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Separation of hypericins and hyperforins in extracts of *Hypericum perforatum* L. using non-aqueous capillary electrophoresis with reversed electro-osmotic flow

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Abstract

The separation of the lipophilic compounds in extracts of *Hypericum perforatum* L. is demonstrated in a non-aqueous capillary electrophoresis system with reversed electro-osmotic flow. Solvent mixtures of methanol, dimethylsulfoxide and *N*-methylformamide were used for the electrophoresis media, with addition of ammonium acetate and sodium acetate as electrolytes. The flow was reversed by the addition of the polycation hexadimethrine bromide, and thus negative voltage was applied. The method shows baseline separation between the four hypericins—protopseudohypericin, pseudohypericin, protohypericin and hypericin—whereas total baseline separation between the two hyperforins—hyperforin and adhyperforin—was not achieved. Using a fused-silica capillary (30 cm × 25 μm ID) and a voltage of −25 kV the analysis time of the hypericins and hyperforins was obtainable within 3 min. Application of the method with a fused-silica capillary of a larger internal diameter (48.5 cm × 50 μm ID) and a voltage of −20 kV resulted in analysis times of 8 min, but also lower limits of detection. The maximal attainable voltage was applied in both cases. Simultaneous separation of the flavonoids—although less efficient—may also be achieved. The technique of non-aqueous capillary electrophoresis with reversed electro-osmotic flow provides a very fast technique to evaluate the composition of hypericins and hyperforins in extracts of *Hypericum perforatum* L.

Keywords: *Hypericum perforatum* L.; Non-aqueous capillary electrophoresis; Reversed electro-osmotic flow; Hyperforins; Hypericins

1. Introduction

The quality of phytopharmaceuticals containing *Hypericum perforatum* L. relies on the content of active constituents in the preparations. However, a complete knowledge of all constituents contributing to the overall antidepressant activity is still incomplete. Previously it was considered that the naphthodianthrone derivatives—the hypericins (Fig. 1)—were the active constituents [1] but later studies have failed to confirm this investigation [2–6]. Despite these later studies, the stan-

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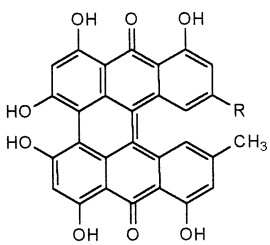
dardization of phytopharmaceuticals containing *H. perforatum* still refers to the content of hypericins, usually 0.3% calculated as total hypericins. Recently, several in vitro and in vivo studies have pointed at the phloroglucinol derivatives hyperforin and adhyperforin (Fig. 1) as the main contributors to the antidepressant activity [7–15].

This new knowledge question the present standardization procedure of phytopharmaceuticals containing *H. perforatum*, since only the content of hypericins is declared and no information regarding the content of hyperforins is available.

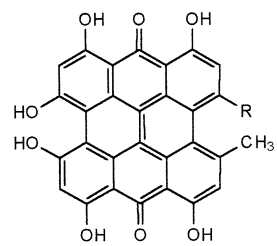
In order to evaluate the composition of these lipophilic constituents in extracts of *H. perforatum* fast and valid analytical methods are desirable. For the determination of hypericins, several different spectroscopic methods as well as high performance liquid chromatographic (HPLC) methods with UV detection at 590 nm have been published [16–21]. Likewise, HPLC methods for the quantitative determination of hyperforins have been developed [22].

Capillary electrophoresis represents a method usable for the separation of constituents in complex mixtures. A number of articles on the separation of flavonoids from various plant extracts have been published using basic aqueous buffer systems [23–29], as most flavonoids are soluble in these media. Separations of anionic substances may lead to extended migration times, since the ions are migrating in the opposite direction of the electro-osmotic flow (EOF) and, therefore, have longer migration times than the EOF.

