Quality Control of *Hypericum perforatum* L.

Analytical challenges and recent progress

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Abstract

Objectives

The most widely applied qualitative and quantitative analytical methods in the quality control of *Hypericum perforatum* extracts will be reviewed, including routine analytical tools and most modern approaches.

Key findings

Biologically active components of *H. perforatum* are chemically diverse, therefore different chromatographic and detection methods are required for the comprehensive analysis of St. John's wort extracts. Naphthodianthrones, phloroglucinols and flavonoids are the most widely analysed metabolites of this plant. For routine quality control, detection of major compounds belonging to these groups seem to be sufficient, however closer characterisation requires the detection of minor compounds as well.

Conclusions

TLC and HPTLC are basic methods in the routine analysis, whereas HPLC-DAD is the most widely applied method for quantitative analysis due to its versatility. LC-MS is gaining importance in pharmacokinetic studies due to its sensitivity. Modern approaches, such as DNA barcoding, NIRS and NMR metabolomics may offer new possibilities for the more detailed characterization of secondary metabolite profile of *Hypericum perforatum* extracts.

Keywords

Hypericum perforatum, St. John's wort, HPLC, TLC, NMR metabolomics, DNA barcoding

Introduction

Hypericum perforatum L. (St. John's wort - SJW) is one of the most important medicinal plants, being the active component of several products. In the modern medicine the aerial parts (Hyperici herba) are applied, usually as extracts. The efficacy of St. John's wort has been studied in several clinical trials and according to the most recent Cochrane review, *Hypericum* products were superior to placebo in patients with major depression and similarly effective as standard antidepressants.[1] The European Medicines Agency granted a community herbal monograph for Hyperici herba extracts,[2] and there are several *Hypericum*-containing medicines on the market with well-established indications as antidepressants. *Hypericum* is marketed as food supplement in different countries of the world, typically with the intention to act on the central nervous system. The majority of products for internal application contain dry extracts; some preparations contain the oily extract of the herb, however these are intended for external use. Many analytical techniques have been established for the quality control of St. John's wort products. The objective of this paper is to review the existing literature on phytochemical analysis of Hyperici herba and dry *Hypericum* extracts and to assess the validity of these methods for everyday use in the relevant industries. This extensive review gives an overview of all the methods that are used in the analysis of St. John's wort-based products.

Pharmacopoeias are the cornerstones of the quality control of medicinal products, since these determine the compounds to be analysed and also the methods to be applied in case of raw materials. The European Pharmacopoeia specifies a minimal (total) hypericin content of 0.08% for Hyperici herba,[3] however for the dry extract (Hyperici herbae extractum siccum quantificatum), the ranges of total hypericin (0.1-0.3%, expressed as hypericin), flavonoids (minimum 6%, expressed as rutoside), and hyperforin (maximum 6%) are defined.[4] The U.S. Pharmacopeia National Formulary contains three *Hypericum* monographs in order to regulate the quality of *Hypericum*-based food supplements. The St. John's wort monograph specifies not less than 0.6% hyperforin content and not less than 0.04% combined hypericin and pseudohypericin content for the herb[5] and the powdered herb as well.[6] For the powdered St. John's wort extract, only the acceptable deviations (90-110%) from the declared hypericin and hyperforin contents are prescribed, there are no upper or lower limits for the concentrations of these analytes.[7] The Chinese Pharmacopoeia defines a lower limit of hyperoside content (0.1%) in the herb.[8]

SJW preparations are usually quantified to their content of hypericin-type compounds, which may be determined by spectrophotometric measurement[9] or for their content of hypericin-derivatives and hyperforin derivatives. Hypericin and pseudohypericin result in red solutions with organic solvents and have characteristic UV spectra with a maximum at 590 nm. One major limitation of spectrophotometric

quantifications is that there is possible interference from other plant metabolites, eg. chlorophylls, that may have absorption overlapping directly with hypericin derivatives. Further, using this method only the total amount of hypericin-derivatives can be determined, the quantification of individual compounds is not possible. Therefore, UV spectrophotometry is not considered as the most appropriate tool for the quality control of SJW products and the plant material. Moreover, it has been shown that by adulterating SJW with food dyes, it is possible to mimic the UV-spectrum and produce substandard material that passes the analytical test.[10] However, the European Pharmacopoeia still prescribes UV spectrophotometry as quantitative assay for Hyperici herba.[11] Other methods, such as TLC and HPTLC may be applied primarily for qualitative analysis of SJW extracts. In recent studies (similarly to pharmacopoeia monographs of dry extracts),[4,7] HPLC-DAD is most widely applied for quantification, whereas for qualitative analysis, primarily LC-MS is used. DNA barcoding and NMR metabolomics belong to the most modern tools of instrumental analysis, which are under development for use also within pharmacopoeias.

Sample preparation

Sample preparation has a major impact on the reliability of analytical experiments. In the case of SJW, the polarity of the extracting solvents and light exposure are the most determinative factors, whereas pH and temperature have less impact on the recovery of analytes. Hypericin, hyperforin and their derivatives are unstable under certain conditions. Light catalysis the transformation of protoderivatives to their respective hypericins (hypericin and pseudohypericin as the main components). Hyperforin is unstable at higher temperatures and in the presence of air and in apolar solvents such as *n*-hexane, resulting in the formation of furohyperforin derivatives. It is more stable in protic solvents.[12] When exposed to light, hyperforin and adhyperforin in a MeOH extract solution degraded rapidly, particularly at pH 7, where within 12 h, complete transformation was observed. Interestingly, hyperforin was more stable in an acidic milieu. When protected from light, the solutions regardless of pH, underwent minimal transformation after 36 h.[13] A 5 min exposure of the crude extract of SJW to sunlight induced a 96% loss of hyperforins.[14] Hypericin and pseudohypericin show low stability to air and light. Rapid degradation of total naphthodianthrone content (only about 30% of the theoretical content) was detected after three months of storage, even if antioxidants were added to the extracts.[15]

In order to simplify and increase the reliability of methods for the determination of hypericins, experiments have been carried out to assess the effect of light exposure on the transformation of protohypericins to hypericins. One method combines on-line, precolumn photochemical conversion followed by photodiode-array detection to allow convenient quantification of hypericins. A photochemical reactor was used in order to transform the light sensitive naphthodianthrones, protohypericin and protopseudohypericin, into hypericin and pseudohypericin, respectively.[16] Using a photo halogen lamp (1000 W), the plateau of the hypericin content expressed as the sum of areas of hypericin and pseudohypericin peaks was achieved after 10 min of light exposure in the liquid extracts of SJW-containing samples.[17] A HPLC method for the determination of hypericin and pseudohypericin included the use of a light reaction coil, installed between the autosampler and the analytical column to convert potentially existing protohypericin and protopseudohypericin into hypericin and pseudohypericin to make quantification more reproducible.[18]

SJW contains marker compounds of different polarity, therefore sample preparation has major influence on the composition of extracts. Different extraction procedures are described in different pharmacopoeias (eg. in the European Pharmacopoeia 80% THF[11]), and quantitative data reported in the literature are obtained from experiments with samples gained by different extraction methods. Avato and Guglielmi performed a systematic study to assess the hypericin content of different SJW extracts. Soxhlet extraction was carried out with MeOH or EtOH (in the latter case, after pre-extraction with diethyl ether). Extracts with solvents of different polarity (petroleum ether, $CHCl_3$, EtOAc and MeOH) were prepared by sonication. Macerate was gained with MeOH. One extract was prepared with 90% aqueous acetone under stirring and one sample was extracted with hot methanol. These experiments revealed, that extracts poor in chlorophylls and relatively rich in hypericins can be obtained by Soxhlet extraction with ethanol (after pre-extraction with diethyl ether) and with 90% aqueous acetone. Hot MeOH and Soxhlet extraction with MeOH resulted in the highest hypericin content. Soxhlet extracts contained the highest amount of hyperforin, whereas ultrasonic extracts were relatively poor in this compound. HPLC analyses of the various extracts provided useful information on the quantities of flavonoids and chlorogenic acid in the extracts. Based on these results the best extraction procedure to obtain an extract representative of all the major metabolites (hypericins, hyperforins and flavonoids) involves the use of a polar solvent such as MeOH or EtOH.[19] Milevskaya et al carried out extensive experiments to study the influence of different factors on extraction efficiency based on the quantification of 15 constituents (phenolcarboxylic acid, flavonoids, naphthodianthrones and phloroglucinols) of SJW. It was concluded that the effects of temperature and microwave radiation, as well as the combination of temperature and pressure offer the greatest degree of extraction.[20] In one experiment, extraction with hot MeOH after pre extraction with $CHCl_3$

(to remove chlorophyll) resulted in an extract with higher flavonoid content than that of a macerate prepared with EtOH.[21]

Optimal conditions for extraction of *H. perforatum* samples in a water bath shaker were determined using response surface methodology. Extraction efficiency was defined by comparing either the total extractable material weight or individual component (rutin, isoquercitrin, quercitrin, quercetin and hypericin) peaks. Of the tested variables, the extraction temperature most significantly affected extraction efficiency, but high temperature also caused decomposition of hypericin. Considering all variables, optimum ranges for extraction time and extraction solvent concentration (percent ethanol in acetone) were 5.0-6.7 h and 44-74% at 23 °C, 5.4-6.9 h and 45-72% at 40 °C, and 5.3-5.9 h and 44-69% ethanol in acetone at 55 °C, respectively.[22] In one experiment, extraction of dried plant material with MeOH in the dark, at room temperature for 2 h, led to a complete recovery of naphthodianthrones but only a partial recovery of the phloroglucinol derivatives. Extraction with water – EtOH 4:6 in a water bath shaker at 80 °C led to the total extraction of hypericins with a 90% recovery of hyperforins.[14] The optimum conditions for extraction of rutin and quercetin from *H. perforatum* were investigated by Biesaga et al. Aqueous methanol (40-80%) is the most efficient extracting solvent. The aglycone quercetin could be obtained from its glycosides most efficiently after 5/10 min hydrolysis wit 2.8/2.1 M HCl.[23]

Pages et al used different chemometric approaches to evaluate the influence of extraction factors on the detectable amount of hypericin. An asymmetric screening design was built in order to evaluate the weight of each level for each factor – sonication duration, magnetic stirring, light exposure duration on the response, the total hypericin content. Stirring has no real impact on efficiency and there is no direct association between the sonication time and hypericin content, however, it was confirmed that light exposure catalyses the breakdown of hypericin.[24] These results point out that the light exposure, recommended in the monograph as sample pretreatment in the European Pharmacopoeia[11], does not permit reproducible quantification of the hypericin content.

