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Metabolome Classification of Commercial *Hypericum perforatum* (St. John's Wort) Preparations via UPLC-qTOF-MS and Chemometrics

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Key words
- *Hypericum perforatum* L.
- Hypericaceae
- UPLC-MS
- hyperforin
- principal component analysis
- quality control
- St. John's wort

Abstract

The growing interest in the efficacy of phytomedicines and herbal supplements but also the increase in legal requirements for safety and reliable contents of active principles drive the development of analytical methods for the quality control of complex, multicomponent mixtures as found in plant extracts of value for the pharmaceutical industry. Here, we describe an ultra-performance liquid chromatography method (UPLC) coupled with quadrupole time of flight mass spectrometry (qTOF-MS) measurements for the large scale analysis of *H. perforatum* plant material and its commercial preparations. Under optimized conditions, we were able to simultaneously quantify and identify 21 metabolites including 4 hyperforins, 3 catechins, 3 naphthodianthrones, 5 flavonoids, 3 fatty acids, and a phenolic acid. Principal component analysis (PCA) was used to ensure good analytical rigorousness and define both similarities and differences among *Hypericum* samples. A selection of batches from 9 commercially available *H. perforatum* products available on the German and Egyptian markets showed variable quality, particularly in hyperforins and fatty acid content. PCA analysis was able to discriminate between various preparations according to their global composition, including differentiation between various batches from the same supplier. To the best of our knowledge, this study provides the first approach utilizing UPLC-MS-based metabolic fingerprinting to reveal secondary metabolite compositional differences in *Hypericum* extract.

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Introduction

The scientific discipline of metabolomics is being established to help us gain a broader insight into the biochemical composition of living organisms at a given state. With the recent developments in plant metabolomics techniques [1–3], it is now possible to detect several hundred metabolites simultaneously and to compare samples reliably for differences and similarities in a semiautomated and essentially, untargeted manner. Metabolomics makes use mostly of hyphenated techniques which rely on chromatographic separation of metabolites using either gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS) to analyze complex mixtures of extracted metabolites. One particularly well accepted platform for untargeted metabolite profiling in plant extracts is UPLC-MS using electrospray ionization (UPLC-ESI-MS). Compared with conventional (LC), ultra-performance liquid chromatography (UPLC) achieves rapid metabolite analysis and can obtain better peak separation than usually possible with standard LC methods. Plant metabolomics using UPLC coupled with high-resolution qTOF-MS is a relatively new technology that can detect chemical compounds with high sensitivity [4,5]. Several studies have applied this technology to look at metabolite profiles in closely allied plant taxa, different cultivars of an individual taxon, or plants at different stages of development [6–9]. The use of UPLC-qTOF-MS for quality control assessment of phytomedicines and commercial plant drug preparations is relatively new; it was used in the last few years, e.g., for analyses of green tea, ginseng, and ephedra [10–12]. Here we detail the metabolome analysis of *Hypericum* plant extracts via UPLC-qTOF-MS and also report on the utility of a UPLC-qTOF-MS/

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No.	Name of preparation	Producer	Declaration
Tablets			
1[a]	Hiperikan	Marcyrl Pharm	*H. perforatum* ext. 300 mg
2[a]	Safamood	Sekem	*H. perforatum* ext. 250 mg, vitamin C 50 mg
3[a]	Safamood forte	Sekem	*H. perforatum* ext. 600 mg
4[a]	Sedaneurin	Sekem	*H. perforatum* ext. 600 mg, valeriana ext.100 mg
5[b]	Johanniskraut	Zirkulin	*H. perforatum* ext. 300 mg
6[b]	Johanniskraut	Altapharma	*H. perforatum* ext. 180 mg
7[b]	Johanniskraut	Kneipp	*H. perforatum* ext. 180 mg
8[b]	Johanniskraut	Das gesunde plus	*H. perforatum* ext. 180 mg
Capsule			
9[b]	Johanniskraut	Abtei	*H. perforatum* ext. 200 mg, Soy lecithin 40 mg

[a] (1, 2, 3, 4) products manufactured in Egypt; [b] (5, 6, 7, 8, 9) products manufactured in Germany

Table 1 Commercial preparations and their contents of *H. perforatum* included in this investigation.

PCA method for metabolome classification of *H. perforatum* commercial preparations.