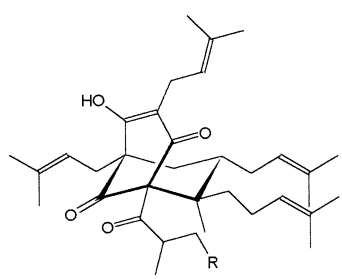
In order to obtain fast migration times of anions the direction of the EOF can be reversed. In aqueous capillary electrophoresis, the addition of cationic surfactants, such as long alkyl chain trimethyl ammonium ions, results in ionic interactions with the capillary wall. The positive charge formed at the inner surface of the capillary is due to hydrophobic interactions of free surfactant molecules with those bound to the wall, and this will result in a reversal of the EOF. By applying a negative voltage, the EOF will still be in the direction of the detection window on the capil-



(**1**) R = CH_2OH Protopseudohypericin
(**2**) R = CH_3 Protohypericin



(**3**) R = CH_2OH Pseudohypericin
(**4**) R = CH_3 Hypericin



(**5**) R = H Hyperforin
(**6**) R = CH_3 Adhyperforin

Fig. 1. Structures of protopseudohypericin (**1**), pseudohypericin (**2**), protohypericin (**3**), hypericin (**4**), hyperforin (**5**) and adhyperforin (**6**).

lary, and anions will migrate in the same direction as the EOF.

In non-aqueous capillary electrophoresis, the interaction between a cationic surfactant and the capillary wall will be very weak and hence the EOF is not reversed. Recently, it has been reported that addition of the cationic polymer hexadimethrine bromide (HDB) in low concentrations (0.001–0.05%) result in a reversed EOF [30–32] even in non-aqueous mode.

The hypericins and hyperforins are lipophilic substances, which cannot be dissolved in aqueous media. In order to analyze these lipophilic constituents obtaining fast migration times, a capillary electrophoresis method using organic solvents as the media and with addition of HDB was developed. The method was used for evaluation of the lipophilic constituents of *H. perforatum*.

2. Experimental

2.1. Chemicals

Extract of *H. perforatum* was a gift from SanoPharm A/S (Vedbæk, Denmark) and flowers and leaves were collected in Denmark, June 2000.

Methanol was supplied by Prolabo (Bois, France), dimethylsulfoxide (DMSO), ammonium acetate and sodium acetate by Merck (Darmstadt, Germany), *N*-methyl formamide (NMF) by Sigma-Aldrich (Steinheim, Germany) and hexadimethrine bromide was purchased from Sigma (St. Louis, MO). Hypericin was obtained from Extrasynthese (Genay, France) and hyperforin and adhyperforin was isolated and characterized according to Jensen et al., 2001 [15].

2.2. Apparatus

An HP^{3D} capillary electrophoresis system (Hewlett-Packard, Walbronn, Germany) equipped with an on-column diode-array detector was used. The detection was set at 300 nm for detection of hyperforins and at 590 nm for the detection of the hypericins. Detection at 350 nm was included for detection of flavonoids. The separations were performed in a fused-silica capillary (48.5 cm × 50 μm ID; 40.0 cm to the detector, or 30 cm × 25 μm ID; 21.5 cm to the detector) from Polymicro Technologies (Phoenix, AZ). The capillaries were thermostated at 25 °C with air and samples were kept at ambient temperature in the autosampler. Injection was performed by applying a pressure of 5 kPa (50 mbar) for 3 s corresponding to an injection volume of approximately 5 nl in the 48.5 cm × 50 μm ID capillary and 0.5 nl in 30 cm × 25 μm ID capillary, when assuming that $\rho = 1$ kg/l. A voltage of -20 kV was applied during analysis with the 50 μm ID capillary and -25 kV was applied using the 25 μm ID capillary.

Fluorescence detection was performed using a fluorescence detector (Argos 250B, Flux Instruments, Switzerland) operated with excitation: 240–400 nm and emission, 495 nm (cut off).

2.3. Electrophoresis medium

The electrophoresis medium consisted of methanol, dimethylsulfoxide (DMSO) and *N*-methylformamide (NMF) (3:2:1, v/v/v) containing 50 mM ammonium acetate, 150 mM sodium acetate and 0.002% (w/v) HDB. Before use, the capillaries were rinsed with 1 M sodium hydroxide for 30 min followed by 0.1 M sodium hydroxide and distilled water for 20 min. Finally, the capillaries were flushed with the electrophoresis medium for 10 min. Between analysis, the capillaries were flushed with the electrophoresis medium for 3 min.