A comparison of sonication, Soxhlet extraction and pressurised-fluid extraction was conducted for several major constituents in SJW. It was confirmed that there is a direct link between sonication time and extraction efficiency. In case of pressurised-fluid extraction, moderate changes in pressure did not significantly affect extraction efficiency. Poor extraction efficiency was observed for the most polar analytes (e.g., chlorogenic acid and flavonoids) with acetone, methylene chloride, and hexane. Acetone was more effective for extraction of the nonpolar naphthodianthrones. The extraction efficiency, especially for non-polar components was relatively constant at 20, 60 and 100 °C, however levels for polar flavonoids were significantly reduced for extractions at 200 °C. Comparing these 3 methods, the

highest recoveries of the major constituents were achieved with Soxhlet extraction.[25]

Optimisation of ultrasonic-assisted extraction of *H. perforatum* for quercetin was carried out using the Box-Behnken design combined with response surface methodology. The effects of temperature (30-70 °C), extraction time (20-80 min), methanol (20-80%), and HCl concentration (0.8-2.0 M) on quercetin concentration were assessed. The optimum conditions were determined as follows: 67 °C, 67 min, 77% MeOH, HCl concentration 1.2 M. The method was validated by experimental confirmation of the predicted quercetin content in the extract.[26]

In case of *in vivo* studies, sample preparation of biological samples usually includes solvent extraction from blood plasma to enrich the analytes. For hyperforin, apolar extracting solvents, such as *n*-hexane-EtOAc 9:1-7:3 are used.[27] In one experiment, solid phase extraction on C8 column was carried out prior to the HPLC analysis of hypericin[28], others used Oasis HLB.[29] In a study biapigenin was extracted from biological tissues using Oasis HLB 1-cc extraction cartridges.[30] Solid phase extraction prior to HPLC is also necessary when analysing oily extracts. From SJW oil (extract prepared with fatty oil), an aminopropyl SPE cartridge may be used. Conditioning was reported sequentially with NaOH, MeOH, acetone, and heptane and rinsing with heptane, elution was carried out with 5 % oxalic acid dihydrate in acetone – MeOH 1:1.[31]

As the result of miniaturisation in analytical chemistry several new liquid–liquid extraction have been developed to reduce the consumption of organic solvents and the time needed for analysis and to facilitate towards automation. In the so-called single-drop liquid-phase microextraction the organic micro droplet is placed into the aqueous sample and the analytes are extracted into the organic droplet based on passive diffusion. This method, with good extraction efficiency, was optimised for the quantification of hypericin, pseudohypericin and hyperforin from biological fluids.[32]

Thin Layer Chromatography

TLC is the method of preference for identification and quality control of *H. perforatum* (both plant and extract) by the European and the United States Pharmacopoeias. Both pharmacopoeias describe the analysis procedure of the SJW plant and extract as well as the compounds that should be seen in their fingerprint. According to the European Pharmacopoeia, both the plant material and the extract are prepared in a concentration of 50 mg/mL in methanol for TLC analysis and the standards rutin and hyperoside are prepared at concentrations of 1 mg/ml for SJW plant and 0.5 mg/ml for SJW extract. The TLC plate is developed with the mobile phase anhydrous formic acid – water – ethyl acetate (6: 9: 90 v/v/v). After the development, the plate is sprayed with solvent 1: 10 g/L diphenylboric acid aminoethyl ester in methanol and solvent 2: 50 g/L macrogol 400 in methanol and is visualised under

UV light at 365 nm. The chromatogram of SJW plant should illustrate the fluorescent bands of rutin, hyperoside, hypericin and pseudohypericin while it is claimed that other bands of yellow or blue colour are visible. The chromatogram of SJW extract needs to have the yellow band of rutin, the blue zone of chlorogenic acid and the yellow band of hyperoside in the lower third of the chromatogram. In the top third of the chromatogram 2 red bands due to hypericin and pseudohypericin and one yellow band due to quercetin have to be visible, while in the middle third, three yellow bands can be seen. The pharmacopoeia states that other fluorescent bands can also be illustrated in the chromatogram of SJW extract.[33]

The United States Pharmacopoeia requires that 100 mg/mL of SJW plant and 50 mg/mL of SJW extract in methanol are analysed. The development solvent proposed is ethyl acetate – glacial acetic acid – formic acid: water (10: 1.1: 1.1: 2.6 v/v/v/v) and the development distance is 18 cm. After development the plate is derivatized with 10 mg/ml solution of diphenylboric acid aminoethyl ester in methanol and 50 mg/mL solution of polyethylene glycol 400 in ethanol and visualized under UV light at 365 nm. The acceptance criteria for SJW plant is the presence of some yellowish bands on the chromatogram, one of which travels at R_f=0.5. The bands of hypericin (R_f=0.85) and pseudohypericin (R_f=0.8) should be present while two blue bands below the yellow hyperoside band are described and correspond to chlorogenic and neochlorogenic acids. The chromatogram of SJW extract should contain the bands of rutin, hyperoside, hypericin and pseudohypericin as described above, but other bands of different colour and intensity might be present in the chromatogram. The USP Pharmacopoeia, unlike other Pharmacopoeias, describe a different solvent system for the analysis of hyperforin, hexane – ethyl acetate (4:1 v/v), while the plate is derivatized with a solution containing 0.38 g ceric ammonium sulfate and 3.8 g ammonium molybdate in 100 mL of 2N sulfuric acid and visualized under UV light (hyperforin is a blue band around R_f=0.54).[34]

TLC published studies have mostly focused on the identification and separation of hypericin and pseudohypericin.[35,36] However there are some TLC studies which analysed the phenolic content of *Hypericum* species, including the study of Jesionek et al and Males et al.[37,38]

Mulinacci et al used TLC-densitometry in combination with HPLC-DAD in order to identify and quantify hypericin in SJW extracts. Hydroethanolic extracts (EtOH 80%) of SJW aerial parts were analysed and the silica gel TLC plates were developed with the solvent system toluene– ethyl acetate – formic acid (50: 40:10 v/v/v). The team used Incremental multiple development in an unsaturated horizontal chamber which means that they developed the plate twice with the same solvent in order to maximize the separation. No dipping or spraying solvents were used, while the densitometric assessment was

conducted under an excitation wavelength of 313 nm. Hypericin and pseudohypericin were well separated and HPLC-DAD were used for their quantification.[35]

Kitanov et al used TLC to identify, and spectrophotometry to quantify, hypericin and pseudohypericin in 36 *Hypericum* species.[36] The different *Hypericum* extracts were applied on silica gel TLC plates and the plates were developed with two mobile phases; toluene – ethyl acetate – formic acid (50: 40: 10 v/v/v), as Mulinacci et al did, and with ethyl acetate: formic acid (50:6 v/v). After development the plates were sprayed with 0.5 N KOH in ethanol and visualised under UV 366 nm. Hypericin and pseudohypericin were well separated and existed in 27 out of 36 *Hypericum* species.

Males et al used TLC not only to separate and analyse flavonoids and phenolic acids from Croatian *Hypericum* species but they were also the first research team to analyse the amino acid content in those species. For the flavonoids and the phenolic acids methanolic solutions of the samples were spotted on TLC silica plates which were developed with the mobile phases ethyl acetate – formic acid – acetic acid – water (100: 11: 11: 26 v/v) and ethyl acetate – formic acid – water (8:1:1 v/v) and derivatized with NP and PEG reagents. For the separation of amino acids, aqueous solutions of the samples were spotted on cellulose TLC plates, which were developed with the mobile phases *n*-butanol – acetone – acetic acid – water (35: 35: 10: 20 v/v) and *n*-butanol – acetic acid – water (40:10 :10 v/v) and derivatized with ninhydrin reagent. UV spectrophotometry was used for quantitative analysis. Overall, 16 amino acids, 10 flavonoids and 3 phenolic acids were separated and *H. perforatum* subspecies were found to be the richest in these constituents. In particular, *H. perforatum* subsp *perforatum* was the richest in rutin, hyperoside and isoquercitrin as well as in tryptophan (which was not detected in the rest of the samples).[38]

Jesionek et al separated and identified phenolic compounds in hydroethanolic (70% EtOH) extracts of five plants including aerial parts of SJW and they optimized the TLC conditions for better separation of those phenolic compounds. In addition TLC was hyphenated to the *(in silico)* DPPH assay to evaluate the antioxidant potential of the compounds. The silica gel TLC plates were developed with 7 different mobile phases and then derivatized with NP reagent and PEG reagent. The research team found that flavonoid aglycons like quercetin were better separated with the system toluene – diethyl ether – acetic acid (60:40:10 v/v/v), the flavonoid glycosides like rutin and hyperoside with the system ethyl acetate – acetic acid – formic acid – water (100: 11: 11: 26 v/v/v/v) and the phenolic acids like chlorogenic acid with the system chloroform – ethyl acetate – acetone – formic acid (40: 30: 20: 10 v/v/v/v).[37]

High performance thin layer chromatography

HPTLC is an improved form of thin layer chromatography, more automated and reproducible, and which provides better separation of compounds and better detection. The European Pharmacopoeia is currently updating the identification method from TLC to HPTLC on the monograph of SJW.[33] In addition, the HPTLC association recommends a well-established method for the identification of compounds in SJW while several studies have been published analysing SJW with HPTLC.

The HPTLC association proposes a method for the analysis of SJW for both crude material and extract. 100 mg/mL and 50 mg/mL methanolic solutions for crude material and extract respectively are prepared as well as the standards rutin and hyperoside at a concentration of 1 mg/mL in methanol. The HPTLC silica gel plates are developed with the solvent system ethyl acetate – dichloromethane – water – formic acid – acetic acid (100: 25: 11: 10: 10 v/v/v/v/v) in a saturated chamber with the humidity set at 33%. After development, the plates are derivatized with Natural Product reagent (NP) and Polyethylene glycol 400 reagent (PEG) for detection of phenolic compounds. The yellow bands of rutin and hyperoside should be seen at R_f=0.1 and R_f=0.25 respectively, as well as the red bands of hypericin and pseudohypericin at R_f= 0.57 and R_f=0.63 respectively. Other yellow bands can be seen between hyperoside and hypericin.[39]

Two HPTLC studies of SJW adulteration have been published.[10,40] Huck-Pezzei et al used a combination of analytical techniques, including TLC, HPLC, MS, NIR (near-infrared) spectroscopy and imaging methods coupled to multivariate data analysis, in an attempt to identify adulteration in 32 SJW samples (both plant material and finished products) and to differentiate between *Hypericum* of European and Chinese origin. HPTLC was used to identify some unusual ingredients present in Chinese samples. Methanolic SJW extracts were applied on HPTLC plates and developed in a saturated chamber with the mobile phase ethyl acetate – water – formic acid (42.5: 2.5: 5 v/v/v). The plates were sprayed with 1% methanolic diphenylboryloxyethylamine and 5% methanolic PEG 400 and were visualized under UV light at 365 nm. They found that SJW of Chinese origin contained a yellow-orange band under hypericin in the chromatogram which they suggested that it might belong to the compounds Kushenol G and H (present in *H. hirsutum* L.) after MS analysis. They also identified different concentrations of phenolic compounds between European and Chinese SJW with European SJW containing higher concentrations of rutin, hyperoside and isoquercitrin.

