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Short communication

High-performance liquid chromatography–off line mass spectrometry analysis of anthraquinones produced by *Geosmithia lavendula*

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ABSTRACT

Lilac coloured species of *Geosmithia lavendula* produce a mixture of polyhydroxylated anthraquinones under condition of submerged fermentation. Three pigments had been isolated and identified earlier as a 1,3,6,8-tetrahydroxyanthraquinone (compound **7**), rhodolamprometrin (1-acetyl 2,4,5,7-tetrahydroxyanthraquinone; compound **5**), and 1-acetyl 2,4,5,7,8-penthahydroxyanthraquinone (compound **4**). A new HPLC method was developed for the separation of three known and ten new anthraquinone pigments. In addition, five new pigments were determined by FTMS as coeluting impurities. The analyses were performed on a reversed phase column using gradient elution with a mobile phase system consisting of phosphate buffer (50 mM; pH = 2.0) and acetonitrile. The structure evaluation was based namely on FTMS and UV–VIS spectrometry. The developed procedure was used for the determination of individual anthraquinones in fermentation broth of *G. lavendula* after 14 days of cultivation. The extractable amount and LOQ (both in $\mu g\ ml^{-1}$) for the two main pigments from *G. lavendula* are 50.02 and 2.15 for compound **4**, and 63.77and 2.75, for compound **5**, respectively.

1. Introduction

Natural anthraquinones (AQs) is an important group of more than two hundreds widely distributed pigments. They are distinguished by a large structural variety exhibiting numerous biological activities which make them good candidates for further biotechnological or pharmacological investigations and other applications (e.g. fabric dyes, additives to mordant, histology stains, repellents or protective devices against a large spectrum of predators like avian pests and insects) [1–3]. A number of studies on emodin (1,3,8-trihydroxy-6-methylanthraquinone) have demonstrated that emodin is capable of inducing cell apoptosis and growth arrest in various cancer cells, such as human lung cancer [4], cervical cancer, leukemia, hepatoma, and prostate cancer cell lines [5–7].

Geosmithia lavendula (Acomycota: Hypocreales) is a filamentous fungus living, similarly as other members of the genus, in symbiosis with bark beetles [8,9]. During the screening of secondary metabolites of this fungus, three anthraquinones 1,3,6,8-tetrahydroxyanthraquinone (**7**), rhodolamprometrin (1-acetyl-2,4,5,7-tetrahydroxyanthraquinone; **5**), and 1-acetyl-2,4,5,7,8-pentahydroxyanthraquinone (**4**) were identified as the most abundant compounds produced into the medium during the submerged cultivation [10].

Many reversed phase HPLC methods analyzing one or several standards of anthraquinones and complex sample matrixes were published [11–20]. In general, good separation without significant peak tailing is achieved in mobile phases consisting of water and organic modifier only for anthraquinones having a lower number of substituents (2–4) [21–23]. In the case of anthraquinones harboring four or more hydroxyl groups it was necessary to add acid to the mobile phase to reach both sufficient solubility and separation. As an acidic modifier formic [11–13,20], acetic [13,15,19], or phosphoric acid [14] is generally used.

There are relatively few reports concerning highly sensitive online HPLC–MS analysis of anthraquinones in difficult complex matrixes obtained from fermentation broth or variety of extracts prepared from herbal samples [13,24,25]. For HPLC–MS analysis the low concentration of free acids in the mobile phase, e.g. formic acid (up to 0.1%), acidic acid (up to 5%), and in some cases TFA acid in concentration 0.01% [25] are often utilized. HPLC–MS method is generally combined with LC–diode array detection (LC–DAD) analysis providing complementary information for the identification of both, known and unknown AQs. Very convenient and rapid method for the simultaneous determination and identification of AQs present in samples is HPLC–DAD–MS procedure [26,27] where an HPLC system is online linked with DAD and MS that enables acquisition of both UV/visible and mass spectra within a single chromatographic run.

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This study was aimed at a development of a method suitable for the isolation and identification of individual AQs in complex mixtures produced by submerged cultures of *Geosmithia* sp. for further testing of their highly interesting biological activities. As found previously, biological activity assay of **4** performed on mammalian cell lines suggested specific modulation of cell physiology which led to alterations in the dynamics of the cell cycle. The most obvious phenotype was accumulation of abnormal metaphase–anaphase transition mitotic phase, which is normally only transient and rare [10]. For the method development the 14-day-old fermentation broth of *G. lavendula* was used as a standard. Further, post-column desalting procedure is described for an application of this method for online liquid chromatography–mass spectrometry in the presence of phosphate buffer.

