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Comprehensive phytochemical characterization of St. John's wort (*Hypericum perforatum* L.) oil macerates obtained by different extraction protocols via analytical tools applicable in routine control

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The aim of the present study was to investigate the impact of crucial process parameters, i.e. of light and temperature conditions, during the preparation of St. John's wort (SJW, *Hypericum perforatum* L.) *Arachis* oil macerates. Extracts were prepared according to a standardized protocol over a period of 28 days. For this purpose, flowering tops of *H. perforatum* were macerated with *Arachis* oil (drug extract ratio, DER_{native} 1:4) under different light and temperature conditions. Spectrophotometric measurements were carried out to quantitate naphthodianthrones and to characterize extract color in the CIE *L*C*h°* system. Moreover, individual plant secondary metabolites were screened by UHPLC-DAD-MS^n measurements following liquid-liquid extraction of the oil macerates with methanol. For quantitation purposes, the chromatographic method was validated using reference standards. This methodology allowed the separation of up to 25 constituents in oily and methanolic SJW extracts, covering hydroxycinnamic acids, flavanols, proanthocyanidins, flavonol glycosides, flavonol aglyca, biflavones, bisanthraquinone glycosides, naphthodianthrones and phloroglucinols. Lowest naphthodianthrone contents were determined in oil macerates recovered at 5 °C, whereas highest amounts were detected upon extraction at 50 °C (both under the exclusion of light). Color shades of the oil macerates differed markedly, revealing e.g. *a**-values ranging from -4.6±0.3 to 42.5±0.3. The flavonoids quercetin, kaempferol and I3, II8-biapigenin as well as the phloroglucinols hyperforin and adhyperforin could be simultaneously detected and quantitated in all oil macerates. Contents of these constituents varied noticeably between macerates prepared under different conditions (quercetin 14.7±1.2 to 21.8±0.6 µg/g, kaempferol 3.0±0.1 to 5.4±0.4 µg/g, I3, II8-biapigenin 4.4±0.2 to 7.4±0.4 µg/g, hyperforin 52.6±46.0 to 451.4±24.9 µg/g, adhyperforin 6.9±5.7 to 74.5±7.1 µg/g). These results confirm that the quality of the resulting plant extracts is largely determined by the respective process parameters, i.e. especially temperature and light conditions, and thus must be thoroughly chosen and monitored to obtain tailor-made preparations.

1. Introduction

With its broad and complex spectrum of plant secondary metabolites, covering among others phenolic acids, flavonoids, naphthodianthrones and phloroglucinols, *Hypericum perforatum* L., commonly known as St. John's wort (SJW), is one of the most prominent traditional medicinal plants (Brolis et al. 1998; Mayer 2014; Wölfle et al. 2014). Beside preparations based on hydro-alcoholic SJW extracts and tinctures, which are used for the symptomatic treatment of mild and moderate depressive episodes today (WHO 2015), oily preparations (*Hyperici oleum*) have been applied in phytotherapy for ages. According to the European Medicines Agency the traditional cutaneous use of oily *Hypericum* macerates is described for the symptomatic treatment of minor inflammations of the skin and subcutaneous tissue disorders (EMA 2009). Moreover, oral use is proposed for the treatment of dyspeptic disorders, nervous gastric diseases, abdominal pain and as a choleretic. In addition to the quality of the plant material and the extractant, the composition and characteristics of the resulting plant extract is markedly determined by the extraction protocol. Consequently, optimal process conditions may be derived from experimental data obtained in systematic trials (Gaedcke 2003). So far, the influence of different extract preparation methods on the composition of *Hypericum* oil macerates has been investigated sporadically. Maisenbacher and Kovar (1992) determined total flavonoid and hyperforin contents of various SJW oil extracts following solid phase extraction on aminopropyl-modified silica gel. SJW sunflower oil extracts were prepared with fresh and dried flowering tops applying three different maceration techniques in order to examine anti-inflammatory and gastroprotective activity (Zdunic et al. 2009). Isacchi et al. (2007) studied SJW oils, prepared with olive oil and considering a range of process factors, which may affect extract composition, such as harvesting time, the use of fresh plant material or dried herbal drug and different heating systems. Arsic et al. (2010) determined the contents of quercetin and total hypericin in extracts prepared with sunflower, olive and palm oils, while Orhan et al. (2013) compared bioactive compounds of homemade and commercially prepared SJW olive oil macerates. However, to the best of our knowledge more systematic studies focusing on a standardized preparation procedure and also considering further lipophilic extractants, such as *Arachis* oil, have not been performed so far. For this reason, our aim was to systematically investigate the impact of different extraction parameters, in particular of light and temperature, on the quantity of plant secondary metabolites in freshly prepared *Arachis* oil macerates and on their optical properties. Consequently, we designed a series of experiments performing extractions under well-defined light exposure conditions, i.e. sunlight, artificial light and absence of light, as well as temperature conditions, i.e. 5 °C, 21 °C, 40 °C, and 50 °C, over a period of 28 days. Detailed light and temperature combinations

are shown in Table 1. Comprehensive quality parameters should be investigated. For this purpose, color measurements according to the CIE *L*C*h°* system were performed, and total hypericin contents were determined. Moreover, we developed a UHPLC-DAD method, which was validated and allowed the separation of a broad range of essential plant secondary metabolites. This powerful tool enables a systematic comparison of different SJW extraction conditions applying various solvent polarities, ranging e.g. from alcoholic extraction to more selective extraction methods with fatty oils.

Table 1: Light and temperature conditions applied during the oil maceration process

Sample designation	Light conditions	Temperature
O1	Darkness (D)	5 °C
O2	Darkness (D)	RT[a]
O3	Artificial light (AL)	20 °C
O4	Sunlight (SL)	RT[a,b]
O5	Darkness (D)	40 °C
O6	Darkness (D)	50 °C

[a] RT, room temperature, 21±3 °C
[b] Process mimicking preparation according to the German Pharmacopoeia

2. Investigations, results and discussion

2.1. Method development and optimization of analytical parameters

For comprehensively analyzing SJW oil macerates, the aim of method development was to cover the broad spectrum of SJW secondary metabolites, displaying a wide range of polarities. For this purpose, the UHPLC-DAD-MS^n method was based on previous literature reports (Brolis et al. 1998; Ganzera et al. 2002; Smelcerovic et al. 2006; Farag and Wessjohann 2012), and separation was optimized by means of a methanolic SJW extract with regard to the simultaneous detection of hydroxycinnamic acids, flavanols, proanthocyanidins, flavonol glycosides and flavonol aglyca, biflavones, bisanthraquinone glucosides, naphthodianthrones and phloroglucinols within one single run. Furthermore, method development focused on the enrichment of the aforementioned target compounds. For this purpose, sample preparation protocols described in the literature (Isacchi et al. 2007; Orhan et al. 2013), which are based on merely dissolving samples in methanol or chloroform/methanol, were developed further by subjecting *Hypericum* oil macerates to liquid-liquid separation with methanol (Arsic et al. 2010). On the one hand, this novel protocol allowed the removal of major parts of the oily matrix, which may interfere with subsequent UHPLC analyses. On the other hand, the compound enrichment ensured the detection of all relevant SJW components and analytes, which may have been overlooked in the past. The developed method also allowed the quantitation of active constituents. Hence, efforts with regard to analysis time and solvent amounts needed were notably reduced without losing resolution, consequently allowing high-throughput sample analysis as a major prerequisite for routine control testing (Chesnut and Salisbury 2007).