Frommenwiler et al used HPTLC to investigate adulteration on crude SJW herbs, commercial finished SJW products and dry SJW extract.[10] The team analysed the samples using the HPTLC association method described above and they detected an extra yellow band at R_f=0.4-0.5 as Huck-Pezzei et al did but additionally they observed the absence of a yellow band at R_f=0.18 for the samples with the extra

yellow band. The samples with the extra band are suspected to be adulterated with Chinese *Hypericum* spp. and in particular with *H. undulatum* Schousb. ex Willd. Some samples that produced green methanolic solutions, were adulterated with the dyes tartrazine, amaranth, sunset yellow and brilliant blue. They confirmed this by reversed phase HPTLC analysis using methanol – 5% aqueous sodium sulfate (3:4 v/v) as the mobile phase. The dyes were also quantified in the samples through densitometry and their average proportions were found 0.043% for tartrazine, 0.21% for amaranth, 0.38% for sunset yellow and 0.20% for brilliant blue.

Marelli et al aimed to assess the chemical variability and the variability in biological activity of four samples of *H. perforatum* subspecies *veronese* (Schrank) H. Lindb collected from 4 different areas of Italy. The chemical variability was investigated through HPTLC. The samples were extracted with 70% ethanol and were applied on HPTLC silica gel plates prewashed with methanol. The rest of the analysis was as described in the HPTLC association. They concluded that the constituents were well separated and easily visualized while the most prominent constituent was found to be chlorogenic acid.[41]

Kirmizibekmez et al achieved separation (by HPTLC) and quantification (by densitometry at 270 nm) of four quercetin glycosides in methanolic solutions of SJW. HPTLC, normal phase silica gel plates were used and the mobile phase for the development of the plates was ethyl acetate – chloroform – formic acid – acetic acid – water (100: 25: 10: 10: 11 v/v/v/v/v). Rutin, miquelianin, hyperoside and quercitrin were well separated and quantified at 0.75%, 1.9%, 4.8% and 1.8% respectively.[42]

Wuthold et al developed a model for the assessment of HPTLC plates and for correlation of HPTLC results with the pharmacological activity of SJW extracts. 27 SJW samples were acquired from 4 different regions, extracted with seven different solvents (different proportions of methanol and ethanol in water) and developed on HPTLC plates with the solvent system *n*-heptane – acetone – t-butylmethyl ether – formic acid (33:35:30:2 v/v). The plates were measured at 200-600 nm by diode-array and three-dimensional chromatograms were obtained and also an opioid binding assay was conducted on cortex of rat brain. Multivariate data analysis (partial least squares regression- PLS-1) of the 3-D chromatograms was used to correlate the phytochemical results with the pharmacological activity of the SJW extracts. The model developed was assessed in seven test SJW samples and was found accurate and reliable for prediction of pharmacological activity of SJW extract and for evaluation of HPTLC plates.[43]

While most of the studies focused on the analysis of flavonoids and naphthodianthrones in SJW, the next two studies focused on the phloroglucinol hyperforin.[44,45] Orth et al used HPTLC to test the identity and purity of the isolated hyperforin. HPTLC silica gel plates were loaded with the samples, two mobile phase systems were used and after development the plates were sprayed with fast blue

salt B 0.5% in water and visualised under UV 254 nm. After development with the solvent *n*-heptane – acetone – t-butylmethyl ether – 96% acetic acid (33: 35: 30: 2 v/v/v/v) hyperforin has an R_f=0.45 and after development with toluene – formic acid ethyl ester – formic acid (5:4:1: v/v/v) hyperforin has an R_f=0.8. Tewari et al used two SJW dry extracts to develop a HPTLC method for quantification of hyperforin. Methanolic solutions of the dried extracts were placed on HPTLC silica gel plates which were developed with the solvent system petroleum ether – ethyl acetate (90:10 v/v) at 65% humidity, they were scanned at 290 nm and then sprayed with 10% sulfuric acid reagent (in methanol). The brown-yellowish hyperforin band was well separated from the rest of the SJW constituents and travelled at R_f=0.32-0.35. Additionally the team found that the minimum detection limit of hyperforin was 100 ng and the quantification limit was 200 ng.

Overall, it seems that HPTLC is a suitable routine method for analysis of SJW both crude material and extract, as its constituents are well separated and quantification is also possible. For the analysis of phenolic compounds, more polar solvents systems were used throughout the literature while for the detection of hyperforin less polar solvent systems were used.

Gas chromatography and GC-MS

In the case of *Hypericum*, gas chromatographic analyis is typically applied for the characterization of essential oils. The essential oil of the plant (which can be obtained by hydrodistillation[46]) is not used in modern medicine, and extraction methods applied in case of orally used products result in products that contain volatile constituents in low amounts. Hence, essential oil components are not considered as relevant analytes in the quality control of such extracts and final products. The use of volatiles could be considered as expedient in case of oily extracts.

GC-FID is a reliable tool for the quantification of essential oil constituents. Identification of peaks in the gas chromatogram may be carried out based on their retention indices, and comparison of fragmentation patterns with literature data. In most experiments, mass spectra were obtained by electron ionisation.[47,48] When possible, co-injection with an authentic standard may reassure the identification. As stationary phases, HP-5, 30-60 m x 0.25 mm[47,49], HP-5 25 x 0.32 mm[50], DB-5 30 m x 0.25 mm[46,51], Silicon DB-1 60 m x 0.25 mm[52], Permabond CW 20M 50 m x 0.25 mm[46], Durabond – DB 1 60 m x 0.25 mm, DB-Wax 60 m x 0.25 mm, CP-Sil 19 CB 25 m x 0.25 mm[53], Elite-5MS 30 m × 0.32 mm[54], HP-FFAP 30 m × 0.25 mm[55,56] are typically used.

HPLC

Characteristic and pharmacologically relevant compounds of SJW are chemically diverse. Therefore, different solvent systems have been reported in the literature to achieve the most efficient separation of analytes. HPLC methods are usually based on the application of C18 stationary phases due to the universality, good selectivity and good resolution of these columns for closely related compounds such as hypericin and hyperforin derivatives. However, typically with the aim of reducing analysis time, other stationary phases, such as monolithic, phenyl-hexyl columns have also been used.[57]

For the determination of more ingredients belonging to different classes of compounds in the extracts, HPLC analysis may require long (up to 60 mins) gradient elution. If the analysis is focused on a specific group of metabolites, shorter analysis time could be achieved. Phloroglucinols and naphthodianthrones are characteristic and pharmacologically active constituents of SJW. HPLC quantification of the SJW extracts usually involves the determination of hyperforin. Further compounds of interest in the analytical assessment of extracts are hypericin and its derivatives. Quantification of hyperforins and hypericins can be carried out with shorter (up to 30 mins) gradient programs. In some cases, short analysis times can be achieved with isocratic elution, as well. Detection is usually based on the registration of UV spectra by PDA detectors, and quantification is carried out by integrating chromatograms at characteristic wavelengths. Hypericins and hyperforins have characteristic UV spectra that facilitate their identification and selective detection. Hyperforins have an absorption maximum around 272-274 nm, whereas hypericins possess λ_{max} values at 548 and 591-593 nm.[20] For hypericin derivative detection, 590 nm, for hyperforins 270 nm is usually applied. Other components (flavonoids, phenolcarboxylic acids) are detected at their characteristic absorption maxima. Fluorescence and ELSD detection may also be used, but the latter is not appropriate for the determination of phloroglucinols.

The applied eluents are usually neutral or acidic. The experiments of Fourneron and Nait-Si showcase the impact of the eluents' pH on the analytical results. The hyperforin signal (both AUC and retention time) is strongly dependent on the pH of the mobile phase, with a major change occurring between pH 3.5 and 2.5. Hyperforin can exist in enol (down to ~ pH 3) or diketone forms depending on pH. The diketone absorbs less strongly, corresponding to the absorption spectrum recorded at low pH values. Although at higher wavelength (290-310 nm) the absorption is highly influenced by pH, at 270 nm, the hyperforin response is not greatly affected.[58]

Hypericin is soluble in alkaline aqueous solutions, and therefore precipitation might occur in the chromatographic system when using acidic eluents. Characteristics of the applied column might play an important role in retaining the compound. Piovan et al assessed the applicability of 6 RP columns

(Jupiter (Phenomenex) 250 x 4.6, 5 µm, 300 Å, Lichrospher (Merck) 150 x 3.2, 5 µm, Lichrosorb (Alltech) 250 x 4.1, 5 µm, Nova-Pak (Waters) 150 x 3.5, 4 µm, 60 Å, Lichrosorb (Merck) 250 x 4.1, 7 µm, µBondapak (Waters) 250 x 4.1, 10 µm) for the quantification of hypericin. Peak areas obtained by LC-MS were compared to that of obtained by flow injection analysis mass spectrometry. All loaded hypericin was retained by the Jupiter column, whereas 90% recovery was observed using the Lichrosorb column. In case of the other columns the recoveries ranged between 31-46%. Polymerization and chelation as an explanation of this phenomenon could be observed.[59]

Pages et al optimized the mobile phase composition using a combined design including three mixture variables and one quantitative variable (temperature) described by a first-degree model. A modification of the European Pharmacopoeia method[11]was proposed to substitute phosphate buffer to acetate buffer. Mobile phase (ethyl acetate/buffer/methanol) was optimized by carrying out a series of HPLC experiments with eluents containing different ratios of the solvents. The first response was the retention time of the last eluted compound (hypericin); the second the resolution between pseudohypericin and protopseudohypericin; the last response is the asymmetry factor. Optimal separation was achieved by using MeOH – acetate buffer – ethyl acetate 69:18:16 as eluent.[60]

The quantitative characterization of SJW extracts was initially based on the determination of hypericin, since this compound was the first supposed active component of the plant and a molecule that can be easily detected due to its characteristic UV spectra. The first analytical reports applying HPLC-UV go back to the 1980's. Reversed phase stationary phases allowed reliable quantification with detection thresholds as low as 0.5 µg/mL.[61] Although recent analytical methods usually focus on multiple metabolites of the plant, some article report methods that were developed primarily to quantify hypericin.[62] Bagdonaie et al reported a method for the determination of four hypericin-type compounds using a C18 column with an analysis time of 30 min.[63] An isocratic method based on the application of a C18 column allowed the separation of hypericin and pseudohypericin with limits of detection for these compounds of 0.1 µg/mL.[64]