H. perforatum, an herbal remedy commonly known as St. John's wort, has become extremely popular in North America as well as in Europe, with increasing sales statistics [13]. St. John's wort has been used traditionally for the treatment of excitability, neuralgia, menopausal neurosis, anxiety, depression, and in topical preparations for the treatment of wounds [14,15]. Today, St. John's wort is best known for its use in the treatment of mild-to-moderately severe depressive disorders [16]. The major active compounds in St. John's wort include a broad range of flavonoids, naphthodianthrones, and phloroglucinols. The antidepressant effect of *H. perforatum* was first attributed to the naphthodianthrones hypericin, pseudohypericin, protohypericin, and protopseudohypericin [17]. Recent studies revealed that the phloroglucinol hyperforin and its derivative adhyperforin inhibit various neurotransmitter receptors and are likely to be responsible for the antidepressant effects in St. John's wort [18–20]. Additionally, flavonoids present in St. John's wort extracts have been shown to have antidepressant activity [21]. Qualitative and quantitative variation in the content of secondary metabolites in *H. perforatum* is influenced by ecological and environmental effects, as well as physiological and genetic factors [22]. It was also found that some components are instead or additionally produced by fungal symbionts [23]. Exposure of the extract to light converts protohypericin into hypericin and also leads to the degradation of hyperforin, which is unstable and extremely sensitive to air oxidation [24]. Since the efficacy of St. John's wort medical preparations is based on a mixture of relevant metabolites (synergism), rather than the presence of a single constituent, the development of quick methods allowing for the analysis of such complex unstable extracts is of high relevance [25–28]. Standardization of commercial *H. perforatum* extracts is only based on the total content of hypericins, as required by the European Pharmacopoeia monograph, which implies that extracts may be variable with respect to other classes of metabolites [26]. A near infrared spectroscopic (NIRS) method was established as an alternative to liquid chromatography for quantitative determination of hypericins and phloroglucinols in St. John's wort extracts [29]. Nuclear magnetic resonance (NMR) based metabolomics used in conjunction with multivariate data analysis, such as principal component analysis (PCA) and parallel factor analysis (PARAFAC) have also been established for the analysis of St. John's wort products based on their global composition [11,25–27]. However, NMR has the problem of its low sensitivity compared to MS and the overlap of ^{1}H-NMR signals that hinders robust metabolite identification. While NMR provides a valuable metabolite signature of a complex plant extract reflecting exactly the relative amounts, UPLC-MS resolves individual chemical components into separate peaks, enhancing the opportunity to mine and uncover novel metabolites. Successful applications of UPLC-MS in phytomedicine quality control analyses include those for ephedra, green tea, and ginseng [8,11,12].

To meet the demand for a quick, sensitive, and untargeted method for St. John's wort analysis, we have developed a UPLC method to achieve rapid metabolite peak separation coupled with qTOF-MS, which can detect chemical compounds with high sensitivity. Aside from generating the first metabolome profile of fresh *H. perforatum* flowers with UPLC, a selection of its commercial preparations available on the German and Egyptian markets (**Table 1**) were also analyzed in parallel, followed by PCA analysis to help to reveal differences in the products chemical composition.

Materials and Methods

Plant material

H. perforatum seedlings were obtained from Staudengärtnerei Gaißmayer GmbH & Co. KG. The identification of the *H. perforatum* plant was done by examining the morphological characteristics of the flowers and leaves secretory structures [30]. A voucher specimen (FPCU5605) is currently deposited at the Herbarium of the Faculty of Pharmacy, Cairo University, Cairo, Egypt. The seedlings were grown on the field of the Leibniz Institute of Plant Biochemistry, Halle, Germany. Flowers were harvested in mid-August 2009 and 2010. The collected material was immediately frozen in liquid nitrogen and kept at −80 °C until further analyzed.

Chemicals and reagents

Acetonitrile and formic acid (LCMS grade) were obtained from J.T. Baker; milliQ water was used for LC analysis. Chromoband C18 (500 mg, 3 mL) cartridge was purchased from Macherey & Nagel. Hyperforin (≥ 97%), hypericin (≥ 98%), pseudohypericin (≥ 98%), rutin (≥ 99%), hyperoside (≥ 99%), isoquercetrin (≥ 99%), and amentaflavone (≥ 99%) were all provided from Chromadex. Umbelliferone (≥ 98%), catechin (≥ 98%), epicatechin (≥ 95%), chlorogenic acid (≥ 95%), and quercetin (≥ 98%) were provided from Sigma Aldrich.

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Extraction procedure and sample preparation for UPLC-PDA-MS analysis

Nine different samples of commercial St. John's wort from Egypt and Germany were obtained from retail stores. Eight preparations were formulated as tablets and one as a capsule. For three of the brands (2–4), different batches were obtained. Details on preparations and its composition are provided in **Table 1**. An amount of powder corresponding to 20 mg of plant extract (according to the labels) was extracted twice with 5 mL of 100% methanol containing umbelliferone (20 µg·mL^{-1}) as the internal standard by sonication for 45 min. The extracts were then vortexed vigorously and centrifuged at 11 000 *g* for 30 min; 500 µL were aliquoted and placed on a (500 mg) C18 cartridge preconditioned with methanol and water. Samples were eluted using 3 mL 100% MeOH; the eluent was evaporated under a nitrogen stream, and the obtained dry residue was resuspended in 500 µL methanol. 3 µL of the supernatant was used for UPLC-MS analysis.

Extracts from *H. perforatum* flowers grown at the Leibniz Institute of Plant Biochemistry were prepared by homogenizing 120 mg of lyophilized powder with 10 mL 100% MeOH using a Turrax mixer (11 000 RPM) for five 20 s periods. To prevent heating, a period of 1 min separated each mixing period. The extracts were then vortexed vigorously and centrifuged at 3000 *g* for 30 min to remove plant debris. Supernatant was evaporated under a nitrogen stream, and 20 mg of the obtained dry residue was placed on a (500 mg) C18 cartridge column and eluted as above. Light was excluded as much as possible during all operations.