2.4. Sample preparation

Different amounts of extract of *H. perforatum* (5.00–200.0 mg) with a know content of hypericins and hyperforins were dissolved in methanol (5.00 ml) and placed in an ultrasonic bath for 15 min. After centrifugation (4000 rpm for 10 min) the samples were directly injected.

Extraction of constituents from fresh flowers and leaves of *H. perforatum* was made with 96% ethanol, placing the samples in an ultrasonic bath for 2 h.

During extraction procedures, the samples were protected from light to prevent conversion of protopseudohypericin and protohypericin to pseu-

dohypericin and hypericins, respectively, and to prevent the degradation of the hyperforins.

3. Results and discussion

Several in vitro and in vivo investigations have pointed at the lipophilic hyperforins as being the main contributor to the antidepressant activity of extract of *H. perforatum*. The standardization is still made according to the content of total hypericins and no information concerning the content of hyperforins is declared. In order to evaluate the composition of both groups of constituents in commercial extracts, a fast analytical method with simultaneous determination of these constituents is desirable.

Capillary electrophoresis enables fast separations and investigations using this technique were carried out. Since the hypercins and hyperforins are rather lipophilic compounds, the use of aqueous capillary electrophoresis was impossible, as the constituents were non-soluble in the aqueous media even at higher pH. Addition of methanol, Tween 20, SDS, glycerol and combinations of these were carried out in order to enhance the solubility of the constituents, but no useful results were obtained, and hence non-aqueous capillary electrophoresis was considered.

Several parameters have to be considered before choosing an organic solvent for non-aqueous capillary electrophoresis, for instance the boiling constant, dipole moment, viscosity, and UV cut off value. In media with low dielectric constant and/or low dipole moment, ions will migrate slower than in water. Therefore, less current and less joule heat is generated. This makes it possible to increase the electric field strength and obtain higher separation efficiency [33,34]. The auto protolysis of organic solvents is often higher than for water, which gives a wider span to operate within compared with aqueous systems. Using a non-aqueous capillary electrophoresis medium would also have the advantage, that the leveling effect on the protolytic behavior of electrolytes exhibited by water is absent or very weak. Therefore, small differences in physicochemical properties between similar analytes are more clearly expressed in organic solvents than in water [33,35]. This effect could be beneficial on the separation of the hypericins and the hyperforins as the differences in the mass to charge ratio in these groups of constituents are very small.

Non-aqueous capillary electrophoresis with 100 mM ammonium acetate in NMF and 25 mM ammonium acetate in methanol/acetonitrile (1:3), respectively, resulted in very broad peaks and bad resolution. Recently, it was shown that acetylsalicylic acid and its three main metabolites could be separated in a non-aqueous capillary electrophoresis system with reversed electro-osmotic flow, using HDB to reverse the flow [32]. Therefore, trials using this principle for the separation of constituents in extracts of *H. perforatum* L were carried out.

3.1. Composition of organic solvents

To separate anions the study by Hansen et al., 1998 suggested a composition of methanol and acetonitrile (1:1 v/v) as the solvent of use. Using this solvent, only two peaks were detected at 590 nm indicating an overlap between the hypericins. Trials with other solvents and mixtures of these were carried out. DMSO and NMF were chosen, DMSO having a high viscosity and a low dielectric constant and NMF with a high viscosity and a very high dielectric constant. Using mixtures of methanol, acetonitrile and NMF four peaks corresponding to the four hypericins could be detected although baseline resolution was not obtained. Replacing acetonitrile for DMSO baseline resolution between the hypericins was obtained, and a ratio of methanol/DMSO/NMF (3:2:1) resulted in the best separation between these constituents. In addition, a shoulder on the peak corresponding to hyperforin could be detected, and standard addition with pure compounds verified that the shoulder corresponded to adhyperforin, the main peak being hyperforin (Fig. 2).