Some methods focus on the quantification of hyperforin. An isocratic HPLC method was developed to quantify hyperforin and adhyperforin in supercritical fluid extracts that are rich in phloroglucinols and void of other metabolites of the plant.[65] For determination of hyperforin content in plant extracts, other methods were also reported with LOD/LOQ on column 10/20 ng.[66]

Validated methods with simultaneous fluorescence and UV detection were developed for the simultaneous determination of hypericins and hyperforin [32,67] and some methods allow the additional quantification of other compounds of interest, such as adhyperforin.[68] One method reported the

baseline separation of hypericin, pseudohypericin, hyperforin and adhyperforin, however, with rather long analysis time (65 min). This method was not validated, LOD and LOQ values were not reported.[19]

In the European Pharmacopoeia, two HPLC methods are included in the monograph of dry *Hypericum* extract (Hyperici herbae extractum siccum quantificatum). As stationary phase, octadecylsilyl silica gel is prescribed (150 x 4.6 mm). In case of the quantification of hypericin, the mobile phase consists of ethyl acetate, 15.6 g/L NaH_2PO_4 solution (pH=2 with H_3PO_4) and methanol (39:41:160, linear). The quantification of hyperforin and flavonoids is based on the measurement of rutoside using gradient elution with H_3PO_4 – H_2O (3:1000, A) and H_3PO_4 – acetonitrile (3:1000, B).[4] The U.S. Pharmacopeia determines the hyperforin, hypericin and pseudohypericin content with a single HPLC run using oxybenzone as standard. The mobile phase consists of H_3PO_4 – water (3:997, A), acetonitrile (B) and methanol (C). Gradient elution is carried out on reversed phase column (250 x 4.6 mm). The major drawbacks of these Pharmacopoeia methods are their duration (15 + 31 min in case of the European, 66 min in case of the U.S. Pharmacopeia) and the fact that quantification of the analytes is not based on the determination of the respective labelled analytes.[5–7] The Chinese Pharmacopoeia prescribes the use of reversed phase (C-18) stationary phase with linear elution using acetonitrile and 0.1% H_3PO_4 solution (16:84) for the quantification of hyperoside.[8]

An HPLC-DAD method for the rapid determination of the major active compounds, naphthodianthrones and phloroglucinols, permits the determination of hypericin, protohypericin, pseudohypericin, protopseudohypericin, hyperforin and adhyperforin in 12 min. Lower levels of quantitative determination were 2 µg/mL for hyperforin and 0.5 µg/mL for hypericin, while detection limits were 0.1 and 0.02 µg/mL, respectively.[69] A simple method for the determination of 4 characteristic bioactive compounds (hyperforin, adhyperforin, hypericin and pseudohypericin) in dietary supplements and functional foods containing SJW was reported with an isocratic method on a C18 column. The limit of detection for hyperforin and adhyperforin was <0.15 µg/g food product and <0.10 µg/g for hypericin and psedohypericin.[70] An RP-HPLC method with a good resolution allows the quantification of the protoforms of the hypericins, hyperforin and adhyperforin in 17 min.[14]

A method, applying a special column (Protein C4) allowed the detection and quantification of the three characteristic classes of constituents of *SJW* (i.e., flavonols, naphthodianthrones, and phloroglucinols) over a 60 minutes period. Hyperforin derivatives (furohyperforin, oxyhyperforin, hyperforin and adhyperforin) were quantified separately.[71] A similar, validated method was reported earlier, however minor hyperforin analogues could not be identified and quantified.[72] A similar, but shorter method allowed the detection of flavonoids, but individual hypericins and hyperforins were not quantified separately.[73] Flavonoids and phenolic acids could be detected and quantified in one, 52

min-long experiment applying gradient elution and a C18 stationary phase.[23] Ganzera et al reported a 35 min long method for the determination of 9 SJW constituents.[74] A very comprehensive RP-HPLC method was reported for the identification and quantification of 14 phenolic compounds, including hyperforin, hypericins, flavonoids and phenolic acids.[75] The analysis of fingerprint chromatograms beside the quantification of marker compounds is a key to the reliable quality control. A major issue in fingerprint analysis is the separation of overlapping peaks. One potential approach to overcome this difficulty is two-dimensional chromatographic separation of the extracts. The major determinant of the successful analysis is the choice of appropriate stationary phases to maximize the distribution of the analytes in the separation space. Allen et al. studied a set of four chemically different conventional bonded reversed phases was used in the first dimension, the second dimension column was either a conventional bonded C18 phase or a carbon-clad phase (CCP). The best resolution (239 detected peaks at 220 nm) was achieved with a Zorbax Bonus-RP column (2.1 mm × 300 mm, 2.5 µm) as the first, and Poroshell 120 carbon-clad silica (33 mm × 2.1 mm, 2.7 µm). As the second dimension 10mM perchloric acid and acetonitrile were used as eluent using gradient elution.[76] An RP-HPLC method was elaborated for the distinction of SJW samples of European and Chinese origin. In European proveniences rutin, hyperoside and isoquercitrin can be found in higher quantities, and the ratio of pseudohypericin and hypericin is >1 (contrary to Chinese samples).[40]

The chromatographic performance of a poly(ethylene glycol) stationary phase for HPLC was assessed and validated for the analysis of the secondary metabolites (chlorogenic acid, flavonoids, phloroglucinols and naphthodianthrones) in extracts of *H. perforatum*.[77] Monolithic columns have also been applied in the analysis of SJW: the major flavonoids (rutin, hyperoside, isoquercitrin and quercitrin) could be quantified within a 7-mins run.[78] One method based on the application of a monolithic column allows the determination of furohyperforin, hyperforin, adhyperforin, pseudohypericin and hypericin.[20] Monolithic columns were favoured since irreversible adsorption of hypericins to the stationary phase is lower than it was suspected for conventional reversed phase columns,[20] however recently the application of C18 columns is almost exclusive nowadays.

To support human studies with SJW, sensitive analytical methods are needed to determine hypericin and hyperforin in human plasma samples. Biber et al reported two methods that are suitable for the analysis of blood samples for hyperforin content. The first, based on HPLC-UV analysis, was not sensitive enough to be applied for the analysis of clinical samples after administering therapeutic doses. Due to its simplicity and specificity, it could be useful for animal studies in which higher doses are applied. The second method, where HPLC was coupled with MS detection, was proved to be adequate tool for the analysis of clinical samples. The limit of detection of this method was 1 ng/mL which is approximately 2 magnitudes lower than the therapeutic hyperforin plasma level. HPLC-UV

experiments were carried out on a C18, LC-MS on a C8 column.[27] A validated isocratic HPLC-UV method was developed to determine hyperforin in human plasma samples. The limit of detection (LOD) of hyperforin was 4 ng/mL in plasma and the limit of quantitation (LOQ) was 10 ng/mL. The hyperforin content was enriched by solid phase extraction.[29]

Beside the most widely applied HPLC-UV and -DAD detection, several publications describe methods coupled with fluorescence detection. These methods are usually applied for the quantification of hypericin derivatives, due to their advantage that co-eluting peaks that may disturb baseline separation in case of UV detection, are not present in the chromatograms detected with this more specific method. Bauer et al developed a validated RP-HPLC method with limits of quantitation of 0.25 ng/ml for hypericin and pseudohypericin and 10 ng/ml for hyperforin. Hypericin and pseudohypericin were detected fluorimetrically, whereas hyperforin was quantified using an UV-detector. This method was sensitive enough to allow determination of marker compounds of SJW in pharmacokinetic studies.[79] A method based on the use of a C8 column and fluorescence detection with very short analysis time (4 min) was reported for the quantification of hypericin with a detection limit 75 pg.[28] The same group developed a method for the determination of hyperforin, using a mixed C18/CN column and with a 4.5 ng detection limit.[80] A comprehensive study compared DAD, FLD and ELSD detection for the analysis of SJW secondary metabolites (chlorogenic acid, rutin, hyperoside, isoquercitrin, quercitrin, quercetin, amentoflavone, pseudohypericin, hypericin). ELSD is particularly useful for analytes that do not have absorbance or fluorescence chromophores. However, hypericin derivatives are not detectable with this method, contrary to FLD and DAD.[81] Hypericin and pseudohypericin were quantified by fluorescence detection from the herbal matrix with a greater degree of specificity than HPLC-UV and comparable sensitivity to some LC-MS methods, with a limit of quantification of 0.18 ng.[82]

Beside the pharmacologically active constituents, SJW contains a wide variety of other components that may play role in the clinical effect by influencing the bioavailability of pharmacophores. Therefore, determination of flavonoid content is the focus of several methods. A validated HPLC method was developed to quantify biapigenin preparations and to investigate its release characteristics in dissolution tests. Detection was performed at a wavelength of 270 nm using a PDA detector with a limit of detection of 0.05 mg/mL.[83] A HPLC method validation study was performed for simultaneous comparison of three different detector systems (ECD, UV, and FLD) for flavonoid analysis of SJW extracts. Eight flavonoids were chosen as the model compounds to undergo the full validation studies. Although the lowest LOQ (21 ppm) could be achieved with FLD, this method is not generally superior in case of flavonoids: for some compounds it is much less sensitive compared with ECD or UV and some

compounds are undetectable. For flavonoid quantification, UV detection seems to be the most suitable.[84]

Some neutral compounds may influence the physical properties of dry extracts, therefore may be of technical importance. Von Eggelkraut-Gottanka et al analysed eight SJW hydromethanolic dry extracts for their sugar content. Analysis was carried out on a Nucleosil CC 100-3 NH_2 (250 × 4 mm) column (Macherey and Nagel, Düren, Germany). Elution was carried out with acetonitrile - water (75:25), adjusted to pH = 3.5 using phosphoric acid (0.7 mL/min, 40 °C). Sugars were detected using a refractive index detector. A lipophilic SPE cartridge, an anion-exchange SPE cartridge, and two cation-exchange SPE cartridges were necessary for sufficient sample clean-up prior to HPLC analysis. The total sugar contents was calculated from the sum amounts of fructose, glucose, saccharose and lactose.[85]

For the quantification of organic acids, an evaporative light scattering detector was used. Separation was done on an Aminex HPX-87-H strong cation-exchange resin column (300×7.8 mm), the mobile phase was 0.02 M TFA (0.6 mL/min). Citric acid and malic acid were quantified and determined in a concentration of 0.9-2.3% and 2.3-3.1% in the extracts, respectively.[85]

In one experiment, hyperforin was detected using atmospheric pressure chemical ionization and precursor ion *m/z* 537 and fragment ion *m/z* 277 were used for quantitation.[27]

Ultra-performance liquid chromatography (UPLC) offers rapid analysis and better separation compared to classical HPLC. An UPLC method coupled with quadrupole time of flight mass spectrometry (qTOF-MS) was developed to simultaneously quantify and identify 21 metabolites including 4 hyperforins, 3 catechins, 3 naphthodianthrones, 5 flavonoids, 3 fatty acids, and a phenolic acid from *H. perforatum*. Principal component analysis (PCA) was used to define characteristics of different SJW samples and based on this to discriminate between various preparations.[86]

Mass spectrometry and associated hyphenated techniques

One of the first LC-MS experiments with SJW were published in 1998. Constituents of the plant were identified by thermospray-MS in positive and ESI-MS in negative mode. Flavonoids, chlorogenic acid derivatives, hypericins and hyperforins were identified based on their characteristic *m/z* values, UV spectra and retention times.[72] A direct infusion ESI-MS (negative ionisation, scan mode from *m/z* 100 to 700) was developed to obtain, in a short time, a mass fingerprint of constituents present in the extracts. $[M-H]^-$ signals of deprotonated compounds are characteristic to SJW extracts.[87] Characteristic compounds of SJW, hypericin, pseudohypericin, hyperforin and adhyperforin may be

detected and identified in ESI-MS-MS experiments based on their molecular ions and fragmentations (**Table 2**).

