Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 45 (2007) 437–442
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Quantitative analysis of iridoids, secoiridoids, xanthones and xanthone glycosides in *Gentiana lutea* L. roots by RP-HPLC and LC–MS

Anita Aberham, Stefan Schwaiger, Hermann Stuppner, Markus Ganzera *

Institute of Pharmacy, University of Innsbruck, Innrain 52, 6020 Innsbruck, Austria

Received 10 May 2007; received in revised form 2 July 2007; accepted 3 July 2007
Available online 10 July 2007

Abstract

The here described HPLC-method enables the determination of all major, currently known bioactive compounds in gentian roots. A separation of iridoids (loganic acid), secoiridoids (swertiamarin, gentiopicroside, amarogentin, sweroside), xanthones (gentisin, isogentisin) and two xanthone glycosides (gentiosides) was possible on RP-18 column material, using 0.025% aqueous TFA, acetonitrile and *n*-propanol as mobile phase. The method is sensitive (LOD ≤ 37 ng/ml and LOQ ≤ 112 ng/ml), accurate (recovery rates of spiked samples were between 96.7 and 101.5%), repeatable ($\sigma_{rel} \leq 1.7\%$) and precise (intra-day variation ≤ 4.6%, inter-day variation ≤ 3.1%). LC–MS experiments performed in negative ESI mode assured peak purity and identity. Analysis of several commercially available *G. lutea* samples showed that gentiopicroside is the most dominant compound in the specimens (4.46–9.53%), followed by loganic acid (0.10–0.76%), swertiamarin (0.21–0.45%) and the xanthone glycosides. Gentisin and isogentisin were found in much lower concentrations between 0.02 and 0.11%, respectively.

Keywords: *Gentiana lutea*; Gentian; Secoiridoids; Xanthones; Reversed-phase chromatography; Mass spectrometry

1. Introduction

The roots of *Gentiana lutea* L. (Gentianaceae), a yellow flowering, perennial plant commonly found in the mountainous regions of central and southern Europe are a popular ingredient in many gastric stimulant herbal preparations [1]. This is mainly due to the occurrence of bitter tasting secoiridoid-glycosides (e.g. swertiamarin (**2**), gentiopicroside (**3**), amarogentin (**4**) and sweroside; see Fig. 1 for structures) in the plant, which revealed cholagogue, hepatoprotective and wound-healing effects in pharmacological studies [2,3]. Not only secoiridoids are relevant for the biological effects of gentian, other constituents such as the iridoid loganic acid (**1**; with anti-inflammatory activity [4]), xanthone glycosides (gentioside and its isomer; **5**, **6**) and xanthones like gentisin (**7**) and isogentisin (**8**) are relevant as well. Gentiosides and xanthones showed a potent inhibition of MAO type A and B in vitro [5], and a recent study revealed that compound **8** (but not **7**) possesses protective effects against endothelial damage caused by cigarette smoking [6].

In order to evaluate quality and efficiency of *G. lutea* plant material it is, therefore, required focusing on all pharmacologically relevant groups of compounds, which are iridoids, secoiridoids and xanthones. Despite the fact that the analysis of secoiridoids in gentian roots by HPLC [7–9], CE [10] and TLC [11] has been described several times previously, there are only two reports of analytical methods for the simultaneous determination of bitter principles and xanthones in *G. lutea* to our knowledge [12,13]. But none of these HPLC-methods allowed the determination of all compounds of interest (e.g. no separation of **7** and **8** was achieved, loganic acid was not assayed) nor were they validated. These missing (and required) facts encouraged us to reevaluate and improve the currently available methodology for *G. lutea* analysis and to utilize this new method for a quality assessment of several commercially available gentian root specimens.

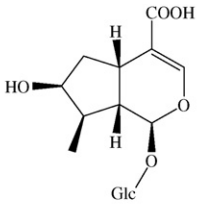
2. Experimental

2.1. Materials

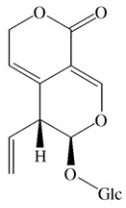
G. lutea plant material for the isolation of compounds **5**–**8** was purchased from Mag. Kottas & Sons (Vienna, Austria; batch:

* Corresponding author. Tel.: +43 512 507 5307; fax: +43 512 507 2939.
E-mail address: Markus.Ganzera@uibk.ac.at (M. Ganzera).