High-resolution UPLC-PDA-MS analysis

Chromatographic separations were performed on an Acquity UPLC system (Waters) equipped with an HSS T3 column (100 × 1.0 mm, particle size 1.8 µm; Waters) applying two elution binary gradients at a flow rate of 150 µL·min^{-1}: (1) 0 to 1 min, isocratic 95% A (water/formic acid, 99.9/0.1 [v/v]), 5% B (acetonitrile/formic acid, 99.9/0.1 [v/v]); 1 to 16 min, linear from 5 to 95% B; 16 to 18 min, isocratic 95% B; 18 to 20 min, isocratic 5% B. The second binary eluent (2) was composed of ammonium acetate 50 mM buffer adjusted to pH 5(A) and 100% acetonitrile (B) using the same elution gradient as above. The injection volume was 3.1 µL (full loop injection). Eluted compounds were detected from *m/z* 100 to 1000 using a MicrOTOF-Q hybrid quadrupole time-of-flight mass spectrometer (Bruker Daltonics) equipped with an Apollo II electrospray ion source in positive and negative ion modes using the following instrument settings: nebulizer gas, nitrogen, 1.6 bar; dry gas, nitrogen, 6 l·min^{-1}, 190 °C; capillary, − 5500 V (+ 4000 V); end plate offset, − 500 V; funnel 1 RF, 200 Vpp; funnel 2 RF, 200 Vpp; in-source CID energy, 0 V; hexapole RF, 100 Vpp; quadrupole ion energy, 5 eV; collision gas, argon; collision energy, 10 eV; collision RF 200/400 Vpp (timing 50/50); transfer time, 70 µs; prepulse storage, 5 µs; pulser frequency, 10 kHz; spectra rate, 3 Hz. Internal mass calibration of each analysis was performed by infusion of 20 µL 10 mM lithium formiate in isopropanol/water, 1/1 (v/v), at a gradient time of 18 min using a diverter valve. Metabolites were characterized by their UV-vis spectra (220–600 nm), retention times relative to external standards, mass spectra, and comparison to our in-house database and reference literature. Quantification of hyperforins, hypericin flavonoids, and chlorogenic acid were calculated from the calibration curve of hyperforin, hypericin hyperoside, and chlorogenic acid standards, respectively, detected using a PDA (photodiode array detection) detector. Standard calibration curves were constructed for each standard using 3 concentrations spanning from 1, 10, and 100 µg·mL^{-1}. Assays were carried out in triplicate.

MS data processing for multivariate analysis

Relative quantification and comparison of *H. perforatum* metabolite profiles after UPLC-MS was performed using XCMS data analysis software, which can be downloaded for free as an R package from the Metlin Metabolite Database (http://137.131.20.83/download/) [31]. This software approach employs peak alignment, matching, and comparison. Native MS files from Bruker Daltonics were first converted into mzData files using the File Converter tool. Files were arranged in one folder that was set as the file source. Peaks were subsequently extracted using XCMS under an R 2.9.2 environment with the signal-to-noise ratio set to 10. After peak extraction and grouping, nonlinear retention time correction of peaks was accomplished in two iterative cycles with descending bandwidth (bw). This was accomplished by manually decreasing the bw parameter (from 30 to 10 s). Evaluation of the XCMS package for similar UPLC-MS datasets showed that bw provided significant changes to the number of peaks detected and the peak area reproducibility [32]. The resulting peak list was further processed using Microsoft Excel software (Microsoft), where the ion features were normalized to the total integrated area (1000) per sample and imported into the R 2.9.2 software package for principal component analysis (PCA). Absolute peak area values were autoscaled as this provides similar weights for all the variables [33]. PCA was then performed on the MS-scaled data to visualize general clustering, trends, and outliers among all samples on the scores plot.

Supporting information

The result of *H. perforatum* preparation analysis using UPLC-PDA-MS (**Fig. 1S**), PCA analysis of different batches for preparations 2–4 (**Fig. 2S**), and the absolute quantification of metabolites in preparations 1–9 (**Table 1S**) are available as Supporting Information.

Results and Discussion

Chemical constituents of *H. perforatum* were analyzed by reversed-phase UPLC/PDA/(−)ESI-qTOF-MS, using a gradient mobile phase consisting of methanol and ammonium acetate 50 mM (pH 5) that allowed for the elution of all analytes, i.e., hyperforins, flavonoids, and naphthodianthrones (hypericins) within 13 min (ca. 800 sec) compared to the 100 min analysis time previously reported [34]. It should be noted that initial attempts to optimize the chromatographic separation for the different classes of compounds using aqueous formic acid (pH 2.9) as eluent in a gradient caused naphthodianthrones peaks to evade detection. One possible explanation is the much more acidic nature of the chosen mobile phase (pH 2.9) compared to ammonium acetate (pH 5) as an eluent, which might not be appropriate for the elution and further detection of these special constituents. Indeed, separation methods reported in the literature using formic acid in aqueous phase failed to detect hypericins in *H. perforatum* extracts [27,35]. UV and MS spectra were recorded for hypericin standard prepared in 0.1% formic acid and injected directly without going through the column, ruling out a problem due to an ionization suppression effect by the eluent or possible degradation.