2.2. Characterization of individual compounds in oily macerates by UHPLC-DAD-MSn

UHPLC-DAD-ESI-MS^n analyses were performed in order to characterize individual SJW constituents. Based on retention time, UV/VIS spectra and fragmentation patterns, individual compounds were assigned by comparison with literature data and analytical reference standards. In methanolic extracts of SJW fresh plant material, 25 constituents were detected (Fig. 1), covering various classes of plant secondary metabolites, namely hydroxycinnamic acids (compounds **1**-**3**), flavanols (compounds **4** and **7**) including proanthocyanidins (compounds **5**-**6**, **8**), flavonol glycosides (compounds **9**-**14**) and flavonol aglyca (compounds **15** and **16**), biflavones (compounds **17** and **18**), a bisanthraquinone glucoside (compound **19**), naphthodianthrones (compounds **20**-**23**) and phloroglucinols (compounds **24** and **25**). Tentative peak assignment is presented in Table 2. Expectedly, in contrast to methanolic extracts of *Hypericum* plant material, the diversity of compound classes in oily macerates was less pronounced. In all SJW oil macerates, the detection of five of the aforementioned compounds was accomplished, i.e. the flavonol aglyca quercetin and kaempferol, the biflavone I3, II8-biapigenin and the phloroglucinols hyperforin and adhyperforin. Contrary to Orhan et al. (2013), who detected hypericin and pseudohypericin in SJW olive oil macerates, these latter two naphthodianthrones were not detected in the oil extracts characterized in the present study. This observation is hardly surprising, since hypericin is known to form lipophilic derivatives upon storage, whereas their exact molecular structure has not yet been described in literature (Maisenbacher and Kovar 1992; Isacchi et al. 2007).

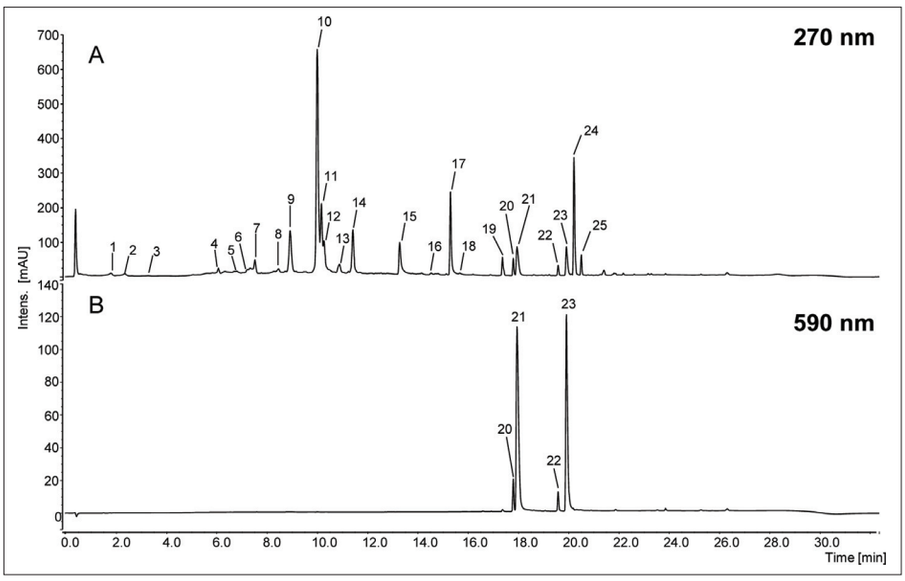

Fig. 1: HPLC fingerprint of a methanolic SJW extract recorded at 270 nm (A) and 590 nm (B). For peak assignment cf. Table 2.

Table 2: Characterization of individual compounds in methanolic SJW extracts: HPLC-DAD and mass spectrometric data, recorded in negative ionization mode: precursor ion represents $[M-H]^-$ species

Peak no.	t_r [min]	Peak assignment	UV/VIS λ_{max} [nm]	MS[a] data [*m/z*]			References
				Precursor ion	MS^2	MS^3	
1	1.9	3-Caffeoylquinic acid (neochlorogenic acid)	296*sh,* 322	*353*	*191*, 179	173	Clifford et al. (2003), Jürgenliemk and Nahrstedt (2002)
2	2.5	5-Caffeoylquinic acid (chlorogenic acid)	292*sh,* 322	*353*	*191*, 192, 179	173	Clifford et al. (2003) [c]
3	3.3	3-*p*-Coumaroylquinic acid	296*sh*, 312	*337*	*163*, 191	-	Clifford et al. (2003), Jürgenliemk and Nahrstedt (2002)[c]
4	6.2	(+)-Catechin	278	*289*	*245*, 205	*203*, 161	Farag and Wessjohann (2012)
5	6.9	Procyanidin dimer	280	*577*	425, *407*	*285*, 281	Ploss et al. (2012)
6	7.3	Procyanidin dimer	278	*577*	*425*	*407*, 408, 273	Ploss et al. (2012)
7	7.6	Epicatechin	278	*289*	245, 205	203	Farag and Wessjohann (2012), Ploss et al. (2012)
8	8.6	Procyanidin trimer	278	*867*[b]	*579*	409, 291	Hellenbrand et al. (2015)
9	9.0	Quercetin-3-*O*-glucuronide (miquelianin)	256, 354	*477*	*301*	*179*, 151	Jürgenliemk and Nahrstedt (2002), Tatsis et al. (2007)
10	10.1	Quercetin-3-*O*-galactoside (hyperoside)	256, 314*sh*, 354	*463*	*301*	*179*, 151	Tatsis et al. (2007)[c]
11	10.3	Quercetin-3-*O*-glucorhamnoside (rutoside)	256, 314*sh*, 354	*609*	*301*	*179*, 151	Jürgenliemk and Nahrstedt (2002)[c]
12	10.4	Quercetin-3-*O*-glucoside (isoquercitrin)	256, 314*sh,* 354	*463*	*301*	*179*, 151	Tatsis et al. (2007)[c]
13	10.9	Quercetin-3-*O*-arabinoside (guaijaverin)	266, 348	*433*	*301*	*271*, 179, 150	Jürgenliemk and Nahrstedt (2002)
14	11.5	Quercetin-3-*O*-rhamnoside (quercitrin)	256, 318*sh,* 348	*447*	*301*	*179*, 151	Tatsis et al. (2007)[c]
15	13.4	Quercetin[a]	256, 370	*301*	*179,* 151	-	Tatsis et al. 2007[c]
16	14.7	Kaempferol[a]	266, 364	*285*	281, 151	-	Silva et al. (2005)[c]
17	15.5	I3, II8-Biapigenin[a]	332, 268	*537*	443, 385	323, *322,* 295	Tatsis et al. (2007)[c]
18	15.8	I3, II8'-Biapigenin (amentoflavone)	326, 270	*537*	*375*, 443	*331*	Berghöfer and Hölzl (1989)
19	17.5	Skyrin-glucopyranoside	452, 298, 260	*699*	*519*	*491*, 492, 447	Wirz et al. (2000)
20	17.9	Protopseudohypericin	538, 372	*521*	477	451, 423, 449	Tatsis et al. (2007)
21	18.1	Pseudohypericin	590, 546, 474, 328	*519*	477, *475*	449, 421	Tatsis et al. (2007)[c]
22	19.7	Protohypericin	538, 372	*505*	*461*	*407*, 435, 433	Tatsis et al. (2007)
23	20.0	Hypericin	590, 564, 472, 330	*503*	459, *461*	433	Tatsis et al. (2007)[c]
24	20.4	Hyperforin[a]	292	*535*	*315*, 383, 466,	*243*, 287	Tatsis et al. (2007)[c]
25	20.7	Adhyperforin[a]	292	*549*	397, *329*	191, 301, 285	Tatsis et al. (2007)[c]