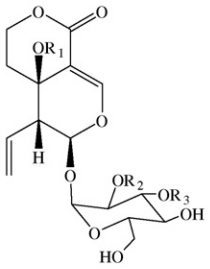
0731-7085/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2007.07.001

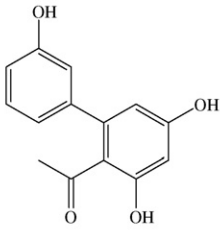


Loganic acid (1)



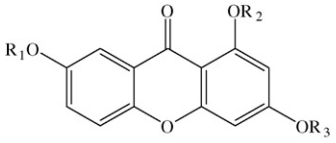
Gentiopicroside (3)





Residue A

	R_1	R_2	R_3
Swertiamarin (2)	OH	H	H
Amarogentin (4)	H	*A*	H
Sweroside (a)	H	H	H



	R_1	R_2	R_3
Gentioside (5)	CH_3	H	*Prim*
Gentioside isomer (6)	*Prim*	H	CH_3
Gentisin (7)	H	H	CH_3
Isogentisin (8)	CH_3	H	H

Prim = Xyl(1,6)Glc-; Primeverose

Fig. 1. Chemical structures of the determined *Gentiana lutea* constituents.

A011167-001; sample: GL-1), isolation was performed by chromatographic methods as described earlier [6]. Identity and purity of the compounds were confirmed by chromatographic (TLC, HPLC) and spectroscopic (1D and 2D NMR, LC–MS) methods in reference to literature values [14]. Reference compound **1** (loganic acid) was purchased from Extrasynthese (Genay, France), secoiridoids **2**–**4** were a gift of Bionorica AG (Neumarkt, Germany). Purity of all standard compounds was ≥97% (determined by HPLC).

Samples GL-2 to GL-12 (*G. lutea* roots or crude cut material) were obtained from diverse vendors in Germany and Austria, and they were authenticated by Prof. Christian Zidorn, from the Institute of Pharmacy, University of Innsbruck, in Austria. Voucher specimens of all samples are deposited at the herbarium of the same institution. Solvents (water, acetonitrile, *n*-propanol and methanol) and the reagents triflouroacetic acid (TFA), formic acid and acetic acid were of HPLC grade and purchased from Merck (Darmstadt, Germany).

2.2. HPLC sample preparation

The finely powdered root material (0.100 g) was extracted three times with 3 ml methanol by sonication (10 min each, at ambient temperature). After centrifugation at 3000 rpm for 5 min the extracts were combined in one 10 ml volumetric flask, which was then filled up to the final volume with extraction solvent. Prior to injection all solutions were filtered trough a 0.45 μm nylon membrane filter (Phenex, Phenomenex, Torrance, CA, USA); each sample solution was assayed in triplicate.

2.3. HPLC and HPLC-MS conditions

Quantitative analyses were performed on an Agilent 1200 series HPLC instrument, equipped with binary pump, autosampler, column oven and photodiode array detector (Agilent, Waldbronn, Germany). A Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm, 5 μm particle size) from Agilent was utilized as stationary phase, the mobile phase comprised 0.025% of TFA in water (A) and a 1:1 mixture of acetonitrile and *n*-propanol (B). Elution was performed using the following gradient: in 20 min from 99A/1B to 70A/30B, then in 0.5 min to 60A/40B, held this composition for 9.5 min, then in 0.5 min to 5A/95B and held at that composition for another 4.5 min (total runtime 35 min). After each injection a re-equilibration period of 10 min followed. The flow rate was adjusted to 1.0 ml/min, the detection wavelength set to 232 nm and 10 μl of sample were injected. All separations were performed at 30 °C.

