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Evaluation of major active components in St. John's Wort dietary supplements by high-performance liquid chromatography with photodiode array detection and electrospray mass spectrometric confirmation

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Abstract

A RP-HPLC method with photodiode array detection and LC–electrospray ionization (ESI) MS confirmation was established for the determination of major active components in St. John's Wort dietary supplement capsules. The samples alternatively were extracted with ethanol–acetone (2:3) using a 55°C water-bath shaker or an ambient temperature ultrasonic bath. Extracts were separated by RP-C_{18} chromatography using a 95-min water–methanol–acetonitrile–trifluoroacetic acid gradient. The major components were identified by photodiode array detection and then confirmed by LC–ESI-MS. The quantification of components was performed using an internal standard (luteolin). This method may serve as a valuable tool for the quality evaluation of St. John's Wort dietary supplement products. © 2000 Published by Elsevier Science B.V.

Keywords: Food analysis; St. John's Wort; *Hypericum peforatum*; Naphthodianthrones; Flavonoids; Phloroglucinols

1. Introduction

The use of herbs as dietary supplements and as over-the-counter (OTC) drugs has increased dramatically in the past few years because of the sense that ''natural'' is better and lower cost in comparison to most western drugs. In Europe, herbal supplements are traditionally regulated as OTC drugs. They are, however, dispensed by licensed pharmacists or other health care professionals [1] and steps have been taken to ensure quality or proper use. The German government has prepared monographs defining quality standards and potency tests for over 350 single plant drugs. France also has officially recognized more than 200 medicinal plants and provided specifications governing their sale [2]. By comparison, in the USA, according to the Dietary Supplement Health and Education Act (DSHEA) issued in 1994, herbal products are generally classified as dietary supplements, a category somewhere between food

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and OTC drugs. Under the DSHEA law, a manufacturer may make structure–function claims for a product on its label provided that these claims are supported by scientific evidence. It is not required, however, that the manufacturers demonstrate either safety or efficacy prior to marketing, and no regulations govern product quality [3].

Dietary supplements represented a US$6.5 billion market in 1996 alone and more than half of Americans use dietary supplements according to a recent market research report [4]. Among these dietary supplements, St. John's Wort is one of the most popular products. In 1997, it is estimated that nearly 7.5 million Americans took St. John's Wort [5]. Sales in the USA increased 20-fold between 1995 and 1997, from US$10 million to US$200 million annually [6]. In Germany, St. John's Wort is the most common antidepressant. It is prescribed four-times more often than fluoxetine hydrochloride [7].

St. John's Wort (*Hypericum peforatum*) is a herbaceous perennial plant that is distributed worldwide. It has been used as a medicinal herb throughout history. It has become very popular because of its reported beneficial effects on the nervous system, especially as an antidepressant [8]. St. John's Wort extracts contain at least 10 constituents or groups of components that could contribute to its pharmacological effects. These components include naphthodianthrones (hypericin, on whose content most of the available preparations are standardized), flavonoids (rutin, hyperoside, isoquercitrin, quercitrin and quercetin), phloroglucinols (hyperforin and adhyperforin), and biflavonoids (biapigenin and amenthoflavon). The mechanism of action of the postulated antidepressant effects is unclear [9].

Because of the fast growing market for St. John's Wort in the USA increasingly more products are sold. The preparations vary widely in content. The amount of authentic plant material is limited and other species with different composition may be substituted. The method generally used for standardization of St. John's Wort products based on the hypericin content is the spectrophotometric determination of naphthodianthrones. It is not as selective as high-performance liquid chromatography (HPLC) methods and can be manipulated easily (e.g., by adding colorants) [10]. In the present work, a method involving solvent extraction, HPLC RP-C_{18} column chromatography, photodiode array detection (DAD), liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) confirmation and internal standard quantitation is developed for determining the major components in St. John's Wort dietary supplements.

2. Experimental

2.1. Chemicals and samples

Rutin, quercitrin, quercetin, hypericin, luteolin and trifluoroacetic acid (TFA) were purchased from Sigma (St. Louis, MO, USA). Hyperoside and isoquercitrin were purchased from Indofine (Belle Mead, NJ, USA). Pseudohypericin, hyperforin and adhyperforin were isolated from St. John's Wort dry plant (purchased from health food store, Little Rock, AR, USA). The purity and structural identity of each isolated compound were chemically characterized by HPLC–DAD, nuclear magnetic resonance (NMR) and MS analysis (unpublished data).