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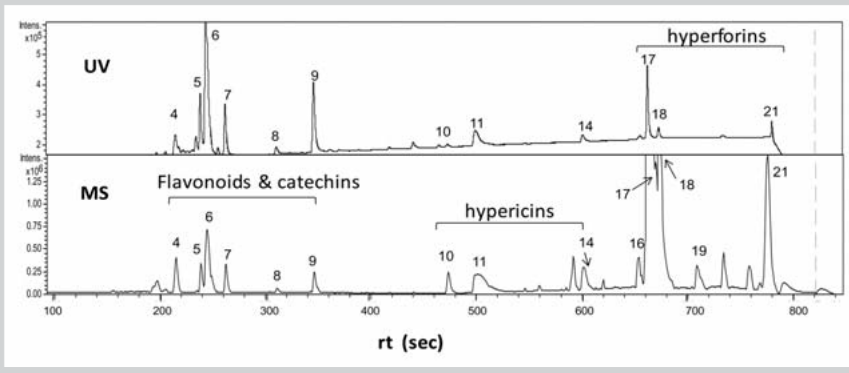

Fig. 1 Representative UPLC-UV-MS traces of *H. perforatum* flower methanol extract characterized by 3 main regions: (150–400 sec) with peaks principally due to flavonoids and catechins, a region (450–600 sec) assigned for hypericins, and a region (640–800) for hyperforins. Assigned peaks: 4, epicatechin; 5, rutin; 6, hyperoside; 7, isoquercetrin; 8, quercetin; 9, amentaflavone; 10, protopseudohypericin; 11, pseudohypericin; 14, hypericin; 16, furohyperforin; 17, hyperforin; 18, adhyperforin; 19, stearic acid; 21, 33-hydroperoxy furohyperforin. Peak numbers follow those listed in ◉ **Table 2** for metabolite identification using UPLC-UV-MS.

Table 2 Compounds tentatively assigned in fresh *H. perforatum* flower methanol extract using UPLC-PDA-MS in negative ionization mode.

Peak	rt (sec)	UV	Name	$[M-H]^-$ (*m/z*)	Error ppm	El. Comp.	MS^n ions
1	154	292 shd, 325	Chlorogenic acid	353.0888	0.5	$C_{16}H_{17}O_9^-$	217 (67), 191 (49)
2	193	275	Catechin	289.0709	3.1	$C_{15}H_{13}O_6^-$	217 (18)
3	203	275	Procyanidin B1	577.1337	2.5	$C_{30}H_{25}O_{12}^-$	425 (25)
4	212	275	Epicatechin	289.0709	3.1	$C_{15}H_{13}O_6^-$	
5	237	255, 345	Rutin	609.1443	3.0	$C_{27}H_{29}O_{16}^-$	477 (5), 301 (15)
6	245	265, 355	Quercetin-3-*O*-galactoside (Hyperoside)	463.0885	0.5	$C_{21}H_{19}O_{12}^-$	301 (8)
7	263	255, 350	Quercetin-3-*O*-rhamnoside (Isoquercetrin)	447.0924	2.0	$C_{21}H_{19}O_{11}^-$	301 (3)
8	311	260 shd, 360	Quercetin	301.0344	3.3	$C_{15}H_9O_7^-$	263 (6), 217 (37)
9	345	270, 335	I3, II8-Biapigenin (Amentaflavone)	537.0806	4.0	$C_{30}H_{17}O_{10}^-$	443 (10)
10	471	370, 535, 575	Protopseudohypericin	521.0857	4.0	$C_{30}H_{17}O_9^-$	
11	499	325, 545, 585	Pseudohypericin	519.0702	3.9	$C_{30}H_{15}O_9^-$	
12	557	nd	Unknown	331.1899	4.7	$C_{20}H_{27}O_4^-$	227 (60)
13	588	nd	Unknown	567.3673	3.3	$C_{35}H_{51}O_6^-$	551 (42)
14	601	330, 545, 585	Hypericin	503.0762	2.1	$C_{30}H_{15}O_8^-$	
15	619	nd	Unknown	507.3464	3.2	$C_{33}H_{47}O_4^-$	413 (21)
16	651	nd	Furohyperforin	551.3733	1.7	$C_{35}H_{51}O_5^-$	481 (13)
17	661	235, 290	Hyperforin	535.3787	4.4	$C_{35}H_{51}O_4^-$	467 (8)
18	671	230, 290	Adhyperforin	549.4020	2.2	$C_{36}H_{53}O_4^-$	535 (55), 481 (9)
19	707	nd	Stearic acid	283.2679	8.5	$C_{18}H_{35}O_2^-$	
20	756	nd	Unknown	635.4507	3.3	$C_{37}H_{63}O_8^-$	567 (40), 471 (100)
21	778	nd	33-Hydroperoxy furohyperforin	567.3689	1.7	$C_{35}H_{51}O_6^-$	471 (100)