Trivial names are given in parenthesis. MS[a] data are ranged in descending order of relative intensity. Parent ions for the next fragmentation step are presented in italics.
[a] compound was detected in SJW methanolic extract and in SJW oil macerates
[b] MS data recorded in positive ionization mode, precursor ion represents $[M+H]^+$ species.
[c] consistent with analytical reference standard
sh shoulder

2.3. Method validation

Herbal products, particularly oily plant extracts, exhibit multi-component profiles with intricate matrix constituents. Due to this complexity, their comprehensive characterization still remains challenging. Hence, the development of analytical methods providing reliable results is a key prerequisite. The validation of the UHPLC method, used for the quantitation of individual constituents of SJW oil macerates, was performed according to ICH guidelines Q2(R1) (2005). For this purpose, tests on linearity, precision, accuracy and robustness were carried out individually with reference standards of quercetin, kaempferol, I3, II8-biapigenin, hyperforin and adhyperforin to warrant reproducibility of quantitative results. This allows the application of the present method in pharmaceutical routine control analysis also of samples characterized by a highly complex matrix, such as oily macerates.

2.3.1. Linearity

For assessing linearity, each calibration sample was injected sixfold. The calibration curves, generated by plotting peak areas against sample concentrations, were linear in a range of 2-50 µg/mL (kaempferol), 10-250 µg/mL (quercetin), 2-250 µg/mL (I3, II8-biapigenin), 13-529 µg/mL (hyperforin) and 1-111µg/mL (adhyperforin), respectively. All correlation coefficients were greater than 0.99. Linear equations were obtained by linear regression analysis, and the regression equations of the calibration curves are specified in Table 3. Additionally, linearity was proven by Mandel's fitting test. For the hyperforin calibration curve, a weighting factor of $1/x^2$ was chosen, according to a minimum sum of the absolute values of the relative errors (Almeida et al. 2002).

2.3.2. Limits of detection (LOD) and quantitation (LOQ)

The limit of detection and the limit of quantitation were based on signal-to-noise ratios. For this purpose, the means of eight blank samples were compared with the lowest concentration of each calibration sample. According to ICH guidelines, a signal-to-noise ratio of 3:1 for LOD and of 10:1 for LOQ determination was applied (Table 3).

2.3.3. Precision

Repeatability, intraday and interday precision were calculated with three concentration levels of each of the aforementioned standard compounds. For proving intraday precision, each sample was injected sixfold on the same day. Interday precision and repeatability were

evidenced by analyzing each concentration level sixfold on two different days. The results for quercetin, kaempferol and I3, II8-biapigenin are presented in Table 4 and for hyperforin and adhyperforin in Table 5, respectively. Standard deviations were < 2% for all injections. Thus, the method is considered to be reliable and reproducible.

2.3.4. Accuracy

For assessing accuracy, 40 µL of a stock solution mixture containing quercetin, kaempferol, I3, II8-biapigenin in concentrations of 500 µg/mL and 250 µL of a stock solution mixture containing hyperforin (265 µg/mL) and adhyperforin (56 µg/mL) were added to 2 g of *Arachis* oil, and sample preparation was performed in the same way as for oil macerate samples by means of twofold solvent extraction with methanol (for a more detailed description see section 3.3.). Recovery was determined by comparing peak areas of spiked samples to those of the appropriate calibration standard prior to spiking. For I3, II8-biapigenin (c = 10 µg/mL) recovery was 87.8 %, for quercetin (c = 10 µg/mL) 76.0%, for kaempferol (c = 10 µg/ml) 79.4 %, for hyperforin (c = 132 µg/mL) 87.2 % and for adhyperforin (c = 28 µg/mL) 85.8 %, respectively. Considering the complex lipophilic matrix of the *Arachis* oil, recovery was in an acceptable range and, thus the method is regarded sufficiently accurate (Xiu-Qin et al. 2009).

2.3.5. Robustness

For sample stability testing, standard solutions of three concentration levels were stored in the autosampler of the HPLC system at ambient temperature. Hyperforin and adhyperforin were found to be stable over three days (Table 6), whereas quercetin and kaempferol showed instability under these conditions. The contents of the latter two compounds significantly decreased within only one day, and higher instability was observed at low concentration levels (Table 7). After two days of storage, I3, II8-biapigenin was also partly degraded, retaining 93.3-96.3 % of the initial amounts. It can therefore be concluded, that standard solutions as well as test solutions should be analyzed immediately after preparation.