3.2. Electro-osmotic flow

Hypericins and hyperforins are negatively charged in alkaline solutions and would in normal

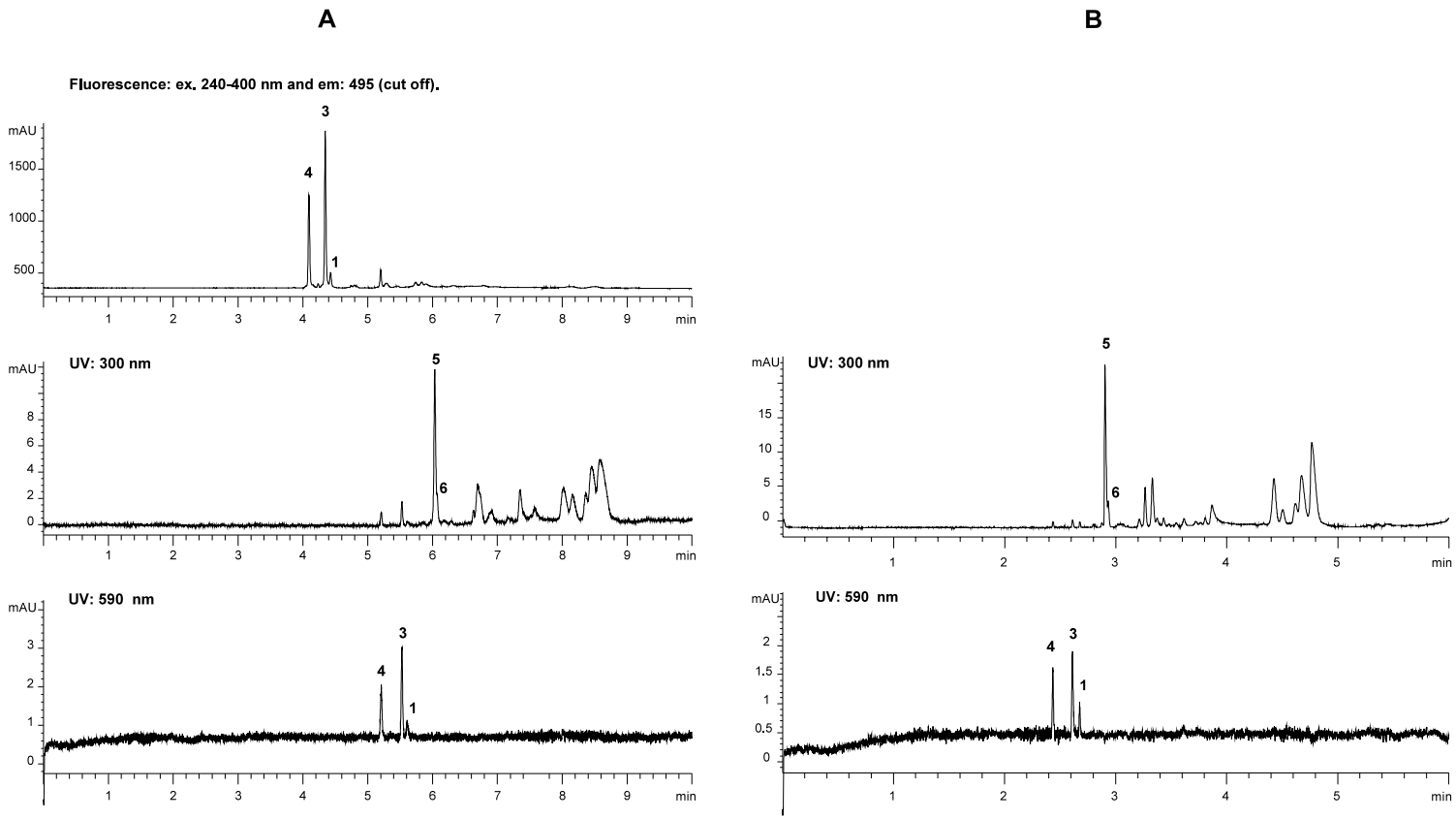


Fig. 2. Electropherogram of an extract of *H. perforatum* in (A) 50 μm ID × 48.5 cm capillary using fluorescence detection (ex.200-400 nm; em. 495 nm cut off) and UV detection (300 and 590 nm) and in (B) 25 μm ID × 30 cm capillary with UV detection at 300 and 590 nm. Electrophoresis medium; 50 mM ammonium acetate, 150 mM sodium acetate in methanol/DMSO/NMF (3:2:1) with 0.002% HDB. Peak identity: Numbers refer to numbers given in Fig. 1.