Simultaneously acquired UPLC–PDA and UPLC–MS base peak intensity chromatograms of *H. perforatum* flower extract are presented in ◉ **Fig. 1**. The chromatogram obtained by MS detection in negative ionization mode was quite similar to the PDA chromatogram for the first half of the chromatograms (100–500 sec), whereas for the later eluents (650–800 sec), the peaks for hyperforins had a much higher response in MS than in UV. The identities, retention times, UV characteristics, and observed molecular and fragment ions for individual components are presented in ◉ **Table 2**. A total of 21 metabolites were detected, and 17 were tentatively identified. Metabolite assignments were made by comparing retention time, UV/Vis spectra, and MS data (accurate mass, isotopic distribution, and fragmentation pattern in both positive and negative ion modes) of the compounds detected with *H. perforatum* compounds reported in the literature and searching in the existing online public databases. Identifications were confirmed with standard compounds whenever available in-house. Identified metabolites belonged to various classes (◉ **Table 2**) including a caffeic acid derivative (i.e., chlorogenic acid), catechins (i.e., catechin, epicatechin), flavonoid glycosides (i.e., isoquercetrin, rutin, hyperoside), naphthodianthrones (i.e., hypericin, pseudohypericin, proto pseudo-hypericin), and phloroglucinols (i.e., hyperforin, adhyperforin), with flavonoids as the most abundant class in *H. perforatum*. Furohyperforin and 33-hydroperoxy furohyperforin, the oxidation products of hyperforin, were detected in *H. perforatum* extract, albeit at much lower levels than the mother compounds, suggesting that hyperforins were not subjected to major chemical degradation [36]. The abundance of furohyperforin (peak 16) was used to identify preparations that have undergone partial decomposition. In comparison, naphthodianthrone compounds are more stable due to their aromatic conjugation [34], except that the protoforms, i.e., protopseudohypericin (open-ring system) are less stable than, i.e., hypericin and pseudohypericin (closed-ring structures). In these preparations, both pseudohypericin and hypericin were the major naphthodianthrones (peaks 11 & 14) versus the proto-form (peak 10). The structures of metabolites com-

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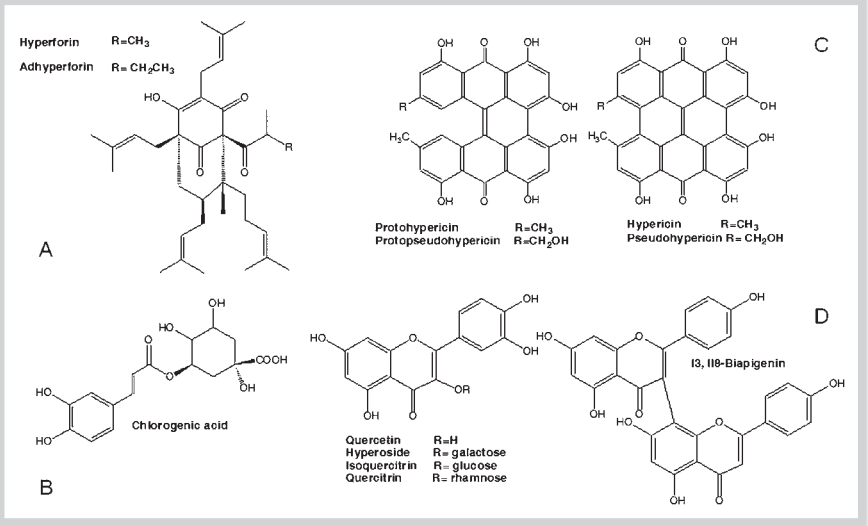

Fig. 2 Major classes of natural products; phloroglucinols (**A**), phenolic acid (**B**), naphthodianthrones (**C**), and flavonoids (**D**) detected in St. John's wort with selected compound(s) discussed in the manuscript.