2.4. Quantitation of secondary metabolites via UHPLC-DAD

The flavonol aglyca quercetin and kaempferol, the biflavone biapigenin as well as the phloroglucinols hyperforin and adhyperforin were detected in all oil macerates prepared in this study (Fig. 2). Quantitation of these compounds was carried out by external calibration with the respective reference compounds. Considering quercetin recovery, oil macerates prepared under illumination with artificial light (O3) (14.7±1.19 µg/g) and sunlight (O4) (14.8±0.84 µg/g) showed lowest amounts (Fig. 3). Highest concentrations were achieved, when extracts were prepared under light exclusion at 50 °C (O6) (21.8±0.62 µg/g). Quercetin contents of oil extracts obtained in the dark at 5 °C, RT and 40 °C amounted to 19.6±0.9 µg/g, 18.3±0.3 µg/g, and 18.4±0.9 µg/g, respectively. In comparison to these findings, kaempferol contents were generally lower, ranging from 3.0±0.1 µg/g (O2) to 5.4±0.4 µg/g (O6). Similar results were obtained for the contents of I3, II8-biapigenin. Oil macerates prepared at 5 °C in the dark (O1) resulted in minimal contents (4.4±0.2 µg/g), whereas an increase in temperature (50 °C, darkness; O6) brought about maximal values (7.4±0.4 µg/g). Hyperforin concentrations ranged from 52.6±46.0 µg/g to 451.4±24.9 µg/g with the highest amounts being determined in O2 (Fig. 4). In contrast, light exposure during the extraction process seemed to deplete hyperforin yields, as can be deduced from concentrations of 52.6±46.0 µg/g (O3) and

Table 3: Calibration curves of I3, II8-biapigenin, quercetin, kaempferol, hyperforin and adhyperforin generated from calibration standard solutions, limit of detection (LOD) and limit of quantitation (LOQ)

Compound	n	Regression equation[a] $y = ax + b$		R^2	LOD (µg/mL)	LOQ (µg/mL)
		Slope (a) (n=6)	Intercept (b) (n=6)			
I3, II8-Biapigenin	7	0.4691	0.7523	0.9999	0.17	0.56
Quercetin	5	0.6167	-6.6461	0.9992	0.41	1.38
Kaempferol	5	0.5218	-1.0472	0.9985	0.34	1.14
Hyperforin	6	0.2632	0.1862	0.9975	0.07	0.22
Adhyperforin	7	0.2643	0.0400	0.9993	0.05	0.17

n, number of calibration levels; R^2, correlation coefficient
[a] y refers to the peak area [mAU × min]; x refers to the concentration of the corresponding reference

Table 4: Repeatability and intermediate precision for I3, II8-biapigenin, quercetin and kaempferol obtained with standard solutions of three different concentrations

Compound	Theoretical sample conc. (µg/mL)	Calculated mean conc. (µg/mL)	RSD intraday (%)	RSD interday (%)	Mean recovery (%)
I3, II8-Biapigenin	5.1	6.0	1.32	1.96	102.5
	25.3	23.4	1.07	0.39	98.7
	101.2	101.5	1.06	1.64	98.5
Quercetin	5.0	-[a]	-[a]	-[a]	-[a]
	24.9	24.7	1.46	0.93	98.2
	99.5	95.9	0.93	1.95	95.0
Kaempferol	5.1	5.2	1.33	1.87	104.7
	25.6	23.7	0.45	0.40	95.2
	102.3	114.5	0.51	1.52	94.6

[a] Data outside of validated linear quantitation range
RSD, relative standard deviation

Table 5: Repeatability and intermediate precision for hyperforin and adhyperforin obtained with standard solutions of three different concentrations

Compound	Theoretical sample conc. (µg/mL)	Calc. mean conc. (µg/mL)	RSD intraday (%)	RSD interday (%)	Mean recovery (%)
Hyperforin	5.3	-[a]	-[a]	-[a]	-[a]
	52.9	57.5	0.26	0.59	100.5
	529.2	486.9	0.18	0.31	101.2
Adhyperforin	1.1	1.0	1.11	1.47	100.1
	11.2	11.9	0.19	0.63	100.5
	111.6	112.0	0.21	0.17	101.3

[a]Data outside of validated linear quantitation range
RSD, relative standard deviation

Table 6: Assessment of robustness by storage of hyperforin and adhyperforin samples in the autosampler for three consecutive days at ambient temperature and subsequent HPLC analysis in triplicate

Compound	Theoretical sample conc. (µg/mL)	RSD (%)	Mean recovery (%)
Hyperforin	5.3	-[a]	-[a]
	52.9	0.33	102.3
	529.2	0.10	101.4
Adhyperforin	1.1	1.61	102.4
	11.2	0.52	101.9
	111.6	0.37	100.8

[a]Data outside of validated linear quantitation range
RSD, relative standard deviation

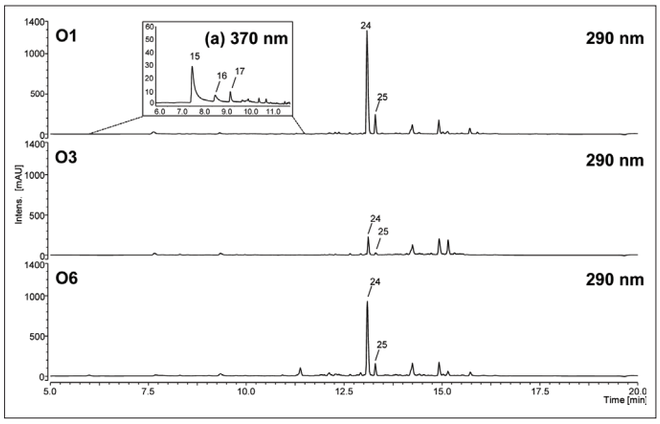

Fig. 2: UHPLC-DAD profile of three SJW *Arachis* oil extracts: O1 (D, 5 °C), O3 (AL, 20 °C), O6 (D, 50 °C), recorded at 290 nm with flavonoids being detected at 370 nm (small insert). Peak assignment: (15) quercetin, (16) kaempferol, (17) I3, II8-biapigenin, (24) hyperforin, (25) adhyperforin.

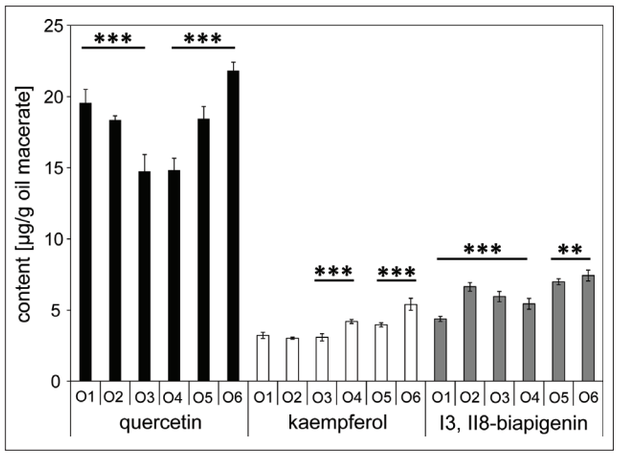

Fig. 3: Contents of flavonols and biflavones obtained upon extraction under different conditions: O1 (D, 5 °C), O2 (D, RT), O3 (AL, 20 °C), O4 (SL, RT), O5 (D, 40 °C), O6 (D, 50 °C). Bars represent means ± standard deviation (n=3). Quantitation of quercetin and kaempferol was carried out at 370 nm, and quantitation of I3, II8-biapigenin at 330 nm, respectively. Sample preparation was performed in duplicate, and each sample was analyzed in duplicate. The asterisks indicate significant differences (**p < 0.05, ***p < 0.01, Tukey's range test).