LC–MS experiments were performed on an Esquire 3000 ion-trap mass spectrometer (Bruker-Daltronics, Bremen, Germany), which was coupled to the LC apparatus (split ratio 1:3). Elu-

Table 1
Calibration data of compounds **1**–**8**, including regression equation, correlation coefficient (R^2), extinction coefficient (EC), linear range (in μg/ml), limit of detection (LOD; in μg/ml) and limit of quantitation (LOQ; in μg/ml)

	Regression equation	R^2	EC[a]	Linear range	LOD	LOQ
1	$y = 16.296\,X$	0.9999	0.352	220–0.9	0.012	0.035
2	$y = 15.048\,X$	0.9999	0.381	210–0.9	0.013	0.038
3	$y = 5.738\,X$	0.9998	1.000	1920–7.9	0.016	0.047
4	$y = 28.284\,X$	0.9999	0.203	240–1.0	0.037	0.112
5	$y = 20.397\,X$	0.9997	0.281	360–1.5	0.017	0.052
6	$y = 20.342\,X$	0.9998	0.282	340–1.4	0.005	0.015
7	$y = 48.588\,X$	0.9998	0.118	240–1.0	0.012	0.038
8	$y = 50.953\,X$	0.9998	0.116	200–0.8	0.014	0.048

[a] Determined at 232 nm, in relation to gentiopicroside (**3**).

Table 3
Intra- and inter-day precision of the developed HPLC-assay using sample GL-1; results are based on peak area, relative standard deviation in parenthesis

Compound	Intra-day ($n = 5$)			Inter-day ($n = 3$)
	Day 1	Day 2	Day 3	
1	950.4 (1.9)	964.5 (1.2)	967.6 (1.4)	960.8 (1.0)
2	415.4 (1.7)	420.2 (0.5)	424.9 (1.6)	420.2 (1.1)
3	3112.4 (2.2)	3146.8 (0.6)	3153.7 (1.7)	3137.6 (0.7)
4	102.3 (2.4)	106.7 (2.5)	105.9 (3.9)	105.0 (2.3)
5	806.7 (2.7)	830.9 (1.5)	816.3 (1.8)	818.0 (1.5)
6	638.6 (2.7)	653.2 (1.6)	647.6 (1.4)	646.5 (1.1)
7	250.0 (3.5)	249.7 (4.6)	257.5 (1.6)	252.4 (1.7)
8	330.0 (2.6)	324.6 (4.4)	344.3 (1.8)	333.0 (3.1)

ent A was changed and comprised a mixture of water, formic acid and acetic acid in the ratio of 99:0.9:0.1; otherwise the same separation conditions as described above were used. For optimum MS results ionization was performed in negative ESI mode. Nebulizer, dry-gas and probe temperature of the mass spectrometer were set to 30 psi (nitrogen), 10 l/min (nitrogen) and 350 °C, respectively; mass scan range was set in the range of *m/z* 100–1000.

2.4. Calibration

A standard stock solution was prepared by dissolving all eight standard compounds in 5.00 ml methanol (1.00 mg of **1**, **2**, **4**, **7**, **8**; 10.00 mg of **3**; 2.00 mg of **5** and **6**). Five additional calibration levels were prepared by diluting this solution 1:2 with methanol; see Table 1 for calibration data. The standard solutions were stable for at least 1 month if stored at 4 °C (confirmed by re-assaying). Compound **a** (sweroside) was quantified based on the calibration data of the structurally similar **2**. Table 1 also contains extinction coefficients determined at 232 nm in relation to compound **3**, which is commercially available.

2.5. Validation

Accuracy was determined by spiking sample GL-1 with three concentrations of standard compounds (low, medium, high spike). For this purpose, known amounts of **1**–**8** were added to the dry, powdered plant material, which was then extracted and assayed as described before. The actually found amounts in relation to the theoretically present ones were expressed as percent of recovery (Table 2).

Precision of the method was deduced from repeatability of multiple injections (see Table 4 for standard deviations, which were below 2% for all compounds) as well as intra- and inter-day variance of the results. For the latter five portions of sample GL-1 were extracted and assayed under optimized conditions on day 1; the same procedure was repeated on two more days. By comparing variations within the same days intra-day precision was determined, by observing differences within the 3 days inter-day precision was deduced (Table 3).

3. Results and discussion

3.1. Method development

The main disadvantage of all analytical methods reported on *G. lutea* so far is their limitation to single compounds or a group of constituents. They will not help to fully ensure quality and pharmacological potency, as recent studies indicated the impact of numerous compounds in that respect. By focusing on the simultaneous analysis of iridoids, secoiridoids and xanthones in gentian, one faces several problems, which are a wide polarity range of the analytes of interest and their very close structural resemblance in part. Therefore, to achieve a satisfactory separation in reasonable time, all separation parameters had to be carefully assessed.