Methanol, ethanol and acetonitrile were HPLC-grade from J.T. Baker (Phillipsburg, NJ, USA). Formic acid (98%) was from Fluka (Milwaukee, WI, USA). Water was purified using a Milli-Q system from Millipore (Milford, MA, USA).

St. John's Wort capsules of five different brands were purchased from local stores in Little Rock, AR, USA; they were assigned code designations C1–C5.

2.2. Apparatus

The HPLC system consisted of a Waters 600 liquid chromatograph equipped with a photodiode array multi-wavelength detector (Waters, Milford, MA, USA). The absorption was measured either as a full spectrum (200–790 nm) or at 284 nm for most components, or at 590 nm for naphthodianthrones. The chromatographic data were recorded and processed by the Waters Millennium 2000 software.

LC–MS was performed on an HP 5989B mass spectrometer equipped with HP 1090 L/M HPLC system and a photodiode array detector (Hewlett-Packard, Palo Alto, CA, USA). The mass spectrometer was operated in positive electrospray mode with a capillary exit voltage of 150 V, high-energy dynode

at 10 kV and multiplier at 2.1 kV. Nitrogen was used as nebulizing gas at 80 p.s.i. and drying gas at 399°C (1 p.s.i.=6894.76 Pa). Full scans were acquired from m/z 50 to 750 at 1.2 scans/s. HP Chemstation software was used to collect concurrent LC–DAD and positive ESI-MS data.

2.3. Chromatographic conditions

Analyses were carried out at 25°C on a LiChrospher end-capped RP-C_{18} column (3 μm, 250×4 mm, Phenomenex, Torrance, CA, USA). Gradients were formed between two helium-purged mobile phases. Mobile phase A was 0.5% TFA in water, and phase B was 0.5% TFA in a mixture of methanol–acetonitrile (13:7). The analysis followed a linear gradient program. Initial conditions were 90% A; 0–20 min, changed to 50% A; 20–40 min, to 40% A; 40–50 min, to 0% A; kept to 60 min; 60–70 min, went back to 90% A; then finally equilibrated until 95 min. The flow-rate was kept constant at 0.6 ml/min, and the injection volume was 10 μl.

For LC–MS, components were separated on a Prodigy ODS (3) column (5 μm, 250×2 mm, 100 Å, Phenomenex). The linear gradient was from acetonitrile–water (5:95) to acetonitrile–water (99:1) with constant 0.1% formic acid in 94 min with a 31 min hold. The flow-rate was 0.2 ml/min.

2.4. Identification and peak purity

Peaks were tentatively identified by comparison of the retention times and UV spectra of the peaks in the extractions with those of the reference standards. Identifications were subsequently confirmed by LC–ESI-MS analysis. The purity of each peak was checked by DAD software routines.

2.5. Calibration curves

Rutin, hyperoside, isoquercitrin, quercitrin, quercetin, pseudohypericin, hypericin, hyperforin and luteolin were dissolved in methanol and diluted to appropriate concentration ranges for the construction of calibration curves. The calibration curves were based on the analysis of duplicate standards at five concentration levels (Table 1). These curves were constructed by plotting the concentration of each compound as a function of the peak area ratio (the compound peak area to the internal standard peak area). The concentration of the internal standard, luteolin, was 1.6 mg/ml.

2.6. Repeatability

Measurements of intra- and inter-day variability were utilized to determine the repeatability of the method. The intra-day repeatability was examined on six individual samples in 1 day, and inter-day repeatability was determined for 3 independent days. The relative standard deviation (RSD) was calculated as a measurement of method repeatability.

Table 1
Linear calibration curves derived for major components in St. John's Wort

Compound	$y=ax+b$, the linear model[a]		Regression coefficient (r^2)	Concentration (mg/ml)
	Slope (a)	Intercept (b)		
Rutin	0.959	−0.499	0.976	0.5, 1.0, 2.0, 3.0 and 4.0
Hyperoside	0.604	−0.195	0.985	0.3, 0.6, 1.2, 1.8 and 2.4
Isoquercitrin	0.604	−0.268	0.988	0.25, 0.5, 1.0, 1.5 and 2.0
Quercitrin	0.558	0.047	0.975	0.2, 0.4, 0.6, 0.8 and 1.0
Quercetin	0.422	0.071	0.974	0.2, 0.4, 0.6, 0.8 and 1.0
Pseudohypericin	0.506	0.098	0.948	0.04, 0.08, 0.12, 0.16 and 0.20
Hypericin	0.477	−0.018	0.993	0.02, 0.04, 0.06, 0.08 and 0.10
Hyperforin	0.622	0.107	0.953	0.2, 0.4, 0.6, 0.8 and 1.0
Adhyperforin	0.743	0.110	0.953	0.2, 0.4, 0.6, 0.8 and 1.0

[a] y: Concentration, x: peak area ratio.