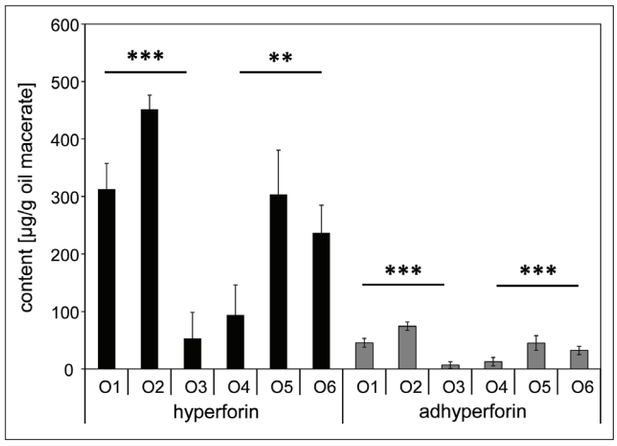

Fig. 4: Phloroglucinol contents obtained upon extraction under different conditions: O1 (D, 5 °C), O2 (D, RT), O3 (AL, 20 °C), O4 (SL, RT), O5 (D, 40 °C), O6 (D, 50 °C). Bars represent means ± standard deviation (n=3). Quantitation of hyperforin and adhyperforin was carried out at 290 nm. Sample preparation was performed in duplicate, and each sample was analyzed in duplicate. The asterisks indicate significant differences (**p < 0.05, ***p < 0.01, Tukey's range test).

Table 7: Assessment of robustness by storage of I3, II8-biapigenin, quercetin and kaempferol samples in the autosampler at ambient temperature and subsequent HPLC analyses after one day and after two days

Compound	Theoretical sample conc. (µg/mL)	RSD (%)		Mean recovery (%)	
		Day 1	Day 2	Day 1	Day 2
I3, II8-Biapigenin	5.1	2.59	2.28	96.66	95.44
	25.3	0.43	1.87	94.90	93.32
	101.2	1.79	1.87	99.11	96.25
Quercetin	5.0[a]	-[a]	-[a]	-[a]	-[a]
	24.9	0.15	1.73	90.34	82.27
	99.5	1.40	2.17	96.94	92.66
Kaempferol	5.1	1.66	1.44	89.62	81.17
	25.6	0.54	0.83	94.04	88.74
	102.3	0.26	0.92	98.95	95.99

[a] Data outside of validated linear quantitation range
RSD, relative standard deviation

93.9±52.2 µg/g (O4). As evidenced by the large standard deviations, phloroglucinols are most unstable when exposed to light (Orth and Schmidt 2000). Adhyperforin revealed the same extraction behavior as hyperforin, even though absolute values were generally lower, ranging from 6.9±5.7 µg/g to 74.5±7.1 µg/g.

2.5. Total naphthodianthrone contents in oil macerates

The characteristic intensive red color of SJW oil macerates presumably originates from lipophilic derivatives of hypericin as a result of the influence of sunlight during maceration (Maisenbacher and Kovar 1992). Its degradation has been described to be due to an extractive photolysis as a result of illumination. Since a reasonable separation of these derivatives via HPLC has not yet succeeded, concentrations of these derivatives have been determined spectrophotometrically, which allows rapid estimation of contents in routine control analyses. In order to compare extracts, which were prepared under different light and temperature conditions, the contents of lipophilic hypericin derivatives were calculated as total hypericin equivalents, based on the specific absorption of hypericin at 590 nm (Ph. Eur. 2014). O6 and O5 revealed highest amounts of total hypericin (3.46±0.095 mg/100 g and 2.23± 0.05 mg/100 g) in contrast to O1, the contents of which were comparatively lower (0.47±0.02 mg/100 g) (Fig. 5). It can, thus, be concluded that temperature exerts the most predominant impact on the recovery of hypericin derivatives. In contrast, extraction under illumination seemed to have only minor effects on total hypericin amounts of

Table 8: Color characteristics (*L, *a**, *b**, *C**, *h*°) of different oil extracts: O1 (D, 5 °C), O2 (D, RT), O3 (AL, 20 °C), O4 (SL, RT), O5 (D, 40 °C), O6 (D, 50 °C), values represent means ± standard deviation (n=3), different letters (vertically) indicate significant differences (p < 0.05, Tukey's range test)**

sample	*L**	*a**	*b**	*C**	*h*°
O1	78.8 ± 0.8 [a]	-4.6 ± 0.3 [f]	43.5 ± 2.7 [a]	43.8 ± 2.7 [b]	96.1 ± 0.8 [a]
O2	59.9 ± 1.1 [b]	5.0 ± 0.3 [e]	43.9 ± 0.1 [a]	44.1 ± 0.1 [b]	83.5 ± 0.4 [b]
O3	56.8 ± 0.7 [c]	19.4 ± 0.7 [c]	25.6 ± 1.1 [d]	32.2 ± 1.0 [d]	52.8 ± 1.4 [e]
O4	56.3 ± 0.8 [c]	15.9 ± 1.3 [d]	34.6 ± 0.7 [c]	38.1 ± 0.3 [c]	65.3 ± 2.2 [c]
O5	41.9 ± 0.2 [d]	24.4 ± 0.6 [b]	38.2 ± 0.7 [b]	45.3 ± 0.4 [b]	57.4 ± 1.0 [d]
O6	31.9 ± 0.6 [e]	42.5 ± 0.3 [a]	36.3 ± 0.6 [bc]	55.9 ± 0.3 [a]	40.5 ± 0.6 [f]

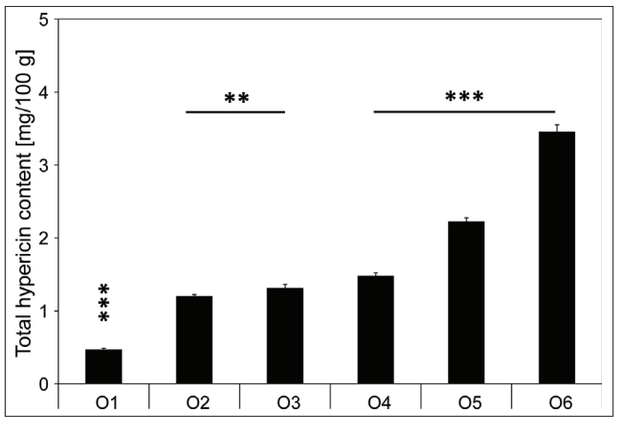

Fig. 5: Total hypericin contents obtained upon extraction under different conditions: O1 (D, 5 °C), O2 (D, RT), O3 (AL, 20 °C), O4 (SL, RT), O5 (D, 40 °C), O6 (D, 50 °C). Bars represent means ± standard deviation (n=3). The asterisks indicate significant differences (**p < 0.05, ***p < 0.01, Tukey's range test).