In order to establish the fragmentation pathway of hyperforin, ESI-MS-MS experiments were undertaken. In the MS spectrum of the molecule an intense signal of the molecular ion $[M+H]^+$ can be detected at *m/z* 537. In the MS–MS spectrum, signals of several fragments (including the major and characteristic signals at *m/z* 469 and 481) can be recorded, due to the losses of the alkylic chains such as isoprene (-68), isobutene (-56) and dimethylketene (-70).[12] The fragmentation pattern of hyperforin and adhyperforin in case of negative ionisation mode can be characterised by molecular ions ion $[M-H]^-$ at *m/z* 535 and 549, respectively, and losses of *m/z* 69, 138 and 152 fragments correspond to $[M-H-C_5H_9]^-$, $[M-H-C_5H_9-C_5H_9]^-$ and $[M-H-C_5H_9-C_6H_{11}]^-$.[88]

Most of the published LC-MS methods were used for identification of analytes but not for quantification. Only some papers report MS methods for quantification. One paper described a method for the quantification of hyperoside, quercitrin, hyperforin and hypericin.[89] Tolonen's method, based on multiple reaction monitoring, offers lower levels of quantitation for hyperforin 0.5 ng/mL and 2 ng/mL for hypericin.[90] An HPLC-ESI-MS method was developed to simultaneously separate, identify and quantify hyperforin, hypericin, pseudohypericin, rutin, hyperoside, isoquercetrin, quercitrin and chlorogenic acid. The method consisted of two protocols: one for the analysis of flavonoids and glycosides and the other for the analysis of the more lipophilic hypericins and hyperforin. As stationary phase, a phenyl-hexyl column was used which provided relatively short separation times (35 min for flavonoids and glycosides and 9 min for hypericins and hyperforin). Using ESI-MS detection in the negative ionisation mode pseudo-molecular ions were detected for all the compounds, with little or no fragmentation. This method was validated with commercial SJW products[57] A sensitive method HLPC-MS-MS method, applying reversed phase monolithic stationary phase was developed and validated, allowing the determination of hyperforin down to a concentration level of 250 pg/mL from biological samples.[91]

A method was developed for the LC-MS determination of apolar compounds in supercritical extracts (and also suitable for the DAD quantification of hyperforin and adhyperforin). 16 hyperforin derivatives were identified by LC-MS in ESI negative and positive ionisation modes, and DAD determination of hyperforin and adhyperforin was also carried out with LOD and LOQ values of 4.1 µg/mL and 2.3 µg/mL, 13.4 µg/mL and 7.8, µg/mL respectively.[92]

Some methods are dedicated to the analysis of biological samples from human trials. A sensitive LC-MS-MS method for the simultaneous determination of hypericin and hyperforin in human plasma depending was validated with plasma samples. The analytes were detected with tandem mass

spectrometry in the selected reaction monitoring mode using an electrospray ion source. The limit of quantification was 0.05 ng/mL for hypericin and 0.035 ng/mL for hyperforin.[93] A HPLC method coupled with tandem mass spectrometry was developed for the quantitative determination of I3,II8-biapigenin to serve pharmacokinetic studies. The procedure includes solid-phase extraction and separation on an XTerra MS C18.[30] A method based on liquid-phase extraction followed by HPLC-ESI-MS was elaborated and validated for quantification of biflavones (amentoflavone and biapigenin) in human plasma.[94] Both methods have similar sensitivities (LLOQ 1 ng/mL).

Electroanalytical methods

Electroanalytical methods have been developed with the aim of achieving shorter analysis time and more sensitive detection than in case of generally applied HPLC-DAD. Capillary electrophoresis (CE) as an alternative separation technique to HPLC offers fast separation and high sensitivity. CE for separation of hypericine and pseudohypericine was established, separation of the two analytes could be achieved within 2 min, but it is ten times less sensitive compared to HPLC-UV (LOD 10 µg/mL). A buffer system consisting of 100 mM borate (pH = 9.50), 40% 2-butanol and 10% acetonitrile is suitable for baseline separation with high peak symmetry.[95]

The electrochemically active behaviour of hypericins initiated the development of a HPLC method with electrochemical detection (ECD), taking advantage of the high sensitivity of ECD, with the aim of application in pharmacokinetic studies on tissues. The developed method is characterized with a lower limit of detection (LLOD) of 0.3 ng/mL and 0.7 ng/mL for hypericin and pseudohypericin and a lower limit of quantification (LLOQ) of 0.5 ng/mL for hypericin and 1 ng/mL for pseudohypericin.[18]

As part of an HPLC method with amperometric detection, the oxidation of analytes was carried out with a glassy carbon electrode at a potential of +1.1 V vs. an Ag–AgCl–KCl reference electrode. The limit of detection was determined to be 0.01 ng on-column for hypericin. The method was applied to the determination of total hypericin (hypericin, pseudohypericin, protohypericin and protopseudohypericin) in herbal extracts after converting the protoforms into hypericin and pseudohypericin by subjecting the samples to artificial light.[96] In the same setting, the limit of detection for hyperforin was 0.05 ng on column.[97] An improved method with amperometric detection allowed the simultaneous determination of total hypericin (protopseudohypericin, pseudohypericin, protohypericin and hypericin) and hyperforin.[98]

A capillary zone electrophoretic method was established for the determination of rutin from *H. perforatum* extracts. The analysis was performed using a fused-silica capillary, the background

electrolyte consisted of 10% ethanol and 20 mM borate buffer (pH = 8.0). Rutin was detected at 200 nm with a detection limit of 2.7 μM.[99]

Near infrared spectroscopy (NIRS)

NIRS is a quick and non-invasive analytical method which has been used for both qualitative and quantitative analysis of plant material.[100–103] Near infrared includes the spectral range of 800-2500 nm (or 12821-4000 cm^{-1}) and NIRS detects the vibrations mainly of the -OH, -CH, -NH, -SH bonds (Roggo et al).[100] However, because of the difficulty of interpretation of the NIRS spectra, the application of chemometrics is required (including regression methods, classification methods and mathematical pretreatment of the data). In addition, Fourier transmission infrared (FT-IR) imaging has been used[40,103] for the acquisition of morphological information of plant material and for the determination of the distribution of chemical entities within the plant.

Rager et al developed a quantitative NIRS method for the analysis of hyperforin and 13,118-biapigenin.[104] No sample preparation was conducted and 35 SJW dry extracts were directly analysed. 3 spectra were taken from each sample in the spectral range of 1100-2498 nm, with 700 data points per spectrum and the data obtained were pre-treated and subjected to analytical regression statistics. Among other calibration models, Root Mean Square Error of Prediction (RMSEP) was used for calibration and validation. HPLC was selected as the reference method. For hyperforin the RMSEP was found 0.22% for a concentration range of 1.0-6.0% while the respective figure for biapigenin was found 0.024% for a concentration range of 0.2-0.55%. They showed that NIRS is a sufficient and fast quality control tool that could be used in the quantification of chemical entities in plant material and could replace traditional techniques, although its accuracy could be questioned in the analysis of low concentration molecules.

Another research group[95] established a NIRS method for the quantification of hypericin and hyperforin in SJW dry extracts. The researchers also aimed to compare NIRS to Liquid Chromatography (LC) and Capillary Electrophoresis (CE) for quality control of SJW. In this case again, reverse-phase LC was used as a reference method for calibration of NIRS. 320 spectra were acquired from 80 SJW dry extracts over the spectral range of 4500-10000 cm^{-1} in transflection mode. The samples were thermo stated at 23 °C (heat increased the reflectance), the number of scans used was 10 and the optical thickness was 1 mm. Chemometrics applied included mathematical pretreatment, partial-least square regression (PLSR) and statistical analysis with PCA. They concluded that NIRS is an effective method which provides robust results in the quantification of hypericin and hyperforin. However, they claimed that for lower concentration molecules, the traditional techniques (LC) are preferred.

Huck-Pezzei et al used FT-IR imaging, light microscopy and multivariate image analysis to obtain morphological and compositional information about SJW plant tissue. Spectra were acquired in MIR transmission over a wavelength range of 4000-750 cm^{-1} and with a resolution of 4 cm^{-1}. They assigned wavelengths to certain ingredients and identified the presence of those ingredients in certain plant tissue. In particular 1084 cm^{-1} was assigned to nucleic acids (present mainly in epidermis and sclerenchyma), 1515 cm^{-1} to lignin (present in xylem and protoxylem) and 2956 cm^{-1} to lipids, nucleic acids, proteins and carbohydrates (present in epidermis and sclerenchyma). It was shown that FT-IR imaging is suitable for semi-quantitative analysis of ingredients in SJW plant tissue and that clustering techniques increase the amount of information obtained from the IR images.[103]

The same research group used NIRS and FT-IR imaging methods alongside with traditional analytical techniques (TLC, MS) for quantification of chemical entities in SJW, for quality control of the plant and for identification of the distribution of certain constituents within the plant.[40] Attenuated-total-reflectance mid infrared (ATR-MIR) and NIR spectra were acquired from 32 SJW samples which were log1/R treated and normalized. PLSR calibration method was used for NIR and ATR-MIR for the compounds rutoside, hypericin, hyperforin and hyperoside. As in previous research, HPLC was the reference method. NIRS was found as a suitable method for the quantification of chemical entities in plant material. FT-IR imaging data were acquired as in their previous research. 3 clustering techniques were coupled to the method; HCA (hierarchical cluster analysis), KMC (k- means clustering) and FCM (fuzzy C-means). Spectra of several plant tissues were obtained (xylem, protoxylem, phloem, sclerenchyma, epidermis) and the distribution of certain ingredients (nucleic acids, lipids, proteins) could be detected semi-quantitatively in them. However, while the discrimination of European and Chinese *Hypericum* was possible via NIRS coupled to PCA, it was not possible through FT-IT imaging.