2.7. Quantification of active components in St. John's Wort capsules

One gram (±0.01 g) of fine powder from inside a capsule was extracted with ethanol–acetone (3:2) solvent three times. Each extraction was performed using either a water-bath shaker (Model 3540, Lab-Line Instruments, Melrose Park, IL, USA) at 55°C for 5.6 h, or an ultrasonic bath (Cole-Parmer, Chicago, IL, USA) at ambient temperature (23°C) for 1.5 h. The extracts were filtered through No. 4 filter paper (Whatman, Maidstone, UK) into a volumetric flask. The total volume of extract was adjusted to 50.00 ml with additional extraction solvent. The detailed procedures for HPLC quantitative analysis were described in a previous paper [11].

3. Results and discussion

Several HPLC methods have been developed for the analysis of St. John's Wort [12–17]. All of these methods either used three mobile phases or inorganic buffer systems. In the current method two mobile phases compatible with common LC–MS systems were used. More than 10 major components were detected (two wavelengths, 284 nm and 590 nm) using a 95-min program. A typical chromatogram is shown in Fig. 1. The LC–DAD–ESI-MS data for standard reference compounds and components in the crude extract (Table 2) correlated directly with retention order, positive ion thermospray MS and UV results previously reported by Brolis et al. [13]. The compounds were identified and confirmed as rutin, hyperoside, isoquercitrin, quercitrin, quercetin and hypericin, while I3,II8-biapigenin, pseudohypericin, hyperforin and adhyperforin were tentatively identified.

Based on comparing the HPLC retention time in the current chromatogram with that in the reported data [13], peak 4 was tentatively identified as 3,3′,4′,5,7-pentahydroxyflavanone 7-*O*-rhamnopyranoside. The ESI-MS ion data were also consistent with this interpretation ($[M+H]^+$ at m/z 451, $[M+Na]^+$ at m/z 473 and a fragment ion at m/z 305). In contrast, the peak had UV absorption maxima at 257.1 and 352.4 nm, which were the typical spectra of a flavonol, not a flavanone. The UV absorptions of peaks 9 and 11 were similar to hyperforin, and both of these compounds had a molecular mass of 552, 16 u unit higher than hyperforin, corresponding to oxidized hyperforin. The hypericin peak (peak 10) was eluted between these two peaks. It was very difficult to observe the hypericin peak when detected at 284 nm, nevertheless because peaks 9 and 11 did not have strong absorbance at 590 nm, the hypericin peak could be observed clearly.

Luteolin was used as an internal standard for quantitative analysis because it has a UV spectrum similar to that of major flavanoids and it could be completely separated from the other components in the extracted solution. The regression coefficient of each calibration curve was greater than 0.9 (Table 1). The concentrations of all the target compounds were within the linear ranges of the respective calibration curves.

Wide ranges of solvent polarities, extraction times and extraction temperatures were tested in the previous study [11]. The optimum conditions used in this experiment were achieved by using response surface methodology for water-bath extraction [11]. The same strategy was used to obtain optimum conditions in the ultrasonic extraction (unpublished data). However, during 1.5 h of ultrasonication, the temperature (monitored by a thermometer) rose from 23 to 65°C, and the increase in the temperature was not constant. Because the ultrasonic bath was placed inside a fume hood without any temperature control device, the surrounding temperature and air flow did affect the ultrasonic bath temperature. Considering that water-bath had temperature control within 1°C, the fluctuating temperature of ultrasonic bath was a drawback. As shown in Tables 3 and 4, water-bath extraction had better repeatability than ultrasonic extraction. The overall intra- and inter-day variations of flavonoids were less than 10% in water-bath extraction, while, in ultrasonic extraction, some of variations were higher than 10%, i.e., day 2. On the other hand, ultrasonic extraction was more efficient than water-bath extraction. The 1.5 h extraction efficiency was comparable to water-bath extraction for 5.6 h (Table 5).