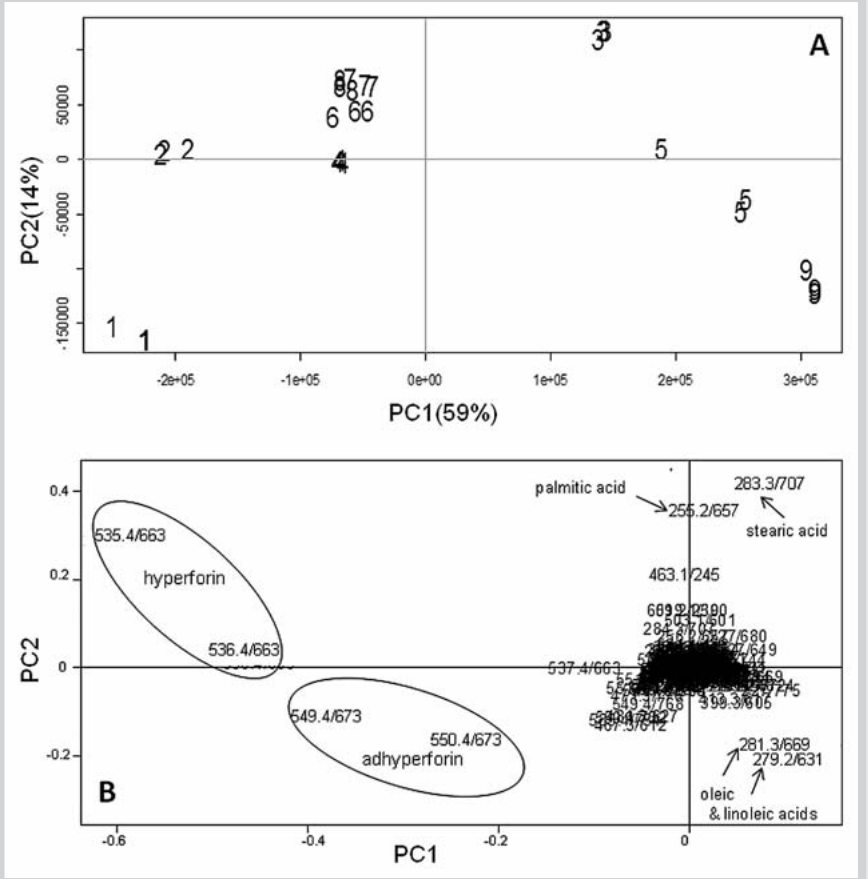

Fig. 3 Principal component analyses of 9 commercially available preparations of *Hypericum* analyzed by UPLC-qTOF-MS (n = 3). The metabolome clusters are located at the distinct positions in two-dimensional space prescribed by two vectors of principal component 1 (PC1 = 59%) and principal component 2 (PC2 = 14%). **A** Score plot of PC1 vs. PC2 scores. **B** Loading plot for PC1 and PC2 contributing mass peaks and their assignments, with each metabolite denoted by its mass/rt(sec) pair. Note that in case of hyperforin and adhyperforin, 2 mass pairs were identified for each metabolite corresponding to its molecular ion $[M-H]^-$ and associated ^{13}C mass isotope. It should be noted that ellipses do not denote statistical significance but are rather for better visibility of clusters as discussed.

monly found in *H. perforatum* and discussed throughout the manuscript are shown in **Fig. 2**.

The UPLC-MS spectra of St. John's wort preparations from the 9 different manufacturers can be examined in **Fig. 1S**, Supporting Information. Except for preparations 9 and 5, which are very different from other samples, it can be seen that the chromatograms are quite similar. Particularly, for preparation 9, major peaks could be attributed to fatty acids being absent from other preparations. Despite this broad similarity, we attempted to analyze UPLC-MS spectra in a more holistic way using PCA to explore the relative variability within the different samples rather than a direct inspection of the chromatograms [37].

From the 9 preparations, 1879 mass signals extracted by XCMS from the UPLC-MS data set acquired in negative ionization mode were used for PCA analysis (**Fig. 3**). Triplicate measurements from the same sample were found to be highly reproducible, as the scores of replicate measurements were more or less superimposed. Four principal components (PCs) were required to capture almost 99% of the variance. The main principal component (PC) to differentiate between samples, i.e., PC1, accounts for 61% of

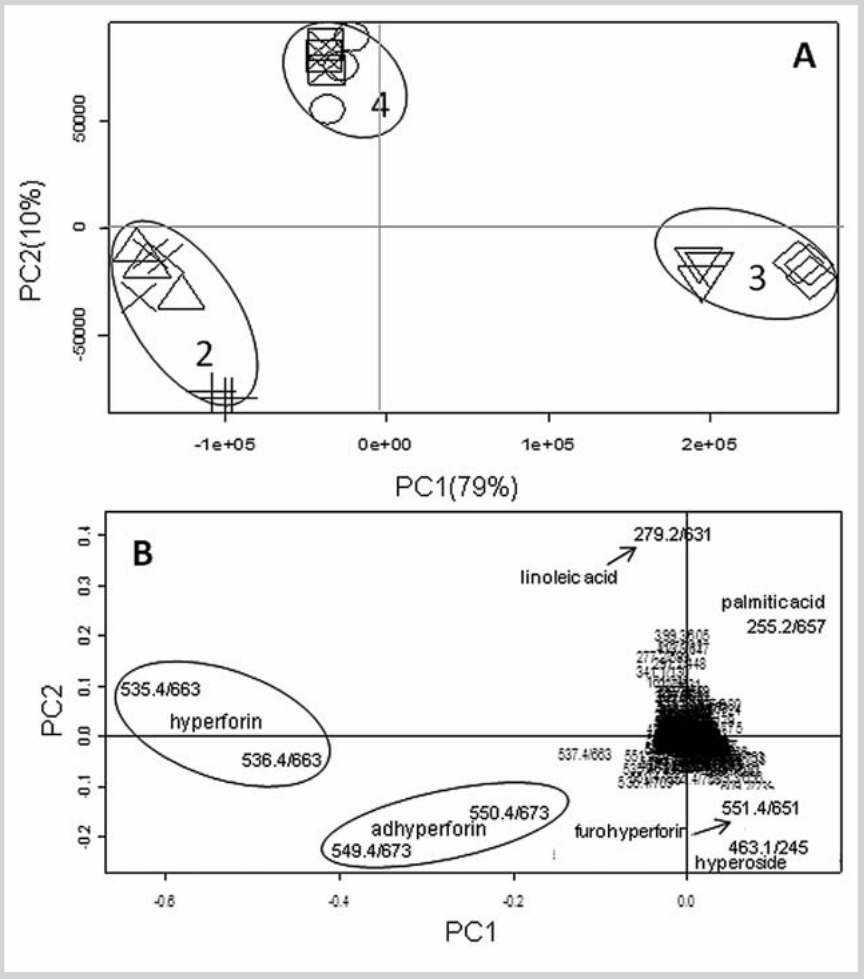

Fig. 4 UPLC-qTOF-MS (*m/z* 100–1000) principal component analyses of 3 commercially available preparations 2, 3, and 4 obtained from the same supplier but with different batch numbers (n = 3). Preparations symbols are as follows: 2a (+), 2b (△), 2c (×); 3a (▽), 3b (◇); 4.1 (□), 4.2 (○) with number denoting for preparations (**Table 1**) and letters for different batches within each preparation. The metabolome clusters are located at the distinct positions in two-dimensional space, described by two vectors of principal component 1 (PC1 = 79%) and principal component 2 (PC2 = 10%). **A** Score plot of PC1 vs. PC2 scores. **B** Loading plot for PC1 and PC2 contributing mass peaks and their assignments, with each metabolite denoted by its mass/rt(sec) pair. Note that for hyperforin and adhyperforin, 2 mass pairs were identified corresponding to molecular ions $[M - H]^-$ and associated ^{13}C mass isotopes. It should be noted that ellipses do not denote statistical significance but are rather for better visibility of clusters as discussed.