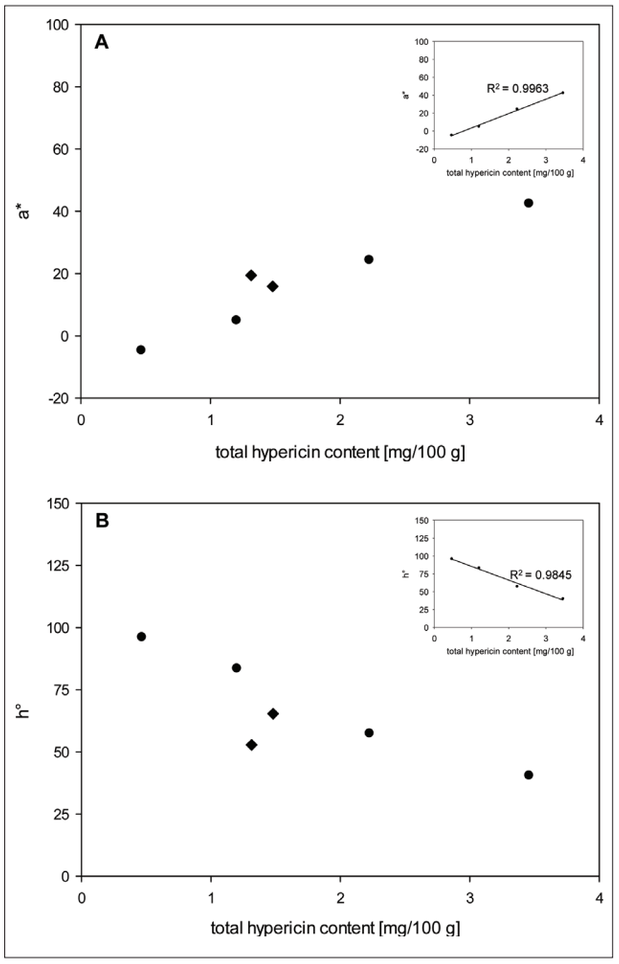

Fig. 6: Correlation between color values and total hypericin contents [mg/100 g] of oil macerates prepared under different conditions. Dots represent macerates prepared under light exclusion: O1 (D, 5 °C), O2 (D, RT), O5 (D, 40 °C) and O6 (D, 50 °C), diamonds represent macerates prepared under illumination: O3 (AL, 20 °C) and O4 (SL, RT). (A) Correlation between *a**-value and total hypericin content. The inserted illustration outlines the linear relationship. (B) Correlation between hue angle *h*° and total hypericin content. The inserted illustration outlines the linear relationship. In both cases the straight line was generated by linear regression analyses with R^2=0.9963 and 0.9845, respectively.

oil macerates, since O2 and O3 only slightly differed with regard to this parameter (1.20±0.02 mg/100 g and 1.31±0.05 mg/100 g). O4 (1.48±0.04 mg/100 g) revealed only marginally higher values as compared to O3 and may be explained by a temperature increase as a result of solar irradiation, which, however, was not monitored. We therefore assume that the conversion of hypericin into the aforementioned lipophilic hypericin derivatives in oil macerates is rather depending on temperature conditions than on light exposure.

2.6. Color measurements

The color impression of the oils prepared under different extraction conditions varied markedly. Consequently, CIE *L*C*h*° color measurements were performed to obtain objective data. As can be seen from Table 8, *a**-values, which are a quantitative measure of red hues, differed within a wide range and were increased with higher extraction temperatures. O1 revealed the lowest *a**-value, indicating only a slightly reddish hue. In contrast, samples of O6 showed the highest *a**-value, which translates into a deep red solution. Quite obviously, sunlight as well as artificial light significantly increase *a**-values. Nevertheless, elevated temperatures (50 °C) seemed to have the most predominant impact on the intensity of the red color of the oil. Furthermore, a large disparity of *b**-values of oils prepared under light exclusion (O1, O2, O5, O6) was not observed. However, a remarkable decrease of *b** may be observed, when light is abundant during extraction. Artificial light seems to have the greatest influence on the decrease of yellowness (*b**), whereas temperatures up to 50 °C do not affect this parameter. Based on these findings for *a** and *b** values, highest color saturation (metric chroma, *C**) was calculated for samples extracted at 50 °C under light exclusion. Lowest *C**-value was obtained, when extraction was performed at 20 °C under illumination with artificial light. Hue angles (*h*°), which are also calculated from *a**- and *b**- values, ranged from 40.5±0.6° to 96.1±0.8°, indicating color shades from magenta-red (0°) to yellow (90°) and bluish green (180°). As far as extraction under light exclusion is concerned, *h*° values considerably decreased with increasing temperature, indicating more intense red color hues.

*2.7. Correlation between CIE L*C*h° color values and hypericin contents*

Oil macerates, which were prepared at 50 °C under light exclusion, revealed greatest hypericin contents, whereas lower temperatures resulted in lower yields. It can therefore be concluded, that elevated temperatures improve the recovery of these compounds. In agreement with these findings, these oils showed highest *a**- and lowest *h*°-values in color measurements. Figure 6 illustrates the correlation between CIE *L*C*h*° color values and hypericin contents. Both *a**- and *h*°-values were found to be proportional to the total hypericin content for oil macerates solely prepared under light exclusion. However, it can also be deduced that there is no direct linear correlation between total hypericin contents and color values of all oil macerates in general, since only hypericin contents of extracts prepared under the exclusion of light were proportional to *a**- and *h*°-values.

2.8. Conclusion

The validated UHPLC method presented in this paper allows the quantitation of characteristic SJW plant secondary metabolites. It therefore provides reliable and reproducible results. All SJW oil macerates characterized in this study were prepared with *Arachis* oil according to a standardized protocol (DER_{native} 1:4), systematically evaluating the effects of light and temperature conditions on the recovery of individual compounds and color evolution. Five oily extract constituents, which have previously been reported in the literature, i.e. quercetin, kaempferol, I3, II8-biapigenin, hyperforin and adhyperforin (Maisenbacher and Kovar 1992; Zdunic et al. 2009; Isacchi et al. 2007, Arsic et al. 2010; Orhan et al. 2013) were identified and quantitated. More polar constituents, such as hydroxycinnamic acids, flavanols including proanthocyanidins and flavonol glycosides, could only be detected in methanolic SJW plant extracts and assigned by UHPLC-DAD-MS^n analyses. The latter compounds were not found in oil macerates, which is assumed to be due to their physicochemical features and the selectivity of *Arachis* oil extraction. Consequently, the yield and profile of secondary metabolites of the corresponding preparations may be modulated by varying the hydrophilicity and lipophilicity of the extractant (Gaedcke 2003). Moreover, lipophilic hypericin derivatives were quantitated as total hypericin based on spectrophotometric analyses. Elevated temperatures led to maximal recovery of hypericin derivatives. However, more detailed analyses are required, since the exact structure of these derivatives still remains unknown. The present study provides valuable information about the SJW extraction process, and markedly expands the knowlege of the influence of light and temperature on the recovery and quantity of flavonols, biflavones, phloroglucinols and napththodianthrones using *Arachis* oil as extractant. The same set-up will also allow the determination of individual compounds in SJW extracts obtained with further apolar and polar solvents, thus being also applicable to the analysis of a wide range of *Hypericum* preparations in routine control.