Table 1
Correlation coefficients and concentration ranges of hypericin, pseudohypericin and hyperforins and the repeatability (CV), $n = 6$

	Correlation coefficients	Concentration range (μg/ml)	Repeatability ($n = 6$)	
			Concentration (μg/ml)	Coefficient of variance (CV), %
Hypericin	0.9952	3–109	26.5	3.70
Pseudohypericin	0.9954	5.7–209.5	51.0	3.05
Hyperforins	0.9917	225–8250	1715.0	1.39

aqueous capillary electrophoresis system with positive voltage applied elute after the EOF, resulting in rather long migration times. Adding HDB to a non-aqueous system will reverse the flow, and by applying a negative voltage, the negatively charged compounds will elute before the EOF, reducing the migration times. A concentration of 0.002% HDB was used. Increasing the concentration to 0.01% did not result in more stable retention times, and by reducing the concentration to 0.001% less repeatable retention times were obtained.

3.3. Choice of capillary

Separations of the constituents in extracts of *H. perforatum* were carried out in capillaries of 50 μm ID (48.5 cm) and 25 μm ID (30 cm), respectively. Using the electrophoresis medium with an organic solvent composition of methanol/DMSO/NMF (3:2:1), separation of the lipophilic constituents could be carried out within 8 min in a capillary of the dimensions 50 μm ID × 48.5 cm by applying a voltage of −20 kV (−48 μA). In a capillary of the dimensions 25 μm ID × 30 cm separation was obtained within 3 min using a voltage of −25 kV (−20 μA), Fig. 2. The samples were introduced in methanol or 96% ethanol and no more than −20 kV could be applied in the 50 μm capillary due to too much heating of the sample, generating air bubbles which resulted in loss of current.

3.4. Composition and concentration of electrolytes

Ammonium acetate was used since it is soluble in many organic solvents. Sodium acetate was added to make the conditions more alkaline, in favour of the anionic form of the constituents. A reduction of the electrolyte concentration resulted in broader peaks and the same was seen by excluding ammonium acetate from the medium. A concentration of 50 mM ammonium acetate and 150 mM sodium acetate seemed appropriate for the separation.

3.5. Validation

A preliminary validation of the method was carried out. Even though some differences in the migration times of analytes were observed, calibration curves with linear correlation and with high r^2 values were obtained for hypericin, pseudohypercin and the sum of hyperforin and adhyperforin defined as total hyperforins in a 50 μm ID capillary (Table 1). Peak normalization was performed in order to compensate for changes in migration times. The calibration curves ($n = 6$) were carried out using a solution of an extract of *H. perforatum* with a known content of hypericin, pseudohypericin, hyperforin and adhyperforin. Previous investigations have shown the concentration ratio between the four hypericin [21], and based on these results peak **1** corresponds to protopseudohypericin, **2** to protohypericin, **3** to pseudohypericin and **4** to hypericin. Furthermore, by spiking a sample with a reference hypericin standard solution the peak corresponding to hypericin was verified. Good repeatability with a coefficient of variance (CV) of less than 4% ($n = 6$) was obtained for the constituents at the chosen level of concentration (Table 1).

Limits of detection (LOD) were determined in a 50 μm ID × 48.5 cm capillary and in a 25 μm ID × 30 cm capillary, respectively. By comparing the LOD by UV detection at 590 nm in the two capillaries, similar values in the range of 10–25 pg on column was found corresponding to 3–30 μg/ml in the sample solution. Connecting a fluorescence detector to the system lowered the LOD for hypericin and pseudohypericin considerably. Absolute values around 150 fg were obtained, lowering the limits by a factor of approximately 100 (Table 2).

The time of injection was for the limits of detection experiments set to values of 2 s for the 50 μm ID capillary and 5 s for the 25 μm ID capillary, respectively. This means that only 0.4–0.5% of the capillaries were loaded with sample.

3.6. Application

The calibration curves were made using a solution of an extract of *H. perforatum*. Thus, the method is suitable for an evaluation of the content of hypericins and hyperforins in commercial extracts. Separation of the flavonoids is also achieved having migration times between 8.5 and 11.5 min, although less efficient (Fig. 3). The separation of the compounds were performed on a different capillary than used for the separation in Fig. 2A, which explains the differences in migration times. Even though commercial extracts are still standardized on the content of hypericins, recent investigations pointing at the hyperforins as being the active constituents will make a simultaneous detection of these groups of constituents relevant.