IR spectroscopy has also been used for the examination of differences between *Hypericum* species.[105] Overall, sixty samples of six *Hypericum* species *(H. perforatum, H. hirsutum, H. montanum, H. dubium, H. maculatum, H. tetrapetrum)* were analysed with four IR spectroscopy modes in order to identify the best mode for species determination. It was found that the KBr transmission mode provided the best results as there were correct species classification by 97%. Spectra were obtained in the spectral range of 450-4000 cm^{-1} with a resolution of 1 cm^{-1}. The research team concluded that IR is a valuable tool for plant species determination but the selection of the best mode should be based on morphological characteristics of the plant material.

Zhu et al. (in Chinese) investigated 20 Chinese *Hypericum* species including HP, and were able to distinguish the species from these taxa, but did not include some of the more common species found outside of China. *H. japonicum* (seen as a possible adulterant) was well separated.[106] Nichita et al used

spectroscopic (UV-VIS-NIR, FT-IR), chemical (chemiluminescence) and chromatographic techniques (TLC) and managed to identify the presence of flavonoid compounds in *H. perforatum*. For the spectroscopic analysis they used UV-VIS-NIR spectroscopy at the wavelength range of 190-2300 nm and FT-IR spectroscopy at the 4000-400 cm^{-1}.[102]

Overall, NIRS coupled to chemometrics has been implemented successfully in the quality control of plant material, quantitative analysis of chemical entities and could even replace the traditional methods which are time consuming and involve complex samples preparation and waste of big amounts of solvents. However, a chromatographic technique (HPLC) is required for cross validation and calibration of the NIRS method. Also, for the analysis of molecules which occur in low concentrations, traditional analytical techniques could be more accurate NMR metabolomics

NMR metabolomics, as other types of metabolomics, comprise preparation and extraction of the samples, identification of the components and interpretation of the spectra through multivariate analysis. NMR based metabolomics have been extensively used for metabolic fingerprinting of plants and organisms since the technique provides an integrated outlook on the majority of the constituents rather than on a single constituent. Many studies focused on the identification of *H. perforatum* constituents both of the crude plant and of commercial products.

Bilia et al applied ^{1}H-NMR, COSY, TOCSY and HMQC spectroscopy on one SJW extract sample with the aim to identify and assess the metabolites present in it. The sample was dissolved in deuterated DMSO and the spectra obtained revealed signals in 4 main regions (a. 9.0-6.0 ppm, b. 5.5-4.5 ppm, c. 4.5-3.0 ppm and d. 2.7-0.7 ppm) which were assigned to flavonols, phloroglucinols, napthodianthrones, polyphenols, chlorogenic acid, lipids and sucrose. This research team identified the whole range of SJW constituents, including hypericins. For verification of results, HPLC was also conducted.[15]

Rasmussen et al obtained both full resolution and integrated ^{1}H-NMR data from 10 commercial SJW preparations and introduced them to Principle Component Analysis (PCA) in order to examine the compositional differences between the preparations. The samples were dissolved in both deuterated methanol and deuterated DMSO and the full spectrum (0-20 ppm) was examined using 128 scans. They concluded that the clustering of products in PCA was caused due to differences in concentration of certain metabolites (quercetin, hyperoside, rutin, fatty acids and quercetin) but not due to hypericins or hyperforins. The PCA did not discriminate between capsules and tablets while the integrated and full resolution NMR data were in agreement.[107]

Porzel et al used both MS and NMR based metabolomics coupled to PCA and HCA (hierarchical cluster analysis) in order to investigate the differences in the metabolome of seven *Hypericum* species, including *H. perforatum*. The clustering of the species occurred mainly due to differences in

hyperforins, shikimic acid, lipids and chlorogenic acid content while hypericins could not be detected (also noticed by Rasmussen et al). The NMR the samples were dissolved in deuterated methanol and were subjected to NMR 600 MHz using 160 transients. The HCA showed that *H. polyphyllum*, *H. tetrapetrum* and *H. perforatum* grouped, indicating that the two first species could substitute SJW.[108]

Two research teams used NMR as a part of many hyphenated techniques in an attempt to study the metabolome of *H. perforatum*.[88,109] Tatsis et al used LC/DAD/SPE/NMR and LC/UV/MS in order to separate the principle components in Greek SJW and to elucidate their structure. Liquid chromatography (LC) was used for separation of constituents, Solid Phase Extraction (SPE) was used to capture the eluent components and NMR (as well as MS) for their structure elucidation. For the spectra acquisition a spectrometer of 400 MHz was used, the samples were dissolved in deuterated acetonitrile and ^{1}H-NMR, COSY and TOCSY spectra were obtained. The constituents that were separated and elucidated were phloroglucinols, naphthodianthrones, flavonoids (including astilbin and miquelianin) and phenolic acids while two novel phloroglucinols (hyperfirin and adhyperfirin) were identified. Similarly, Schmidt et al used NMR as a part of hyphenated techniques but on 24 commercial products, unlike Tatsis et al who used it on crude material. HPLC-PDA was used for compound separation, SPE for eluent capturing while NMR and MS coupled to the PCA-type method PARAFAC (parallel factor analysis) were used to elucidate the structures of 12 constituents and investigate the metabolomic diversity between the products based on those constituents. ^{1}H-NMR, COSY, HSQC and HMBC experiments were conducted at a 600 MHz spectrometer using 128-512 transients.

Overall, NMR metabolomics have been successfully used to identify the metabolome of SJW and to cluster crude materials and commercial finished products, based on the presence and the concentration of certain metabolites (when coupled to PCA).

DNA barcoding

Based on morphological characteristics Robson's theory claims that *H. perforatum* L. could be a hybrid of *H. attenuatum* Fisch. ex Choisy and *H. maculatum* Crantz.[110] This theory is also supported by the fact that both *H. attenuatum* and *H. maculatum* have 16 chromosomes (2n=16) while *H. perforatum* contains 32 chromosomes (2n=32).[111,112] Alternatively, based on DNA differences with *H. attenuatum*,[113,114] suggest *H. perforatum* may have originated from *H. maculatum* alone The only way to shed light to the *H. perforatum* origin is DNA techniques**.**

While chemical techniques, like HPTLC/TLC and NMR, provide an overview of the phytochemistry of plant material they cannot always be accurate in the identification of plant species and subspecies[115] as the chemistry of a plant is susceptible to many factors. Plant DNA however, does not depend on the

plant's habitat, age or even tissue damage so it could be useful and give accurate results in plant identification. DNA barcoding is the use of a small and certain DNA sequence in the plants' genome as a distinctive area for plant species identification[116] and it seems to contribute significantly in this area in the last decade.

According to Howard et al the nuclear ribosomal Internal transcribed spacer (ITS1 and ITS2) DNA region is the most suitable ''barcode'' for primer creation (through PCR), plant species distinguishing and detection of adulterants.[117] Many DNA barcoding studies on *Hypericum* species identification have been conducted including those of Crockett et al,[115] Park and Kim,[118] Howard et al,[116] Pilepic et al[119] and Costa et al .[120]

Crockett et al used PCR-based ITS sequence analysis to discriminate *H. perforatum* from 50 *Hypericum* taxa native to Europe, Asia and America. Both ITS 1 and ITS 2 regions were sequenced, introduced to PCR and compared for all the 50 taxa and *H. perforatum* was successfully discriminated from the rest of the species. The authors proposed this technique for the authentication of SJW in commercial products (species level) but they concluded that the technique was not sufficient on subspecies level. The technique was also useful for evaluation of phylogenetic affinities within the genus.[115]

Park and Kim used the same technique (nr ITS) on 36 *Hypericum* species from Korea and Japan to study their phylogeny. After DNA isolation, PCR and sequencing it was found that *Hypericum* section is a polyphyletic cluster while sections Trigynobrathys, Roscyna and Sampsonia are monophyletic. They also found that *Hypericum* species from these countries reside in Trigynobrathys and Hypericum sections.[118]

Howard et al used the ITS 1 region of eight *Hypericum* species to create PCR primers which are specific to *H. perforatum*. Those primers were tested with SJW voucher samples and with other eight *Hypericum* species samples and it was found that only the DNA of *H. perforatum* and *H. delphicum* Boiss. & Heldr was amplified. *H. perforatum* and *H. delphicum* sequences were found similar by 90% while *H. athoum* Boiss. & Orph and *H. maculatum* Crantz showed a considerable sequence similarity with *H. perforatum* as well. The technique was also used to identify *H. perforatum* among commercial *Hypericum* ornamental plants and among supplement marketed as SJW.[116]

Pipelić et al used ITS sequencing to examine the phylogenetic links between 34 *Hypericum* species. Their findings that *H. perforatum* has a distance from *H. maculatum* and *H. attenuatum* in the parsimony analysis opposed Robson's theory about *H. perforatum* hybrid nature.[119]

Costa et al used ITS1 and matK region sequencing coupled to High Resolution Melting (HRM) analysis to distinguish *H. perforatum* from *H. androsaemum* in infusions. The technique successfully identified

and discriminated the two species and could be generally used in the authentication of plant species. From the two DNA regions, matK was better suitable for identifying the two species while ITS1 exhibited intra-species diversity which was problematic for HRM analysis.[120]

is Amplified Fragment Length Polymorphism (AFLP) was also used for the authentication of *Hypericum* species[121,122] Percifield et al used AFLP analysis to describe the genetic variability among 56 *Hypericum* samples from three continents of which 42 were *H. perforatum* samples both wildly collected and cultivated. 298 markers were generated from the samples (after DNA extraction, AFLP analysis and amplifications) of which 17 are found in all *Hypericum* samples examined, while 2 markers were found exclusively in *H. perforatum samples.* Therefore, the technique could be used in the authentication of plant material.[121] Aziz et al applied AFLP to 11 species and subspecies of *Hypericum* to obtain their DNA fingerprints and also applied HPLC to obtain their phytochemical fingerprints. The genetic and chemical profiles were correlated for an integrated perception of each species identity and it was concluded that AFLP is able to differentiate closely genetically related *Hypericum* species.[122]

Finally, the transcriptome of *H. perforatum* was *de novo* sequenced to detect specific genes responsible for certain activities.[123,124]

He et al used the technique (coupled to de novo software) to detect genes in *H. perforatum* responsible for the biosynthesis of hypericin (12 unigenes), hyperforin (91 unigenes) and melatonin (66 unigenes). Overall, 59.184 unigenes were acquired of which the 68.86% were interpreted and annotated.[123]

Galla et al used the technique to detect genes in *H. perforatum* flower responsible for the plant reproduction. The research team managed to detect and annotate 36.988 transcripts present either in female or in male reproductive organs.[124]

Overall, these genetic approaches have been shown to be useful in separating species, but so far this has not been translated into routine quality control protocols. Of course, DNA barcoding will only allow the verification of the correct species, but cannot help with the quality assessment of the drug material as such (e.g. in terms of other contaminants like dyes) or the use of the wrong plant part.