3. Experimental

3.1. Plant material

SJW fresh plant material was collected manually in Bad Boll, Germany, in June 2014. For this purpose, the top 6 cm of the plants, mainly comprising the flowers, were cut and frozen at -80 °C immediately after harvesting. The plant material was identified by Prof. Dr. O. Spring (Institute of Botany, Hohenheim University, Stuttgart, Germany), and a sample item with the voucher number HOH – 0140241 was deposited at the herbarium of Hohenheim University.

3.2. Extraction of the plant material and further sample preparation

3.2.1. Preparation of methanolic extracts

Methanol (MeOH, 25 mL) was added to 2.5 g of frozen plant material. The mixture was homogenized with an Ultraturrax® (2 min, 21,000 rpm, IKA Werke GmbH & Co. KG, Staufen, Germany). The first extraction step was performed at room temperature for 2 h under light exclusion. Separation of the supernatant and solid residues was achieved by centrifugation (10 min; 4,570*xg;* Heraeus Multifuge 1S, Thermo Fisher Scientific GmbH, Dreieich, Germany). Subsequently, the remaining plant material was re-extracted with 25 mL of MeOH under the same conditions as previously described for further 16 h and centrifuged. The combined supernatants were made up with MeOH to a final volume of 50.0 mL. All samples were prepared in triplicate.

3.2.2. Preparation of oil extracts

A standardized protocol for the preparation of oil macerates of SJW plant material was developed, which was in accordance with the Supplement to the German Pharmacopoeia (DAB, Erg.-B. 1941). For this purpose, 25 g of frozen plant material were minced in a variable speed laboratory blender (Waring Laboratory, Torrington, CT, USA) prior to maceration in 100 g of refined Arachis oil (Pharmacopoea Europaea, Ph. Eur., Henry Lamotte Oils GmbH, Germany). After 28 days of extraction in transparent glass vessels (clear glass according to DIN/ISO 719, hydrolytic class III, transmission in the range of 350 nm to 1100 nm > 90%, Heinz-Glas, Kleintettau, Germany), the samples were manually pressed through a cotton cloth and dried over anhydrous sodium sulfate. Extraction was performed in three individually prepared batches. Individual extraction conditions for obtaining the different oil macerates are specified in Table 1. Extraction under illumination with artificial light was operated in a climate chamber (Binder, Tuttlingen, Germany) continuously controlling temperature (20 °C) and a 24 h/day illumination (L36W/840 and LT36W/009, Osram, Munich, Germany) to guarantee reproducible conditions. To ensure homogeneous light exposure, samples were rotated recurrently within the climate chamber. In contrast, for extraction under sunlight conditions, thus mimicking preparation according to the German Pharmacopoeia, samples were exposed for 28 days to the natural day/night cycle at ambient temperature (21±3 °C) on a windowsill facing towards south (November, 85 h of sunshine duration in total).

3.3. Sample preparation for UHPLC-DAD and HPLC-DAD-MS/MS analyses

Method development was focused on the enrichment of the target compounds, which was accomplished by solvent partition. At the same time, this approach allowed the removal of major parts of the oil matrix, which may interfere with subsequent UHPLC analyses. Thus, aliquots of 2 g of oil extracts were combined with 10 mL of MeOH and vortexed for 2 min. After centrifugation (10 min, 4,570*xg*; Heraeus Multifuge 1S, Thermo Fisher Scientific GmbH, Dreieich, Germany), the residue was re-extracted with 10 mL of MeOH to ensure quantitative recovery of the analytes and vortexed for another 2 min followed by centrifugation. The combined supernatants were evaporated *in vacuo* (T = 38 °C). The residues were dissolved in 2 mL of MeOH, vortex mixed and centrifuged (10 min; 20,800*xg*; Centrifuge 5417 C, Eppendorf AG, Hamburg, Germany). All samples were extracted in duplicate and filtered through a syringe filter (pore size 0.2 µm, PTFE; WICOM, Heppenheim, Germany) prior to chromatographic analyses.

3.4. Chemicals and reagents

Chemicals for sample extraction, namely analytical grade methanol (MeOH) and anhydrous sodium sulfate were purchased from Merck (Darmstadt, Germany). For chromatographic analysis, acetonitrile (HPLC grade) was obtained from J.T. Baker (Deventer, Netherlands). Acetonitrile and acetic acid (both LC-MS grade) were purchased from Sigma Aldrich (St. Louis, MO, USA) and from Fluka (Buchs, Switzerland). Purified water (0.055 µS/cm) was used throughout and obtained from a Purelab Option-Q system (Elga Berkefeld GmbH, Celle, Germany). Chemicals used as reference standards for quantitation and/or for qualification purposes were I3, II8-biapigenin, as well as hyperforin and adhyperforin as dicyclohexylammonium salts (Phytolab GmbH, Vestenbergsgreuth, Germany), quercetin (USP reference standard, Rockville, USA) and kaempferol (Sigma Aldrich, Steinheim, Germany).

3.5. Stock solutions and calibration standards

Stock solutions of quercetin, kaempferol and I3, II8-biapigenin were separately prepared by dissolving 10 mg of each reference standard in 5 ml of MeOH. These three stock solutions were combined to obtain a stock solution mixture containing quercetin, kaempferol, and I3, II8-biapigenin at concentrations of 500 µg/mL each. Subsequently, calibration samples with concentrations ranging from 2 to 250 µg/mL were provided by diluting the stock solution mixture with MeOH. Stock solutions of hyperforin and adhyperforin were prepared by diluting 10 mg of a reference standard consisting of hyperforin and adhyperforin as a stable dicyclohexylammonium salt with 5 ml of MeOH. Calibration samples were prepared by dilution of the stock solution with MeOH to obtain hyperforin and adhyperforin solutions with concentrations ranging from 13 to 529 µg/mL and 1 to 111 µg/mL, respectively.