The method was applied in an investigation of the relative distribution of these groups of constituents in fresh samples of leaves and flowers of *H. perforatum* collected in Denmark (Fig. 3). Two samples of plants of *H. perforatum* were collected in different areas. The plant material was divided into groups of flowers, buds and leaves, directly extracted with 96% ethanol and analyzed using the non-aqueous capillary electrophoresis system. The results indicate, that the content of the different constituents in the two samples differs between the flowers, buds and leaves (Table 3). No or very little amounts of the protoforms are detectable in the leaves and major differences in the flavonoid patterns are seen between flowers and leaves. This could reflect a variance between different sorts of *H. perforatum* and/or the time of harvesting, and shows the importance of further investigations of the relative distribution of constituents with respect to these parameters.

The contents of protohypericin and protopseudohypericin are based on the calibration curves of hypericin and pseudohypericin, respectively. Since the molar absorptivity of the proto-forms at 590 nm are lower than those of hypericin and pseudohypericin this results in too low results of the proto-forms [21]. By exposing the samples to

Table 2
Limits of detection of hypericin and pseudohypericin by UV detection and fluorescence detection (ex. 200–400 nm; em. 495 nm cut off) in a 50 μm × 48.5 cm capillary (injection time 2 s) and a 25 μm × 30 cm capillary (injection time 5 s), respectively

		Hypericin	Pseudohypericin
50 μm × 48.5 cm capillary			
UV detection at 590 nm	Relative LOD	2.8 μg/ml, 5.5 μM	3.2 μg/ml, 6.2 μM
	Absolute LOD	9.6 pg	11.2 pg
Fluorescence detection Ex. 240–400 nm; Em. 495 (cut off)	Relative LOD	44 ng/ml, 87.3 nM	50 ng/ml, 96.2 nM
	Absolute LOD	155 fg	176 fg
25 μm × 30 cm capillary			
UV detection at 590 nm	Relative LOD	19.1 μg/ml	31.7 μg/ml
	Absolute LOD	14.8 pg	24.5 pg