In comparison with flavonoids, the intra- and inter-day variations of hyperforin and naphthodianthrones analysis were high by both extraction methods

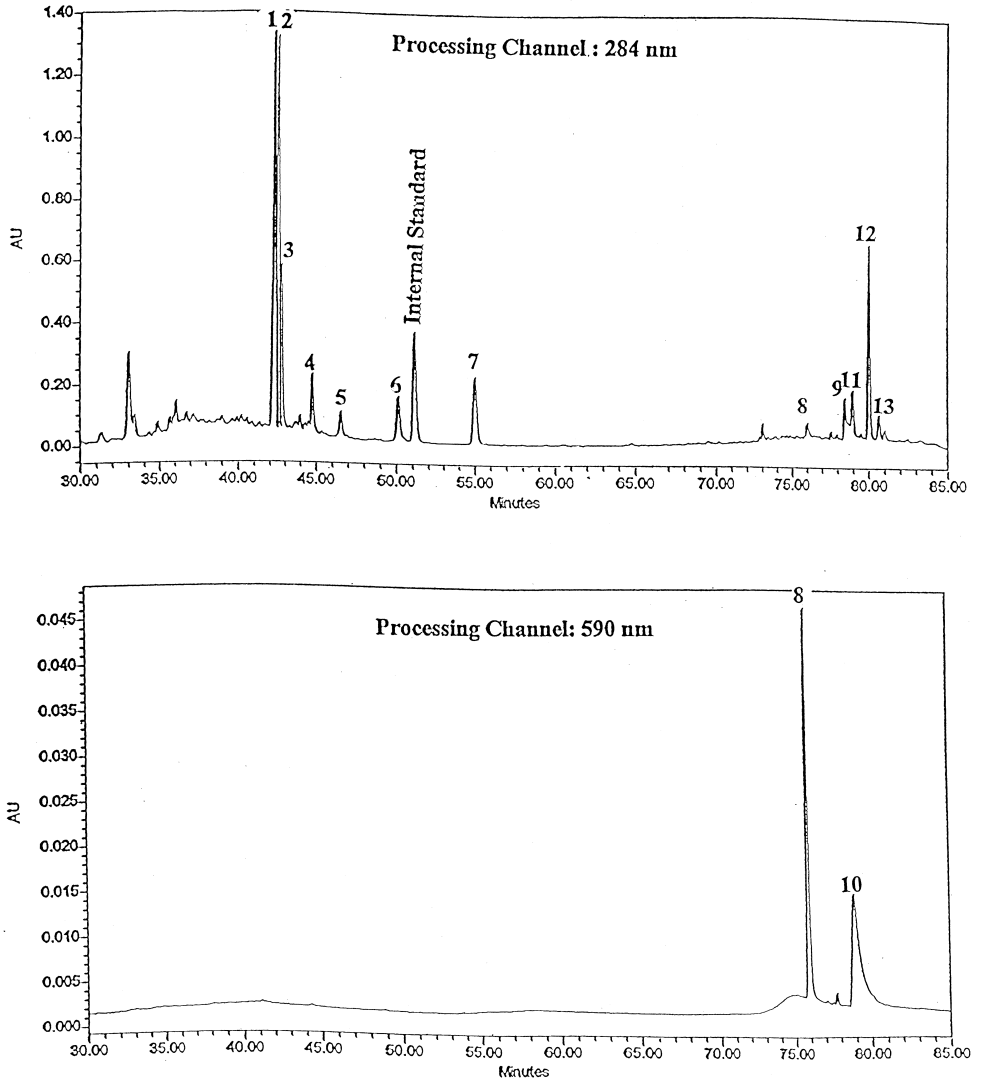

Fig. 1. Typical HPLC chromatograms at 284 and 590 nm of St. John's Wort dietary supplement extracted solution. 1=Rutin; 2=hyperoside; 3=isoquericitrin; 5=quercitrin; 6=quercetin; 7=I3,II8-biapigenin; 8=pseudohypericin; 10=hypericin; 12=hyperforin; 13=adhyperforin; 4, 9, and 11=unknown.

(Tables 3 and 4). Hyperforin is unstable, oxidizing and decomposing at room temperature [18]. The oxidation products were tentatively identified in the current study as peaks 9 and 11 (Fig. 1). The change of hyperforin during and after extraction resulted in the variation of measurement.