Conclusion

A huge number of technically very diverse techniques have been developed for the analytical characterization of *Hypericum perforatum*. *Hypericum*-based products usually contain hydroalcoholic extracts of St. John's wort. Considering the physicochemical characteristics of secondary metabolites of this plant, extracts used for medicinal purposes can be characterised by their phloroglucinol, naphthodianthrone and flavonoid content. However, considering that oily extracts and essential oils

are also utilized, it is not possible to identify a single technique suitable for all applications. Depending on the demands and regulations, quality control may be based on simple and quick techniques (TLC, HPTLC), allowing the detection of key marker compounds, or on the very precise instrumental identification and quantitative measurement of minor constituents (LC-MS). HPLC-DAD/UV is the cornerstone of routine analysis, since the most widely quantified marker compounds (phloroglucinols, naphthodianthrones and flavonoids) can be reliably detected at different wavelengths. UPLC-DAD may allow quick analysis and therefore may be a useful tool in routine quality control. As a rational compromise, robust but not very selective methods (UV, NIRS) are often applied in routine analysis. For industrial analysis HPTLC- and HPLC-based techniques seem to be the most suitable ones and despite of high hopes, DNA-barcoding is not yet at a stage where it can be accepted for use in a regulated context of quality control. Considering the fact that sample preparation has major impact on the composition of the extracts, validation of analytical methods should focus on this issue.

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Table 1. HPLC methods for the analysis of SJW extracts

Analytes (in the order of detection)	Column	Eluent	Detection	Run time, flow, temperature	Reference
Pseudohyperidin, hypericin	ODS (150 x 4.6 mm)	MeOH – NaH_2PO_4 buffer at 15.6 g/L (pH = 4.4) − EtOAc 160:41:39	590 nm	1 mL/min	[4]
Protopseudohypericin, pseudohypericin, protohypericin, hypericin	Lichrospher RP18 (250 × 4.6 mm, 5 µm), Lichrospher RP18e (250 × 4.6 mm, 5 µm), Select B (250 × 4.6 mm 5 µm), Merck.	MeOH – acetate buffer (pH = 4.4) – EtOAc 66:18:16	590 nm	0.7 mL/min	[60]
Pseudohypericin, hypericin	Discovery HS PEG (150 x 4.6 mm, 5 µm, Supelco)	MeOH – THF – 75 mM phosphate buffer (pH = 2.8) 45:25:30	amperometrical detection +0.93 V, 35 °C	0.4 mL/min, 35 °C	[18]
Chlorogenic acid, rutin, hyperoside, isoquercitrin, quercitrin, quercetin, I3, II18 biapigenin, pseudohypericin, hypericin, hyperforin, adhyperforin	Nova-Pack RP-18 column (150 x 3.9 mm; 4 µm; Waters)	H_2O + H_3PO_4, pH 4.0 (A), MeCN (B), MeOH (C) 0 min: 100% A, 10min: 85% A, 15% B; 30min: 70% A, 20% B; 40min: 10% A, 75% B; 55min: 5% A, 80% B	270 nm, 590 nm	65 min, 1 mL/min, room temperature	[19]
Hypericin, pseudohypericin	Hypersil C18 (100 x 4.6 mm; 3.0 µm; Phenomenex)	0.1 M triethylammonium acetate – MeCN 33:67	588 nm	1 mL/min	[64]
Pseudohypericin, hyperforin, hypericin	C18	MeCN – 0.3% H_3PO_4 9:1	273 nm, FLD 315/590 nm (ex/em)	10 min, 1.5 mL/min	[67]
Pseudohypericin, hyperforin, hypericin	Hypersil C18 (ThermoFinnigan)	MeOH - phopshate buffer (pH = 2.2) 95:5	276 nm, FLD 322/593 nm (ex/em)	6 min, 1 mL/min	[32]
Pseudohypericin, hyperforin,	Hypersil C18 (100 × 4.60	MeCN – 0.1 M triethylammoni	284 nm, 490/570 nm (ex/em)	1 mL/min	[68]

adhyperforn, hypericin	mm, 3 µm, Phenomenex)	um acetate 8:2 (pH = 7.0)			
Pseudohypericin, hypericin	Ultrasphere ODS (250 x 4.6 mm, 5 µm, Beckman Instruments)	A: MeOH–MeCN 5:4, B: triethylammonium acetate 0 min: 70% A; 2 min: 70% A, 10 min: 90% A, 14 min: 90% A, 16 min: 100% A, 21 min: 100% A, 22 min: 70% A, 26 min: 70% A	fluorescence detection 236/592 nm,(ex/em)	1 mL/min	[82]
Rutin, hyperoside, quercitrin, quercetin, biapigenin, protopseudohypericin, pseudohypericin, protohypericin, hypericin	Separon SGX C 18 (4 x 150 mm, 7 µm, Tessek)	MeCN – H_2O - H_3PO_4 19:80:1 (A), MeCN (B) 0 min: 5% B, 3 min: 5% B10 min: 55% B, 20 min: 100% B, 25 min: 5% B	590 nm	30 min 0.5 mL/min, room temperature	[63]
Rutin, quercetin-3-*O*-glucoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-rhamnoside, quercetin, pseudohypericin, adhyperforin, hyperforin, hypericin	Symmetry C18 (250×4.6 mm)	30 mM NaH_2PO_4 (pH = 3) (A), MeCN (B) 0 min: 10% B; 40 min: 40%; 50 min: 90 70 min: 90% B	280 and 590 nm	1.8 mL/min	[21]
Rutin, isoquercitrin, quercitrin, quercetin, hypericin	S5 ODS2 column (250 × 4.6 mm, 5 µm, Phase Separations)	0.5% TFA (A), MeOH – MeCN – TFA 60:39.5:0.5 (B) 0 min: 10% B; 15 min: 22% B; 44 min: 38% B; 49 min: 100% B; 54 min: 100% B; 63 min: 64% B; 69 min: 10% B; 86 min: 10% B	284 nm, 580 nm for hypericin	1 mL/min	[22]
Rutin, hyperoside, isoquericitrin, quercitrin,	LiChrospher RP-C18	0.5% TFA (A), 0.5% TFA in	284 nm, 590 nm for hypericins	0.6 mL/min	[125]

quercetin, I3,II8-biapigenin, pseudohypericin, hypericin, hyperforin, adhyperforin	column (250 x 4 mm, 3 µm)	MeCN – MeOH 7: 13 (B) 0 min: 10% B; 20 min: 50% B; 40 min: 60% B;50 min: 100% B; 60 min: 100% B; 70 min: 10% B; 95 min: 10% B			
Rutin, hyperoside, isoquercitrin, quercitrin, quercetin, pseudohypericin, hyperforin, hypericin	YMC ODS-AQE RP-18 (250 x 4.6 mm, 5 µm, Waters)	H_2O – MeOH – TFA 79.5:20:0.5 (A), MeCN – MeOH – TFA 89.5:10:0.5 0 min: 10% B; 20 min: 70% B;25 min: 90% B; 30 min: 100% B; 60 min: 1000% B; 65 min: 10% B	270 nm, 590 nm for hypericin and pesudohypericin	1 mL/min	[126]
Hyperoside, quercitrin, hyperforin, hypericin	Luna C18 100 A (150 x 2 mm, 3 µm, Phenomenex)	10 mM NH_4OAc, pH adjusted to pH = 5 with CH_3COOH (A), MeCN – MeOH 9:1 (B) 0 min: 13 B; 10 min: 17% B, 35 min: 90% B, 40 min: 100% min	ESI negative ionisation mode	0.25 mL/min, 24 °C	[89]
Hypericin, pseudohypericin	LiChrospher RP Select B (250 x 4.6 mm, 5 µm, VDS Optilab)	H_2O (+ H_3PO_4, pH adjusted to 4.0 with NaOH) – MeOH – THF 3:4.5:2.5	fluorescence detection 315/590 nm (ex/em)	0.75 mL/min, 60 °C	[79]
Hyperforin	Luna C18(2) (250 x 4.6 mm, 5 µm, Phenomenex)	MeCN – 0.01 M Na_2HPO_4 buffer (pH =2.4) 9:1	273 nm	1.5 mL/min, 50 °C	[79]
Rutin, hyperoside, isoquercitrin, quercitrin, quercetin, biapigenin, pseudohypericin, hypericin, furohyperforin, oxyhyperforin,	Protein C4 (250 x 0.5 mm, 5 µm 0.5, Vydac Separation Group)	H_2O + 85% H_3PO_4 99.7:0.3) (A), MeCN (B), MeOH (C) 0 min: 100% A; 10 min: 85% A, 15% C; 30 min: 70% A, 20% C; 40 min: 25% A,	230, 254, 270, 350, and 590 nm	60 min, 1 mL/min, 26 °C	[71]

hyperforin, adhyperforin		65% C; 55 min 20% A, 70% C; 57 min: 5% A, 80% C; 60 min: 100% A			
Rutin, hyperoside, isoquercitrin, quercitrin, quercetin, total hypericins, total hyperforins	Protein C4 (250 x 0.5 mm, 5 µm 0.5, Vydac Separation Group)	MeCN (A), MeOH (B), H_2O (pH 3.2, H_3PO_4) (C) 0 min: 15% A, 85% C; 12 min: 15% A, 80% C; 20 min: 75% A, 10% C; 27 min: 80% A, 5% C; 30 min: 15% A, 85% C	230, 254, 270, 350 and 590 nm	30 min, 1 mL/min	[73]
Chlorogenic acid, caffeic acid, hyperoside, rutin, quercitrin, quercetin	Luna C-18(2) (250 x 4.6 mm, Phenomenex)	25 mM phosphate buffer, pH = 2.5 (A), MeOH (B) 0min: 5% B; 35 min: 10% B; 50 min: 80% B; 52 min: 100% B	280 and 350 nm	52 min, 1 mL/min, room temperature	[23]
Chlorogenic acid, rutin, hyperoside, isoquercitrin, quercitrin, quercetin, amentoflavone, pseudohypericin, hypericin	Luna C-18(2) (250 x 4.6 mm, Phenomenex)	1% triethylamine in (pH = 4.5 CH_3COOH) (A), MeCN (B) 0 min: 5% B; 40 min: 55% B; 60 min: 100% B; 80 min: 100% B	340 and 590 nm FLD: 470/590 (ex/em) evaporative light scattering detection (ELSD)	1 mL/min	[81]
Rutin, hyperoside, isoquercitrin, quercitrin	Chromolith TM Performance C18 (100 x 4.6, Merck)	H_2O (pH 2.5) – MeCN 85:15	270 nm	7 min, 2 mL/min	[78]
Furohyperforin, hyperforin, adhyperforin, pseudohypericin and hypericin	Onyx Monolithic C18 (50 × 2.0 mm, Phenomenex)	MeCN (A), 0.1% HCOOH in H_2O (B) 0 min: 0% B; 10 min: 20% B; 20 min: 0% B; 20 min: 20% B; 25 min: 20% B	190-280 nm	0.6 mL/min, 40 °C	[20]
Rutin, hyperoside, isoquercitrin, quercitrin, quercetin, I3,II8-	Synergi MAX-RP 80 A (150 x 4.6 nm, 4 µm, Phenomenex)	A: 10 mM NH_4OAc pH = 5.0, B: MeCN – MeOH 9:1	270 nm	1 mL/min, 40 °C	[74]