Table 2
Accuracy of the developed HPLC-assay, based on recovery experiments (low, medium and high spike) utilizing sample GL-1; quantitative values in μg/ml

	Amount in sample	Low spike		Medium spike		High spike	
		Added	Recovery (%)	Added	Recovery (%)	Added	Recovery (%)
1	28.23	2.2	98.11	11.0	98.40	22.0	98.75
2	34.03	2.1	96.80	10.5	97.05	21.0	96.71
3	521.47	19.2	97.17	96.0	97.20	192.0	98.12
4	7.28	2.4	97.76	12.0	99.82	24.0	100.98
5	27.67	3.6	98.05	18.0	101.20	36.0	98.63
6	18.75	3.4	100.75	17.0	101.52	34.0	100.07
7	7.02	2.4	98.02	12.0	97.97	24.0	98.25
8	10.41	2.0	99.49	10.0	100.47	20.0	99.97

Two facts were obvious from initial screening experiments already; firstly, for a good separation the mobile phase had to be acidic (0.025% TFA were added to solvent A), and secondly, by using methanol or acetonitrile the two xanthone aglyca could not be resolved regardless which stationary phase (C-8, C-12, C-18 or phenyl-hexyl) was used. Different particle-sizes (3–5 μm), pore-sizes (80–300 Å) and additives to the mobile phase (e.g. buffers or THF) did not improve the results either. By replacing pure acetonitrile with a 1:1 mixture of ACN and *n*-propanol much better results were obtained, a trend that could further be enhanced by setting the separation temperature to 30 °C. Other temperatures were less advantageous, as either **3** and **a** (≤30 °C) or **7** and **8** (≥30 °C) started to merge again. A Zorbax Eclipse XDB-C18 from Agilent was finally selected as optimum stationary phase as it resulted in the best resolution and peak symmetry; the wavelength of 232 nm showed to be suitable for the sensitive detection of all compounds of interest. With this system a baseline separation of **1**–**8** was possible within less than 30 min (Fig. 2). For LC–MS experiments only the mobile phase (eluent A) had to be changed slightly in order to ionize and detect all compounds of interest in one analytical run (Fig. 4).

3.2. Method validation

Suitability of the developed method for its intended use can be concluded from several analytical parameters. The detector signal was linear in the tested range, with a correlation coefficient of 0.997 and higher (Table 1). The determined limits of detection (S/N ratio of three, based on a 10 μl injection) and limits of quantitation (S/N ratio of ten) were found to be below 37 ng/ml and 112 ng/ml, and indicated the methods sensitivity. MS- and UV-spectra (the latter were obtained using the system containing TFA) revealed that all peaks of interest were free of co-elution and impurities; this and the fact that structurally closely related compounds were baseline resolved confirmed selectivity of the assay.

Accuracy was determined in recovery experiments, in which plant material was spiked with three different concentrations of standard compounds. As seen in Table 2 all results were within the usually required recovery range of 100 ± 5%, with maximum deviations reached for compound **2** (recovery at high spike: 96.7%) and compound **6** (recovery at medium spike: 101.5%), respectively. Relative standard deviations below 1.8% and very stable retention times over the whole study period (approximately 500 injections) indicated the methods repeatability. Finally, over a period of 3 days precision of the assay was determined (Table 3). These results were very consistent as well, and showed maximum deviations of 4.6% (intra-day precision for compound **7**, on day 2) and 3.1% (inter-day precision for **8**) only.