In addition to hypericin and pseudohypericin, other naphthodianthrones were found in St. John's Wort, including protohypericin, protopseudohypericin and cyclopseudohypericin [10]. Photons can cause the chemical rearrangements that change protohypericin to hypericin and protopseudohypericin to pseudohypericin. In basic methanol solution of NH_3, pseudohypericin further changes into cyclopseudohypericin [10]. Although the extractions were performed under a yellow light, there still might be some light effects. Complete conversion of proto-forms to hypericin and pseudo-

Table 2
Absorbance in UV spectra and ions in ESI mass spectra[a] for identification and confirmation of compounds in St. John's Wort

Compound	M_r	UV absorbance (nm)	ESI ion (m/z)[b]
Rutin	610	224.0, 257.1 352.4	303, 465, 611, 633
Hyperoside	464	224.0, 257.1 352.4	303, 465, 487
Isoquercitrin	464	214.6, 257.1 357.1	303, 465, 487
Quercitrin	448	257.1, 357.1	303, 449, 471
Quercetin	302	257.1, 374.9	303
I3,II8-Biapigenin	538	271.3, 333.2	435, 539
Pseudohypericin	520	228.7, 280.8, 323.6, 457.0, 539.8, 583.7	521
Hypericin	504	228.7, 280.8, 323.6, 457.0, 539.8, 583.7	505
Hyperforin	536	276.0	227, 411, 469, 537
Adhyperforin	550	276.0	291, 411, 493, 551

[a] UV absorbances and ESI ions for each compound were found in both extract solutions and standard solutions. Reference standards were available for all compounds listed in this table except I3,II8-biapigenin.
[b] m/z values include sodiated ions as well as protonated molecules, and fragments. Protonated molecules are underlined.

hypericin by exposure of samples to light allows better reproducibility [10]. However, this approach was not suitable for the current study, because another important compound, hyperforin, is unstable under this condition [18].

Five brands of St. John's Wort dietary supplement capsules were randomly selected from local markets and analyzed using the current methods. The results showed (Table 5) significant variations among the different brands even though all were labeled as St. John's Wort. Rutin, the component with the highest content in St. John's Wort, ranged from 6.7 to 23.1 mg/g by water-bath and from 7.0 to 21.9 mg/g by ultrasonic extraction, respectively. The other high concentration component, hyperoside, ranged from 3.5 to 15.8 mg/g by both extraction methods. Hyperforin, which has been reported as a major active antidepressant component [19], ranged from 1.9 to 10.0 mg/g.

In conclusion, two extraction methods were evaluated. Water-bath extraction had more consistent results than ultrasonic extraction, while the latter was less time consuming. A reversed-phase HPLC method was developed to quantify simultaneously eight

Table 3
Intra- and inter-day repeatability for the major components in St. John's Wort dietary supplements by water-bath extraction

Compound	Intra-day (n=6)						Inter-day (n=3)	
	Day 1		Day 2		Day 3		Mean±SD[a]	RSD (%)
	Mean±SD[a]	RSD (%)	Mean±SD[a]	RSD (%)	Mean±SD[a]	RSD (%)		
Rutin	23.4±2.3	9.7	20.0±1.2	5.9	21.3±1.9	9.0	21.6±1.7	7.9
Hyperoside	14.4±1.0	6.9	11.3±1.1	8.4	13.9±0.6	4.1	13.8±0.7	5.4
Isoquercitrin	3.9±0.2	5.4	3.4±0.3	8.8	3.4±0.3	9.0	3.6±0.3	9.1
Quercitrin	2.7±0.2	6.9	2.3±0.1	4.0	2.5±0.1	4.7	2.5±0.2	8.6
Quercetin	2.8±0.3	0.1	2.3±0.1	3.9	2.5±0.1	5.7	2.5±0.2	9.8
Pseudohypericin	1.8±0.2	10.2	1.5±0.1	6.9	1.6±0.1	4.4	1.6±0.1	8.8
Hypericin	0.9±0.2	17.0	0.7±0.0	3.7	0.7±0.1	8.2	0.8±0.1	16.4
Hyperforin	9.4±0.3	3.4	7.7±0.6	7.7	8.3±0.5	5.9	8.5±0.9	10.8
EMW[b]	448.0±18.9	4.2	439.8±12.2	2.8	459.6±13.2	2.9	449.1±10.0	2.2

[a] Unit: mg/g.
[b] EMW: Total extractable material mass.

