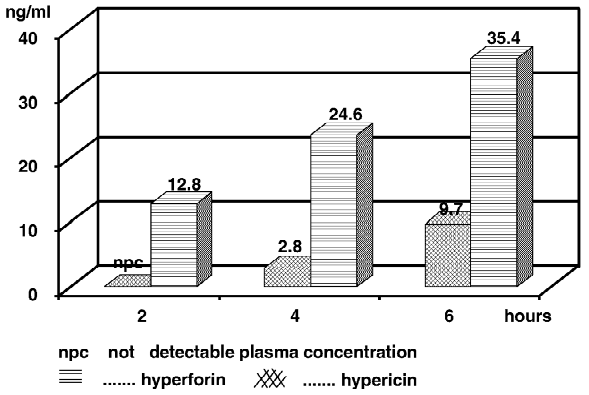


Fig. 6. Time dependent concentrations of hypericin and hyperforin in real blood samples.

## References

[1] C.M. Hawken, St. John's Wort, Woodland Publishing, Pleasant Grove, 1997.
[2] G. Lavie, Y. Mazur, D. Lavie, D. Meruelo, Med. Res. Rev. 15 (1995) 111.
[3] K. Linde, G. Ramirez, C.D. Mulrow, A. Paulus, W. Weidehammer, D. Melchart, Br. Med. J. 313 (1996) 253.
[4] Ch.M. Schempp, K. Pelz, A. Wittmer, E. Schöpf, J.C. Simon, Lancet 353 (1999) 2129.
[5] N. Rosenthal, St. John's Wort. The Herbal Way to Feeling Good, Harper Collins, New York, 1995.
[6] J. Deltito, D. Beyer, J. Affect. Disord. 51 (1998) 345.
[7] J. Chang, Biochem. Pharmacol. 59 (2000) 211.
[8] G. Micali, F. Lanuzza, P. Currò, J. Chromatogr. A 731 (1996) 336.
[9] G. Laakmann, C. Schüle, T. Baghai, M. Kieser, Pharmacopsychiatry 31 (1998) 54.
[10] S.S. Chatterjee, M. Nölder, E. Koch, C. Erdelmeier, Pharmacopsychiatry 31 (1998) 7.
[11] A. Biber, H. Fischer, A. Romer, S.S. Chatterjee, Pharmacopsychiatry 31 (1998) 36.
[12] C.A.J. Erdelmeier, Pharmacopsychiatry 31 (1998) 2.
[13] K. Das, A.V. Smirnov, J. Wen, P. Miskovsky, J.W. Petrich, Photochem. Photobiol. 69 (1999) 633.
[14] F. Brinker, Herbal Gram 46 (1999) 43.
[15] M. Agrosí, S. Mischiatti, P.C. Harrasser, D. Savio, Phytomedicine 7 (2000) 1.
[16] L. Liebes, Y. Mazur, D. Freeman, D. Lavie, G. Lavie, N. Kudler, S. Mendoza, B. Levin, H. Hochster, D. Meruelo, Anal. Biochem. 195 (1991) 77.
[17] J.D. Chi, M. Franklin, J. Chromatogr. B 724 (1999) 195.
[18] C. Etzelstorfer, H. Falk, N. Müller, W. Schmitzberger, U.G. Wagner, Monatsh. Chem. 28 (1989) 1289.
[19] Z. Diwu, Phytochem. Photobiol. 61 (1995) 529.
[20] F. Gai, M.J. Fehr, J.W. Petrich, J. Phys. Chem. 98 (1994) 5784.
[21] T. Yamazaki, N. Ohta, I. Yamazaki, P.S. Song, J. Phys. Chem. 97 (1993) 7870.
[22] H. Falk, E. Mayr, Monatsh. Chem. 128 (1997) 353.
[23] W.E. Freytag, Dtsch. Apoth. Ztg. 124 (1984) 2383.
[24] N. Schelosky, Analytische Untersuchungen an Johanniskraut, Ph.D. Thesis, University of Innsbruck, 1998.
[25] H.C.J. Orth, C. Rentel, P.C. Schmidt, J. Pharm. Pharmacol. 51 (1999) 193.