St. John's wort variance. Most of the preparations are located on the left side of the vertical line representing PC1 (negative PC1 values), whereas preparations 3, 5, and 9 were positioned on the right side (positive PC1 values), **Fig. 3A**. This group can still be separated along PC2, with preparation 9 (capsule) being the most distant, likely due to a different dosage form and fatty acid content versus tablets in other preparations. This indicated that these 3 preparations were chemically distinctively different from the remaining samples. The separation observed in PCA can be explained in terms of the identified compounds, using the loading plots for PC1 signals that expose those peaks (compounds) that have the largest effect on the respective principle component. Two major groups stand out in this plot, **Fig. 3B**. The first corresponds to the MS signals of hyperforin and adhyperforin, contributing negatively to PC1 and accounting for the most in sample discrimination. The second, from MS signals assigned for fatty acids, i.e., palmitic, linoleic, oleic, and stearic acids, as evident from high-resolution masses 255.2351, 279.2359, 281.2520, and 283.2679 with predicted molecular formulae of $C_{16}H_{31}O_2^-$, $C_{18}H_{31}O_2^-$, $C_{18}H_{33}O_2^-$, and $C_{18}H_{35}O_2^-$, respectively. Fatty acids are likely to be derived from soy oil, present as an additive in preparation 9. Nevertheless, these fatty acids were also identified in preparations 3 and 5, although not declared by manufacturers. Except for stearic acid, fatty acids were not identified in the fresh *H. perforatum* flower profiling experiment (**Fig. 1**) suggesting that they are either derived from other organs (i.e., seeds) or used as additives during extraction or in preparations.

PC2 explains 14% of the variation captured between samples and was related to hyperoside and rutin, both contributing positively to PC2 (data not shown). No clustering of data attributable to differences in hypericins or catechins was observed in this study, certainly caused by the fact that *H. perforatum* commercial preparations are likely all standardized using hypericins and are expected to be fairly constant in these. Previous studies using NMR metabolomics revealed variation in flavonoid content among *H. perforatum* preparations [26]. Nevertheless, hyperforins accounted more for the preparation segregation in our study, but evaded detection by NMR [26] with no signals for hyperforin or adhyperforin recognized. While NMR provides the valuable metabolite fingerprint, UPLC-MS resolves individual chemical components into separate peaks, enhancing the opportunity to detect metabolites present at lower levels. These results suggest the importance of combining both NMR and MS techniques to obtain the most complete metabolite profile of *Hypericum* pharmaceuticals and/or other phytomedicines [38]. It should be noted that our homemade fresh extract from *H. perforatum* flowers was not included in PCA, considering that it showed 15- and 10-fold levels of hyperforins and hypericins, respectively, versus the commercial products. If this sample had been included, it would have appeared as the most distant in the score plot and overly affected the other preparations clustering. Metabolite enrichment in our home-prepared extract is likely due to the selective extraction from flowers only, the most enriched part in hyperforins and hypericins, and to the fact that the extract was freshly prepared.

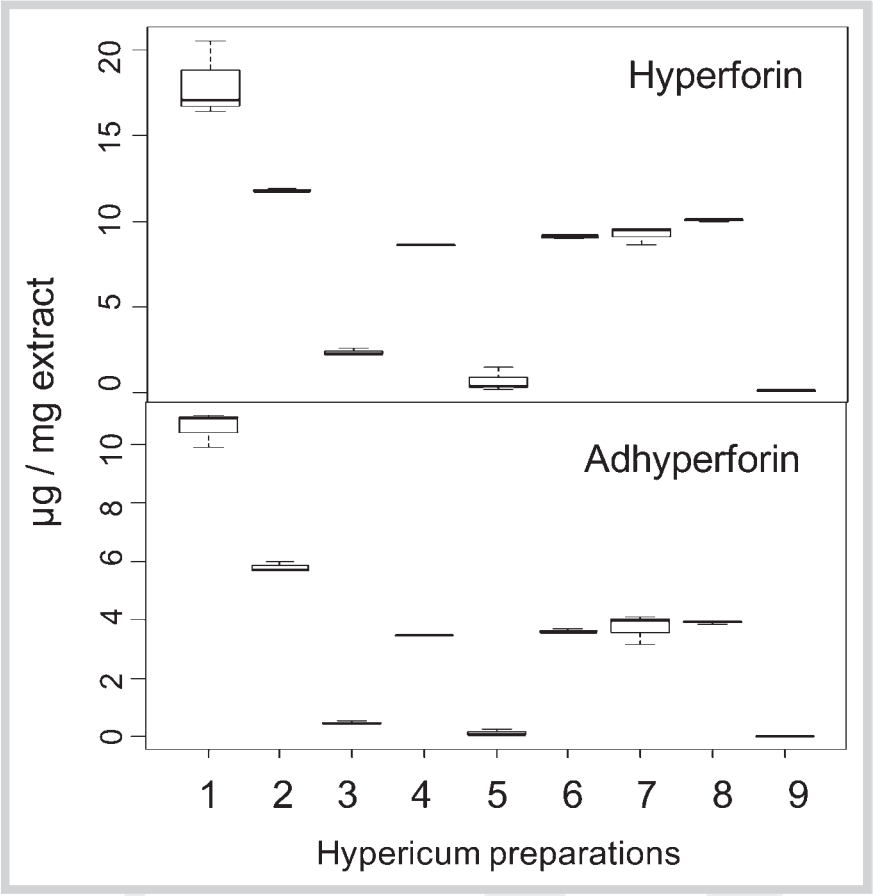

Fig. 5 Box plot showing hyperforin and adhyperforin contents in *H. perforatum* preparations expressed as $\mu g \cdot mg^{-1}$ extract. These secondary metabolites were identified by UPLC-MS and are responsible for the differentiation in PCA. (Line = mean; box = standard error; whisker = standard deviation.)