Table 4
Intra- and inter-day repeatability for the major components in St. John's Wort dietary supplements by ultrasonic extraction

Compound	Intra-day (n=6)						Inter-day (n=3)	
	Day 1		Day 2		Day 3		Mean±SD[a]	RSD (%)
	Mean±SD[a]	RSD (%)	Mean±SD[a]	RSD (%)	Mean±SD[a]	RSD (%)		
Rutin	15.7±1.0	6.2	17.4±2.4	13.9	16.2±1.8	10.9	16.4±0.9	5.2
Hyperoside	13.9±1.2	8.9	15.1±1.6	10.8	12.2±1.2	9.8	13.8±1.4	10.6
Isoquercitrin	3.4±0.3	8.8	2.8±0.3	11.6	2.9±0.3	12.0	3.1±0.3	9.9
Quercitrin	2.0±0.2	8.3	2.2±0.3	13.0	1.9±0.1	6.6	2.1±0.1	6.1
Quercetin	1.5±9.4	6.4	1.9±0.2	11.4	1.7±0.1	4.6	1.7±0.2	12.6
Pseudohypericin	1.1±0.1	6.0	1.4±0.1	9.2	1.5±0.1	4.5	1.3±0.2	15.1
Hypericin	0.6±0.1	14.4	0.5±0.1	12.0	0.5±0.1	12.3	0.5±0.1	16.7
Hyperforin	5.2±0.8	16.2	5.2±0.7	14.2	5.1±0.8	15.3	5.2±0.1	1.2
EMW[b]	355.8±23.2	6.5	357.4±34.2	9.6	401.1±13.5	3.3	371.5±25.6	6.9

[a] Unit: mg/g.
[b] EMW: Total extractable material mass.

major biologically active components in St. John's Wort. It was successfully applied to the analysis of five St. John's Wort dietary supplements. The results indicated that the HPLC method could be utilized for quality control of St. John's Wort dietary supplements.

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Table 5
Quantities of major components in St. John's Wort dietary supplements from five different sources

Compound	Extraction method[a]	Dietary supplement[b]				
		C1	C2	C3	C4	C5
Rutin	W	21.6±2.3	23.1±1.2	14.5±0.2	6.7±0.8	12.1±0.7
	S	17.7±0.9	16.4±1.8	21.9±1.8	7.0±0.3	12.0±1.4
Hyperoside	W	13.8±1.0	15.0±1.0	15.8±0.7	3.7±0.2	4.9±0.4
	S	11.0±0.3	13.8±1.7	15.0±1.0	3.5±0.4	4.6±0.3
Isoquercitrin	W	3.6±0.4	3.9±0.3	9.1±0.8	1.4±0.2	1.8±0.1
	S	3.4±0.3	3.1±0.4	5.1±0.5	1.3±0.1	1.7±0.3
Quercitrin	W	2.5±0.2	2.5±0.0	2.9±0.1	0.6±0.0	0.7±0.0
	S	2.1±0.2	2.1±0.2	1.6±0.2	0.6±0.1	0.7±0.1
Quercetin	W	2.5±0.3	2.1±0.1	2.6±0.0	0.9±0.1	1.2±0.1
	S	2.1±0.0	1.7±0.2	2.3±0.2	0.9±0.0	1.2±0.1
Pseudohypercin	W	1.6±0.2	1.5±0.1	1.7±0.1	0.3±0.0	0.6±0.0
	S	1.5±0.1	1.3±0.2	1.7±0.1	0.3±0.0	0.4±0.0
Hypericin	W	0.8±0.1	0.5±0.2	0.9±0.1	0.2±0.0	0.6±0.1
	S	0.7±0.0	0.5±0.1	0.5±0.0	0.2±0.0	0.4±0.1
Hyperforin	W	8.5±0.9	3.2±0.2	9.3±0.5	2.5±0.2	2.4±0.1
	S	7.6±0.8	5.2±0.7	10.0±0.2	1.9±0.1	2.3±0.0
Extractable material mass	W	449.1±16.2	412.1±7.9	448.1±15.4	129.2±6.5	236.5±1.7
	S	388.3±23.0	370.5±31.5	396.3±24.5	188.8±4.0	258.6±16.0

[a] W: Water-bath; S: sonication.
[b] Data are presented as mg/g dry powder inside the capsules, and expressed as mean±SD of triplicate analyses for each sample.

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