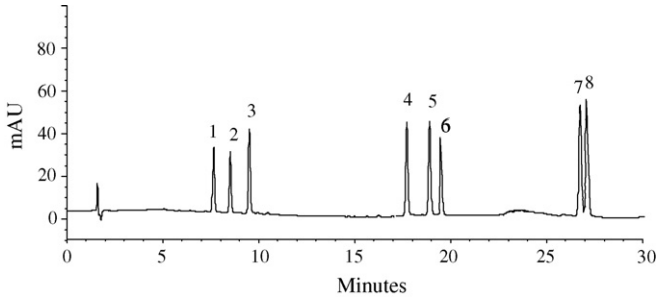


Fig. 2. Separation of a standard mixture of compounds **1**–**8** obtained under optimized HPLC conditions (column: Zorbax Eclipse XDB-C18 column, 150 mm × 4.6 mm, 5 μm; mobile phase: 0.025% TFA in water (A), acetonitrile:*n*-propanol = 1:1 (B)); gradient: 99A/1B in 20 min to 70A/30B, in 0.5 min 60A/40B, hold this composition for 9.5 min, in 0.5 min to 5A/95B, and left at that composition for 4.5 min; flow rate: 1.0 ml/min; sample volume: 10 μl; temperature: 30 °C; detection: 232 nm).

3.3. Analysis of samples

Prior to sample analysis the optimum extraction conditions were determined. Different solvents (methanol, acetonitrile, water and mixtures thereof) and extraction procedures (sonication, shaking or refluxing) were evaluated for this purpose; most efficient and rapid showed to be a repeated sonication of the plant material with methanol (three times sonication for 10 min each). Following this procedure assures a nearly exhaustive extraction, as after the third repetition between 97.9 (compound **5**) and 99.1% (**7**) of the relevant compounds are in solution already; data not shown in detail.

The chromatograms shown in Fig. 3 represent two typical sample preparations. All of the quantified compounds were well resolved and could be assigned without any problem by comparing their retention times and UV-spectra with respective standards. Compound **a** (sweroside) was tentatively identified by a good agreement of its UV-spectra with that of the structurally closely related **2** and by LC–MS studies. For the latter an iontrap mass spectrometer was used, which gave the best results when selecting the negative ESI mode for analysis (Fig. 4); in order to present the results in a clear form the EIC (extracted ion current) mode was selected for presentation. The MS sig-

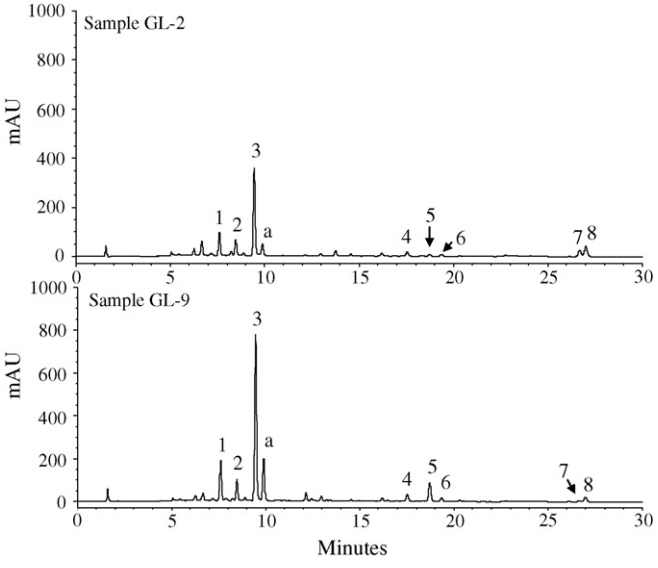


Fig. 3. Separation of samples GL-2 and GL-9 under optimized conditions; peak assignment according to Fig. 1, analytical conditions according to Fig. 2.

Table 4
Quantitative results in percent for samples GL-1 to GL-12; relative standard deviations in parenthesis ($n = 3$)