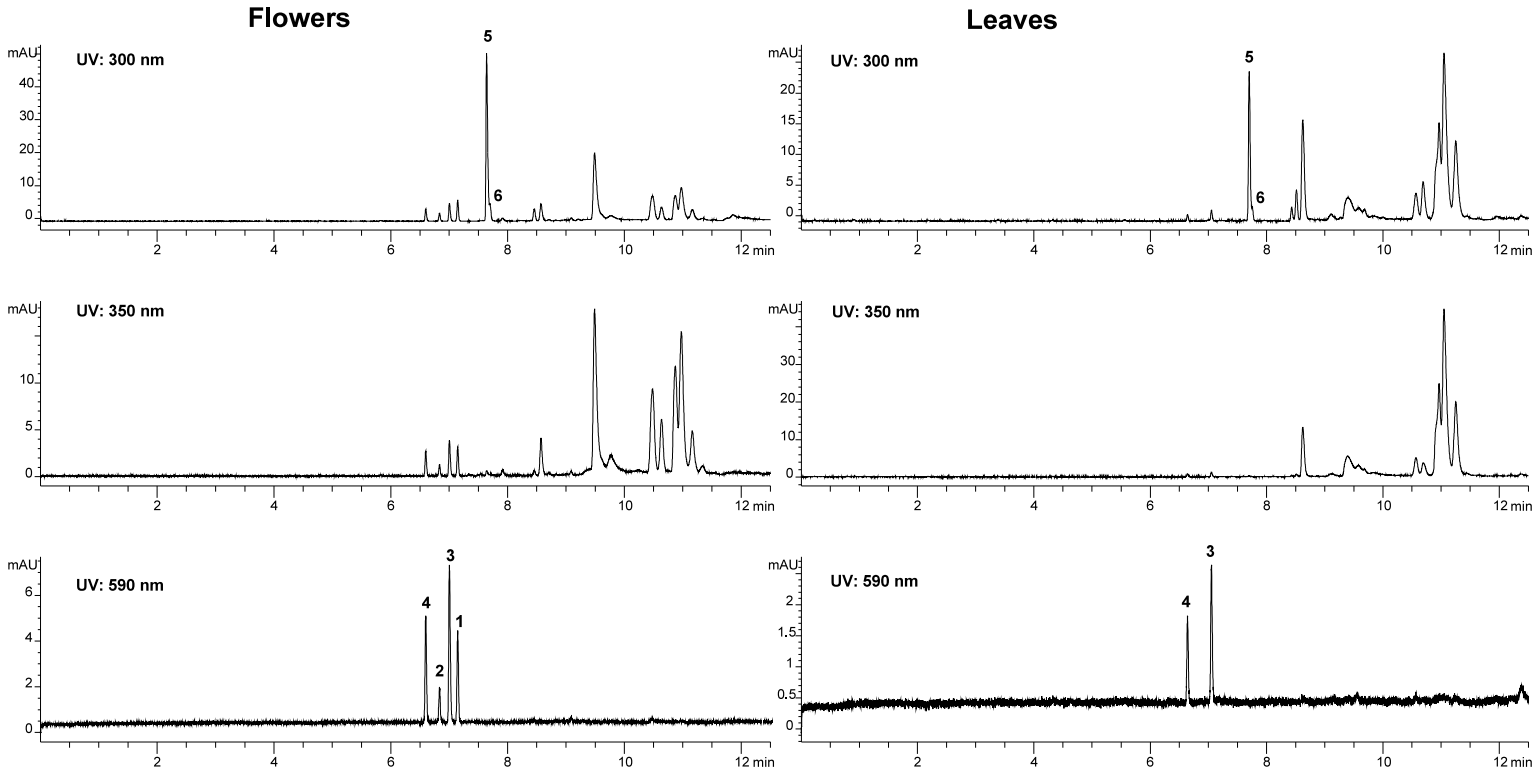


Fig. 3. Electropherogram of extracts of fresh leaves and flowers of *H. perforatum* collected in Denmark. Conditions and peak identity as in Fig. 2.In addition detection at 350 nm is performed to obtain a better detection of the flavonoid pattern.

Table 3
Content of hypericins—hypericin, protohypericin, pseudohypericin and protopseudohypericin—and hyperforins in flowers, buds and leaves of two different samples of *H. perforatum* collected in Denmark

	% (g/100 g of fresh plant material)				
	Hypericin	Protohypericin	Pseudo-hypericin	Protopseudo-hypericin	Hyperforins
Sample 1					
Flowers	0.080 ± 0.002	0.013 ± 0.001	0.096 ± 0.004	0.036 ± 0.002	11.68 ± 0.58
Buds	0.047 ± 0.001	0.028 ± 0.0001	0.117 ± 0.001	0.086 ± 0.001	10.35 ± 0.24
Leaves	0.051 ± 0.004	0.008 ± 0.001	0.075 ± 0.004	0.016 ± 0.001	10.41 ± 0.04
Sample 2					
Flowers	0.121 ± 0.001	0.015 ± 0.001	0.129 ± 0.003	0.046 ± 0.002	9.08 ± 0.02
Buds	0.108 ± 0.005	0.036 ± 0.001	0.180 ± 0.011	0.104 ± 0.004	7.81 ± 0.009
Leaves	0.036 ± 0.001	<0.0056	0.066 ± 0.001	<0.0064	3.84 ± 0.05

The values (%) are means ± S.D. of two determinations.

light, the proto-forms are converted to hypericin and pseudohypericin and the total content of hypericin could be determined as the sum of hypericin and pseudohypericin. However, exposing the samples to light will influence the stability of hyperforins, since hyperforin degrade in samples exposed to light [36].

4. Conclusion

Fast separations of the lipophilic constituents-hypericins and hyperforins-in extracts of *H. perforatum* are demonstrated in a non-aqueous capillary electrophoresis system with a mixture of methanol, DMSO and NMF (3:2:1 v/v/v) as solvent, with 50 mM ammonium acetate and 150 mM sodium acetate added as the electrolytes and an addition of HDB to reverse the flow. The method is appropriate for an evaluation of the quality of plant material as well as of commercial extract.

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