biapigenin, pseudohypericin, hypericin, hyperforin		0 min: 13% B; 10 min: 17% B; 35 min: 100 B			
Chlorogenic acid, rutin, hyperoside, isoquercitrin, quercitrin, quercetin, biapigenin, pseudohypericin, hypericin, hyperforin, adhyperforin	201 TP 54 RP-18 (250 x 4.6 mm, 5 µm, Vydac Separation Group)	H_2O + 85% H_3PO_4 99.7:0.3) (A), MeCN (B), MeOH (C) 0 min: 100% A; 10 min: 85% A, 15% C; 30 min: 70% A, 20% C; 40 min: 10% A, 15% C; 55 min 5% A, 15% C; 56 min: 100% A; 65 min: 100% A	270 nm	65 min 1 mL/min	[72]
Chlorogenic acid, rutin, hyperoside, isoquercitrin, quercitrin, quercetin, hyperforin, amentoflavone, hypericin	Discovery HS PEG (150 × 4.6 mm, 5 µm, Supelco)	0.1 M CH_3COOH (pH 2.8) (A), MeOH-MeCN 5:4 (B) 0 min: 10% B; 18 min: 30% B; 25 min: 90% B; 40 min: 90% B	270 and 590 nm	1 mL/min	[77]
Rutin, isoquercitrin, luteolin-4-*O*-glucoside, quercetin-4-*O*-glucoside, quercetin, naringenin, luteolin, apigenin	Hichrom 5 C18 (300 x 7.75 mm, 5 µm)	MeOH - 0.01 M H_3PO_4 1:1	UV: 230 nm FLD: 450/250 nm (ex/em) Chronoamperometry	80 min, 1 mL/min, 20 °C	[84]
Rutin, hyperoside, isoquercitrin, quercetin, biapigenin	Hypersil BDS (250 x 4 mm, 5 µm, Thermo Scientific)	880.0 g H_2O + 80.0 g MeCN + 2mL 85% H_3PO_4 (pH = 2.80 with triethylamine) (A), 50.0 g H_2O + 275.25 g MeCN + 85.04 g MeOH + 1 mL 85% H_3PO_4 (pH = 6.10 with triethylamine) 0 min: 0% B; 10 min: 60% B; 45	200-600 nm	1 mL/min, 30 °C	[40]

		min: 60% B; 53 min: 100% B			
Biapigenin	LiChroCart 125-4, RP-18 (5 µm)	5% CH_3COOH (A), MeCN – MeOH 3:1 (B) 0 min: 30% B; 7 min: 100% B; 10 min: 100% B	270 nm	1 mL/min	[83]
Neochlorogenic acid, protocatechuic acid, coumaroylquinic acid, cryptochlorogenic acid, rutin, hyperoside, isoquercitrin, miquelianin, astilbin, guaijaverin, quercitrin, quercetin galactoside, quercetin, biapigenin, pseudohypericin, hyeforin, hypericin	Discovery C-18 (250 x 4.6 mm, 5 µm, Supelco)	H_2O – THF – TFA 97:2:1 (A), MeCN – THF – TFA 97:2:1 (B) 0 min: 0% B, 0.5 mL/min; 7 min: 10% B, 0.5 mL/min; 15 min: 15% B, 0.5 mL/min),28 min: 20% B, 0.5 mL/min; 35 min: 50% B, 0.55 mL/min; 40 min: 65% B, 0.6 mL/min; 45 min: 75%, 0.6 mL/min, 50 min: 100% B, 0.7 mL/min; 75 min: 100% B, 0.7 mL/min	284 nm		[75]
Hypericin	Capital C8 (150 x 4.6 mm, 5 µm)	0.03 M KH_2PO_4 pH = 7.0 – MeOH 3:7	fluorescence detection 315/519 nm (ex/em)	1 mL/min, 60 °C	[28]
Hypericin, pseudohypericin	Wakosil-II 5C18 (150 x 4.6 mm, Wako)	0.1% $(NH_4)_3PO_4$ (pH = 7.0)/MeCN 7:3 (A), MeCN/H_2O 7:3 0 min: 0% B; 10 min: 100% B; 15 min: 0% B; 20 min: 0% B	FLD 470/600 nm (ex/em)	1.2 mL/min	[25]
Hypericin	Diazem-phenyl (250 x 4.6 mm, 5 µm, Metachem)	MeCN – MeOH – H_2O – H_3PO_4 48:40:10:2	590 nm	15 min, 1 mL/min, 30 °C	[62]
Hypericin, hyperforin	LiChroCart 125-4 Purospher RP18	0.5 M NH_4OAc /CH_3COOH buffer (pH =	electrochemical detection	0.8 mL/min, 22 °C	[96,97]

	endcapped (5 µm)	3.7) – MeOH – MeCN 1:4:5			
Hyperforin	Capital C18 /CN (150 x 4.6 mm, 5 µm)	MeCN - H_2O pH = 4.5 (with 1 M H_3PO_4)	272 nm	1.2 mL/min,	[80]
Hyperforin	Hypersil H5ODS-150A (150 x 4.6 mm, Hichrom)	H_2O containing 0.1% TFA - MeCN containing 0.1% TFA 25:75	270 nm	1.5 mL/min	[127]
Hyperforin	Kromasil 100 C18 (150 x 4.6 mm, 3.5 µm, Teknokroma)	H_20 containing 0.2% TFA - MeOH containing 0.2% TFA 0 min: 90% B; 30 min: 100% B; 35 min: 100% B; 36 min: 90% B; 45 min: 90% B	270 nm	1.0 mL/min, 25 °C	[66]
Hyperforin	Luna C18 (150 x 4.6 mm, 3 µm, Phenomenex)	MeOH/MeCN (3:2) – H_2O 92:8, pH = 3.2, with HCOOH and triethylamine	287 nm	14 min, 1 mL/min	[29]
Hyperforin, adhyperforin	Luna C18 (150 × 4.6 mm, 3 µm, Phenomenex)	MeOH/MeCN (3:2) – H_2O (containing 0.1% HCOOH) 92:8	270 nm	20 min, 1 mL/min	[65]
Hyperforin adhyperforin	Zorbax SB-C18(4.6 × 150 mm, 3.5 µm, Agilent)	0.01% TFA (A), 0.0% TFA in MeCN (B) 0 min: 90% B; 10 min: 90% B; 15 min: 98% B; 20 min: 98% B	272 nm	1.0 mL/min, 23 °C	[128]
Protopseudohypericin, pseudohypericin, protohypericin, hypericin, hyperforin, adhyperforin	Nucleosil-100 RP18 (125 x 4.6 10 µm, Macherey Nagel)	MeCN (A), H_2O/85% H_3PO_4 99.7:0.3 (B), MeOH (C) 0 min: 60% B, 10% C; 5 min: 20% B, 25% C; 7 min: 5% B, 25% C, 10 min: 5% B, 25% C; 16 min: 60% B,	270 nm, 590 nm	1.2 mL/min, 40°C	[14]

		10% C; 17 min: 60% B, 10% C			
Hypericin, protohypericin, pseudohypericin, protopseudohypericin, hyperforin and adhyperforin	Waters XTerra RP18 (50 × 2.1 mm, 3.5 µm)	5 nM NH_4OAc (A), MeCN (B) 0 min: 50% B; 5 min: 100% B; 8 min: 100 B; 12 min: 50% B	270 nm, 590 nm	0.5 mL/min, 44 °C	[69]
Chlorogenic acid, rutin, hyperoside, isoquercitrin, quercitrin, quercetin,	Luna phenyl hexyl (150 x 4.6 mm, 5 µm	MeCN (A), H_2O with 0.5% HCOOH (B) 0 min: 16% A; 27min: 16% A; 30min: 32% A; 35min: 32% A	selective ion monitoring, ESI negative mode	1 mL/min, 30 °C	[57]
Hyperforin, pseudohypericin, hypericin	Luna phenyl hexyl (150 x 4.6 mm, 5 µm	MeCN – H_2O – HCOOH – MeOH 70:4.95:0.05:25	selective ion monitoring, ESI negative mode	15 min, 1.2 mL/min, 30 °C	[57]
I3,II8-Biapigenin	XTerra MS C8 column (150 x 2.1 mm, 3.5 µm)	MeCN – H_2O containing 10 mM NH_4OAc buffer pH = 5 35:65	MS–MS, ESI negative mode	0.2 mL/min, 30 °C	[30]
Amentoflavone, I3,II8-biapigenin	XTerra MS C18 column (150 x 2.1 mm, 3.5 mm)	MeCN – H_2O containing 5.0 mM $HCOONH_4$ (pH = 3.0 with HCOOH) 1:1	ESI negative mode	10 min, 0.2 mL/min	[94]
Hyperforin	Chromolith PerformanceRod (100 x 4.6 mm, Merck)	MeCN - H_2O (88:12 containing 3.5 mM HCOOH and 2 mM $HCOONH_4$	MS-MS, ESI negative mode	3 mL/min	[91]
Hypericin, hyperforin	Waters Xterra RP18 (50 x 2.1 mm, 3.5 µm)	20 mM NH_4OAc (A), MeCN (B) 0 min: 50% B; 5 min: 100% B; 9 min: 100% B; 14 min: 50% B	ESI negative ionisation	35 °C	[90]

Table 2. ESI-MS-MS data for hyperforins and hypericins [87,88]

Compound	**Negative ions (m/z)**		**Positive ions (m/z)**	
	$[M-H]^-$	Fragments	$[M+Na]^+$	Fragments
Hypericin	503	-	-	-
Pseudohypericin	519	-	-	-
Hyperforin	535	466/397/383/313	559	277
Adhyperforin	549	480/411/397/313	573	291