Sample	1	2	3	a	4	5	6	7	8
GL-1	0.56 (0.14)	0.26 (0.04)	5.24 (0.01)	0.09 (0.17)	0.03 (0.27)	0.37 (0.06)	0.29 (0.13)	0.05 (0.14)	0.06 (0.23)
GL-2	0.37 (0.32)	0.28 (1.33)	4.46 (0.27)	0.26 (0.37)	0.05 (0.33)	0.03 (0.55)	0.03 (0.14)	0.06 (0.18)	0.10 (0.35)
GL-3	0.63 (0.35)	0.21 (0.15)	4.55 (0.21)	0.06 (0.23)	0.03 (0.28)	0.35 (0.07)	0.25 (0.21)	0.04 (0.33)	0.05 (0.20)
GL-4	0.70 (0.17)	0.25 (0.15)	9.26 (0.15)	0.17 (0.15)	0.03 (0.35)	0.11 (0.18)	0.08 (0.09)	0.07 (0.14)	0.08 (0.15)
GL-5	0.59 (1.09)	0.28 (1.14)	6.86 (1.17)	0.05 (1.11)	0.01 (1.72)	0.32 (1.21)	0.28 (1.25)	0.04 (1.07)	0.04 (1.03)
GL-6[a]	0.44 (0.29)	0.37 (1.25)	5.25 (1.29)	0.09 (1.45)	0.02 (1.23)	0.33 (1.40)	0.24 (1.22)	0.05 (1.17)	0.08 (1.22)
GL-7[a]	0.40 (0.05)	0.36 (0.06)	5.00 (0.05)	0.06 (0.16)	0.01 (0.80)	0.21 (0.08)	0.13 (0.04)	0.07 (0.15)	0.11 (0.14)
GL-8[a]	0.67 (1.16)	0.25 (1.25)	5.66 (1.23)	0.06 (1.66)	0.03 (1.22)	0.43 (1.45)	0.32 (1.20)	0.03 (1.47)	0.05 (1.19)
GL-9[a]	0.76 (0.09)	0.45 (0.24)	9.53 (0.08)	0.93 (0.15)	0.09 (0.54)	0.34 (0.01)	0.07 (0.04)	0.02 (0.27)	0.05 (0.20)
GL-10[a]	0.26 (1.26)	0.32 (1.17)	6.95 (1.14)	0.04 (1.03)	0.03 (1.14)	0.35 (1.26)	0.31 (1.25)	0.05 (1.33)	0.08 (1.48)
GL-11[a]	0.10 (0.25)	0.31 (0.07)	8.91 (0.12)	0.05 (1.12)	0.04 (0.57)	0.12 (0.36)	0.08 (0.27)	0.05 (0.13)	0.11 (0.13)
GL-12[a]	0.28 (1.03)	0.34 (1.14)	5.21 (1.08)	0.20 (1.00)	0.07 (1.11)	0.28 (0.98)	0.19 (1.05)	0.07 (1.07)	0.10 (0.97)

[a] Same supplier, but different batches.

nals of each compound were assignable as $[M-H]^-$ ions or as adducts with formic acid (compounds **2**, **3** and **a**) and the deduced molecular masses are in good agreement to reported literature values. Compound **a** is currently being isolated in our laboratory to assure its final structure based on NMR-data.

Twelve commercial samples of *G. lutea* roots were analyzed by the newly developed HPLC-assay, samples GL-1 to GL-5 came from different vendors, GL-6 to GL-12 were different batches obtained from one source. The following trends were obvious when rating the quantitative results shown in Table 4: loganic acid (**1**) and sweroside (**a**) showed the broadest deviation of all compounds, differing from 0.10 to 0.76%, and 0.04 to 0.93%, respectively. Interestingly, these variations were observed within different batches coming from the same supplier, samples from various sources were more homogeneous (e.g. from 0.37 to 0.70% for **1**). In contrary, the occurrence of swertiamarin (0.21–0.45%), and the most dominant compound gentiopicroside (up to 9.53% in the samples) was more consistent. The bitterest natural product till date, amarogentin, was found below 0.09% in all samples. With the exemption of sample GL-2, which contained the lowest amounts of **5** (0.03%) and **6** (0.03%), individual xanthone glycosides were present from 0.07 to 0.43%, with compound **5** always being the dominant one. Respective aglyca were less prevalent (0.02–0.11%), with a higher content of isogentisin compared to gentisin.