A further sample set was used to investigate whether PCA can distinguish between different batches of preparations from the same supplier. Our goal was also to help identify variation in other metabolites, aside from hyperforins and fatty acids, showing the highest variance across samples. For this dataset, two to three different batch numbers from preparations 2, 3, and 4 found on the Egyptian market were analyzed separately, and their scores plot is shown in **Fig. 4A**. The main principal component (PC) to differentiate between samples, i.e., PC1, accounts for 79% of samples variance. The PC1/PC2 scores plot (**Fig. 4A**) shows that 3 major distinct clusters are formed, corresponding to the 3 samples studied. On the right side of the plot, preparation 3 is positioned, whereas on the far left side, preparation 2 is located. The other preparation 4 was plotted in between and in agreement with results observed in the previous uncut dataset (**Fig. 3A**). Regarding batch variation within each product and except for preparation 4, a slight separation was achieved for the different batches from preparations 2 and 3 along PC1. It can be clearly seen, even for these samples that should have very similar composition, that batch variation can still be recognized from PCA score plots. Two major groups stand out in this loading plot (**Fig. 4B**). The first corresponds to the MS signals of hyperforin and adhyperforin, contributing negatively to PC1 and accounting for the most sample discrimination, similarly to that observed in **Fig. 3A**. The second, coming from MS signals assigned to furohyperforin and hyperoside, contributes positively to PC1. Interestingly, preparation 2, fortified with vitamin C, exhibited the highest ratio of hyperforin (unoxidized form)/furohyperforin (oxidized form) compared with other preparations (**Table 1S**, Supporting Information). Vitamin C is a potent antioxidant and might have prevented hyperforins from oxidation and further degradation.

Preparations 2, 3, and 4 with their different batch numbers appeared distant when plotted altogether so that the detection of differences among batches within each preparation as in 4 could have been evaded. To help reveal for inter-batch variation, batches from each preparation 2, 3, and 4 were modeled separately (**Fig. 2S**, Supporting Information). Separation could now be achieved among preparations, with 4 different batch numbers, less readily distinguished in the previous example (**Fig. 4B**), suggesting that the use of multivariate data analysis can readily discriminate between very close St. John's wort samples.

To confirm that the discrimination between samples is mostly caused by hyperforin content, absolute quantifications were attempted for hyperforin and adhyperforin using UV detection. In agreement with the PCA results, the lowest levels of hyperforin and adhyperforin were found in preparations 3, 5, and 9, whereas other preparations showed comparable levels of these metabolites, with preparation 1 being the one most enriched in both metabolites (**Fig. 5**). Details on the quantifications for all major compounds found in these preparations detected using UV is provided in **Table 1S**, Supporting Information. Rutin and hyperoside, major flavonoids in all preparations, were present at an average of 15–30 $\mu g \cdot mg^{-1}$ and showed similar variation patterns among the different products. Hypericins, the biologically most relevant metabolites in St. John's wort, showed less variation among samples, though with ca. 1–5 $\mu g \cdot mg^{-1}$ present at much lower levels than flavonoids. The fact that furohyperforin (oxidative product of hyperforin) was detected at levels well below

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$2\,\mu g \cdot mg^{-1}$ suggests that hyperforins were not subjected to major chemical degradation in these preparations [36].

Overall, a new UPLC-UV-MS approach coupled with multivariate data analysis was adopted to reveal compositional differences in secondary metabolites among St. John's wort preparations. The enhanced peak resolution in UPLC seems to be very promising for QC metabolomic studies, overcoming the low peak resolution limiting factor in LC [38]. Whether the development of 2-dimensional UPLC would also provide a powerful strategy for resolving complex plant extracts, superior to current 2D-LC techniques has yet to be explored. UPLC-UV-MS appears suitable to determine sample relationships, can provide absolute metabolite quantifications and identify samples that have undergone partial decomposition. Differences mainly in the content of hyperforins, but also of fatty acids and hyperoside accounted for the clustering of preparations. The differences in content of hyperforins, a relevant class of St. John's wort active constituents, can inevitably impact product efficacy. Standardization of St. John's wort preparations based on a detailed analysis of hyperforins using UPLC-MS and an investigation of the effects of tissue type, storage, harvesting time, and/or seasonal variation on *H. perforatum* secondary metabolite composition and its pharmacological effect has also yet to be completed. These differences may well contribute for inconsistent results in clinical trials with herbal medicines as a correlation with the full spectrum of metabolites is usually not performed.

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Conflict of Interest

The authors declare no actual or potential conflict of interests including any financial, personal, or other relationships with other people or organizations.

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