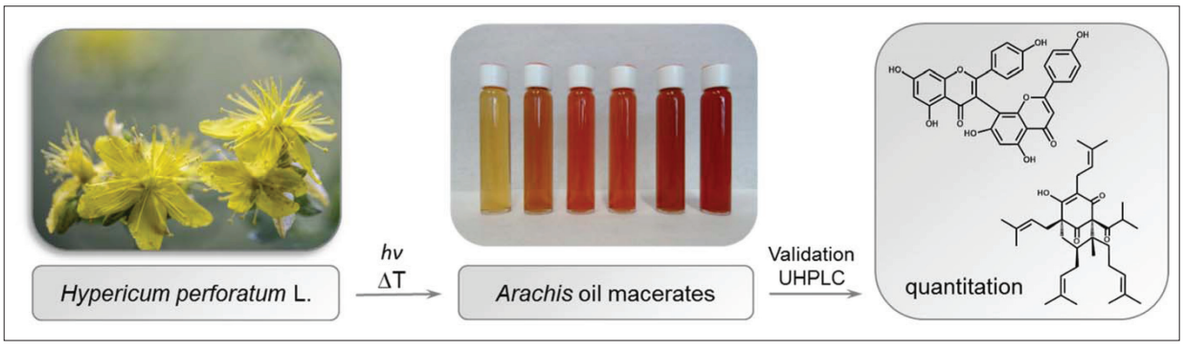

Fig. 7: Study design

3.6. Instrumental analyses

3.6.1. Spectrophotometric measurements

Total hypericin contents were determined by spectrophotometric analyses using a UV-Vis spectrophotometer (Lambda 35, Perkin Elmer, Rodgau-Jüdesheim, Germany) equipped with a UV-Vis software (UV Winlab ES Version 6.0.2). For this purpose, aliquots of 1 g of oil macerate were diluted with 4 g of *Arachis* oil und mixed thoroughly. Subsequently, absorption was measured at 590 nm in a 1 cm path length cuvette against pure *Arachis* oil as a blank. For the calculation of total hypericin contents as hypericin equivalents per 100 g oil macerate, a specific absorption coefficient of 870 [1/ (% × cm)], reported for hypericin in methanolic solution (Ph. Eur. 2014) was applied. Quantitation was performed using the following eq.:

hypericin content [mg/100g] = $((w_T + w_S)/w_S) \times (A \times 1000/870))$

with w_T being the weight of the test sample, w_S the weight of the solvent and A the measured absorption at the specific wavelength of 590 nm.

3.6.2. Color measurements

Color analyses of oil macerates were carried out using a UV-Vis spectrophotometer (Lambda 35, Perkin Elmer, Rodgau-Jüdesheim, Germany) equipped with a UV-Vis (UV Winlab ES Version 6.0.2) and color software (Color version 3.00, both Perkin-Elmer, Shelton, Conn. USA). The samples were centrifuged (10 min; 20,800*xg*; Centrifuge 5417 C, Eppendorf AG, Hamburg) prior to analyses. According to the International Commission of Illumination (CIE), objective color values were determined based on transmission measurements covering the range from 350 nm to 800 nm. Chroma [$C^* = (a^{*2} + b^{*2})^{1/2}$] and hue angle [$h^\circ = (\arctan b^*/a^*)$] were calculated using illuminant D65 and a 10° observer angle (Gonnet 1998).

3.6.3. LC-MS^n analyses

Separation was performed using an Agilent 1200 HPLC system (Agilent, Waldbronn, Germany) with a vacuum degasser, a binary gradient pump, an autosampler, a thermostatic column compartment and a diode array detector. A Hypersil Gold RP C18 column (50 x 2.1 mm i.d., 1.9 µm particle size; Thermo Scientific, Dreieich, Germany), protected with a C18 Guard Cartridge System SecurityGuard ULTRA (2.1 mm i.d.; Phenomenex, Aschaffenburg, Germany) was operated at 38 °C using a binary mobile phase gradient consisting of 10 mM ammonium acetate buffer (adjusted to pH 5 with acetic acid) as eluent A and acetonitrile as eluent B. The flow rate was set at 0.45 mL/min applying the following gradient system: 0-1 min, 0 % B; 1-2.2 min, 0-5 % B; 2.2-9 min, 5-17 % B; 9-12.3 min, 17-30 % B; 12.3-21 min, 30-100 % B; 21-25.7 min, 100 % B; 25.7-27.7 min, 100-0 % B; 27.7-32.4 min, 0 % B. Injection volume was 5 µL. DAD spectra were recorded in a wavelength range of 200 - 700 nm. The LC system was coupled to an HCT ultra ion trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) interfaced with an electrospray ionization (ESI) ion source, which was operated in the positive as well as in negative ionization mode. The following acquisition parameters were applied: capillary voltage, -4000 V (positive ion polarity) and +4000 V (negative ion polarity), dry gas flow (N_2), 9 L/min; nebulizer pressure, 40 psi and capillary temperature, 365 °C. Mass spectra were recorded in a range of *m/z* 150 - 1,200. MS^n experiments were carried out with a compound stability and trap drive level of 100% in the auto MS/MS mode. Data acquisition and evaluation were monitored using Agilent ChemStation (Rev. B.01.03 SR1) and Bruker Daltonik esquire control software (v. 6.1).

3.6.4. UHPLC-DAD analyses

Quantitation of individual compounds were carried out on a Thermo Fisher Scientific Dionex Ultimate 3000 RSLC system (Thermo Fisher Scientific GmbH, Dreieich, Germany) equipped with a vacuum degasser, a binary gradient pump, an autosampler, a thermostatic column compartment operated at 38 °C and a diode array detector (DAD). To speed up chromatographic separation allowing the quantitative analysis of large sample numbers, the flow rate was slightly increased to 0.6 mL/min, applying the following adapted gradient system: 0-0.9 min, 0-5 % B; 0.9-6 min, 5-17 % B; 6-8.5 min, 17-30 % B; 8.5-15 min, 30-100 % B; 15-18.5 min, 100 % B; 18.5-20 min, 100-0 % B; 20-21.3 min, 0 % B without affecting resolution as compared to the chromatographic system described under 3.5.3. Injection volume was 5 µL, using the same analytical column and the identical solvent composition as aforementioned. Detection was performed at 270, 290, 330, 370, and 590 nm, with UV/Vis spectra being recorded in a wavelength range of 200 to 700 nm. Chromeleon software (v. 7.1, Dionex, Idstein, Germany) was used for system control and data processing.

3.6.5. Statistical analyses

For statistical analysis, data were processed with Minitab software, Version 17 (Minitab Inc., State College, PA, USA). Significant differences ($p < 0.05$ and $p < 0.01$, respectively) were determined by one-way ANOVA followed by Tukey's range test, thus comparing all possible pairs of means. Values are presented as means ± SD for the indicated number of independent experiments.

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