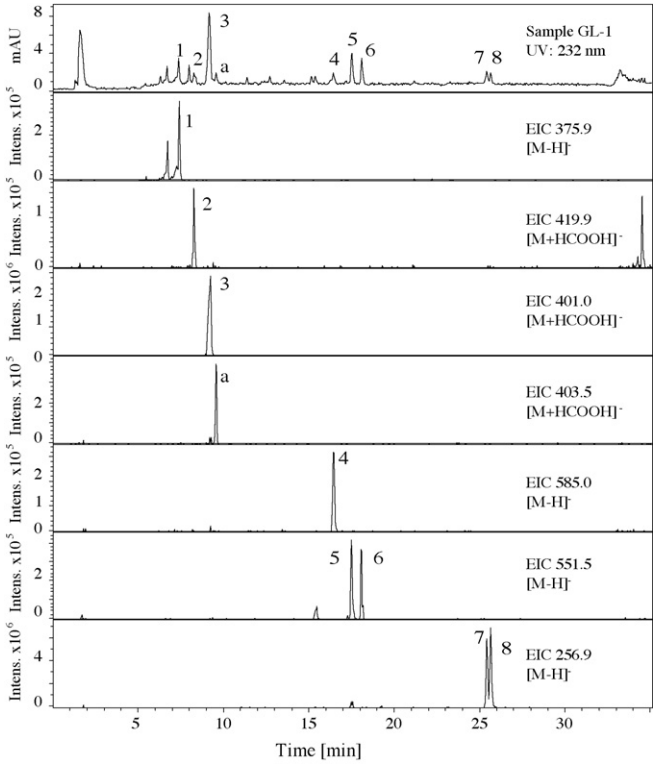


Fig. 4. LC–MS analysis of sample GL-1; LC-conditions according to Fig. 2 except solvent A (0.9% formic acid and 0.1% acetic acid in water); MS-conditions: negative ESI, nebulizer 30 psi, dry-gas 10 l/min, probe temperature 350 °C, split ratio 1:3.

4. Conclusions

The requirements for analytical methods intended for quality control also depend on the knowledge about (pharmacologically) relevant compounds present in the samples. From this perspective the here presented HPLC-assay denotes a major improvement, as it enables for the first time the validated determination of all currently known, major bioactive compounds in *G. lutea*. Owing to the importance of this medicinal plant and the ongoing research this method will definitely be very useful for commercial as well as academic purposes.

References

[1] T. Dingermann, K. Hiller, G. Schneider, I. Zündorf, Arzneidrogen, fifth ed., Elsevier—Spektrum Akademischer Verlag, München, 2004.
[2] C.A. Newall, L.A. Anderson, J.D. Phillipson, Herbal Medicines, first ed., The Pharmaceutical Press, London, 1996.
[3] N. Ozturk, S. Korkmaz, Y. Ozturk, K.H.C. Baser, Planta Med. 72 (2006) 289–294.
[4] M.C. Recio, R.M. Giner, S. Mez, J.L. Rios, Planta Med. 60 (1994) 232–234.
[5] H. Haraguchi, Y. Tanaka, A. Kabbash, T. Fujioka, T. Ishizu, A. Yagi, Phytochemistry 65 (2004) 2255–2260.
[6] A. Schmieder, S. Schwaiger, A. Csordas, A. Backovic, B. Messner, G. Wick, H. Stuppner, D. Bernhard, Atherosclerosis 194 (2007) 317–325.

[7] R.W. Jiang, K.L. Wong, Y.M. Chan, H.X. Xu, P.P. But, P.C. Shaw, Phytochemistry 66 (2005) 2674–2680.
[8] W. Chen, S. Zhang, C. Guan, P. Yu, Zhongguo Yixue Jianyan Zazhi 5 (2004) 246–247.
[9] F.S. Chueh, C.C. Chen, A.P. Sagare, H.S. Tsay, Planta Med. 67 (2001) 70–73.
[10] Z. Glatz, J. Pospisilova, P. Musil, J. Liq. Chromatogr. R.T. 23 (2000) 1831–1839.
[11] A. Krupinska, E. Segiet-Kujawa, L. Skrypczak, M. Ellnain-Wojtaszel, Sci. Pharm. 59 (1991) 135–138.
[12] T. Hayashi, Y. Minamiyama, T. Miura, T. Yamagishi, H. Kaneshima, Hokkaidoritsu Eisei Kenkyushoho 40 (1990) 103–106.
[13] Z. Szücs, B. Danos, S. Nyiredy, Chormatographia 56 (2002) S19–S23.
[14] T. Hayashi, T. Yamagishi, Phytochemistry 27 (1988) 3696–3699.