2. Experimental

2.1. Reagents and standards

Methanol and acetonitrile were purchased from Chromservis (absolute LC/MS, BIOSOLVE, the Netherlands). Potassium phosphate, acetic acid, trifluoroacetic acid (TFA), and phosphoric acid (H_3PO_4) were purchased from Sigma–Aldrich (Steinheim, Germany). Three anthraquinone standards, i.e. 1,3,6,8-tetrahydroxyanthraquinone (**7**), rhodolamprometrin (1-acetyl-2,4,5,7-tetrahydroxyanthraquinone; **5**), and 1-acetyl-2,4,5,7,8-pentahydroxyanthraquinone (**4**) were isolated from fermentation broth of *G. lavendula* as described previously [10]. Briefly, fermentation broth of *G. lavendula* was centrifuged and three times extracted with equal volume of ethyl acetate/acetic acid (20:1, v/v). The pooled extracts were evaporated to dryness. The dry mixture was chromatographed on silica gel column (Kieselgel 60, 70–230 mesh ASTM; *Merck*, Germany) washed with *n*-hexane/ethyl acetate 3:2 (v/v) followed by *n*-hexane/ethyl acetate/acetic acid (3:2:1, v/v/v) and finally with *n*-hexane/ethyl acetate/trifluoroacetic acid (6:4:3, v/v/v) as eluents. The compounds eluted in the following sequence: **7** (yellow), **5** (orange), **4** (red).

2.2. Cultivation conditions

Fungal strain *G. lavendula* (CCM 8366) was isolated from *Hypoborus ficus* (Coleoptera: Scolytinae) Italy, 2004 [8,9]. The stock culture of monosporic strain was maintained on malt agar slants (MA; Malt extract 20.0 g l^{-1}, agar 20.0 g l^{-1}) and cultivated on Czapek-Dox medium (CZD; sucrose 30.0 g l^{-1}, sodium nitrate 3.0 g l^{-1}, magnesium sulfate 0.5 g l^{-1}, potassium chloride 0.5 g l^{-1}, iron(III)sulfate 0.01 g l^{-1}, di-potassium hydrogen phosphate 1.0 g l^{-1}, agar 20.0 g l^{-1}, pH = 6.5) in 250 ml Erlenmeyer flasks on a rotary shaker (3.4 Hz) for the period of 14 days at 24 °C in the dark.

2.3. Pigment extraction

Crude pigments were extracted as described previously [10]. Prior to HPLC analysis dry crude extract was reconstituted in methanol containing 1% TFA (v/v).

2.4. HPLC

The HPLC system (Waters, Milford, MA) consisted of a pump equipped with a 600E system controller, autosampler 717, and dual UV detector 2487. Data were processed with Empower 2 software. Water containing mobile phases was filtered through a 0.22 μM GS filter (Millipore, UK) and degassed in an ultrasonic bath for 10 min before use. The gradient mobile phases were degassed continuously by sparking with helium at a rate of 40 ml min^{-1}. UV detection was carried out at 302 and 464 nm, respectively.

Gemini 5 μ C18 column (250 mm × 4.6 mm, Phenomenex) with a guard column was used for the isolation of individual AQs. Mobile phase consisted of water (A) and methanol (B), both containing 1% TFA. Gradient elution started at 30% B (0 min), increasing linearly to 100% B within 40 min. Each analysis was followed by a column washing (100% B, 10 min) and equilibration step (15 min), resulting in total analysis time 65 min. The flow rate was kept at 1.0 ml min^{-1}. Fractions containing individual compounds were collected, evaporated to dryness under reduced pressure and used for further FTMS analysis.

The Kinetex 2.6 μ C18 column (150 mm × 4.6 mm, Phenomenex) was used for the HPLC method development. Gradient elution (0 min, 20% B; 30 min, 50% B) with mobile phase consisting of phosphate buffer (50 mM; pH = 2.0)/acetonitrile (9:1, v/v; solvent A) and acetonitrile (solvent B); flow rate, 0.7 ml min^{-1}; injection volume, 3 μl; UV detection at 302 nm. Fractions containing individual compounds were desalted and used for detailed FTMS analysis.

2.5. Calibration experiments/quantitative determination of anthraquinones

Standard solutions of pigments **4**, **5**, **7** were prepared in methanol containing 1% TFA (v/v) at final concentrations of 1.25, 2.5, 5, 10, 25, 50, 100, 250, and 1000 μg ml^{-1} (3 μl injected in triplicate). The calibration graphs were constructed by plotting the integrated peak areas of individual compounds versus concentration. The parameters of linear regression equations (a, b), correlation (r), and determination (r^2) coefficients obtained for Kinetex column were **4**, a, 8.97e+003; b, 1.10e+005; 0.999; 0.997; **5**, a, 1.19e+004; b, 8.25e+004; 0.9998; 0.9996; and **7**, a, 9.50e+003; b, 1.61e+004; 0.9999; 0.9999, respectively.

2.6. LOQ

Limit of quantification (LOQ) was determined as the lowest point of the calibration curves with a precision (expressed as % RSD) less than 20% and accuracy of 80–120% in six replicates.

2.7. Mass spectrometry

Mass spectrometric (MS) experiments were performed on a Fourier transform ion cyclotron resonance instrument (FTMS) (9.4T APEX-Ultra, Bruker Daltonics, Billerica, MA). The instrument was operated in a negative ion mode. Spectra were collected over the mass range 150–2000 m/z at 1 M data points resulting in a maximum resolution of 200,000 at 400 m/z. Dried samples were dissolved in 1 ml of MeOH–H_2O (1:1, v/v), diluted 50× and introduced to MS by direct infusion via electrospray ion source. The flow rate was 1.5 μl min^{-1} and the temperature of drying gas (nitrogen) was set to 230 °C. The species of interest were isolated in the gas-phase with a 3.0 m/z window and fragmentation was induced by dropping the potential of the collision cell (16–22 V depending on the compound). The accumulation time was set at 0.5 s, the cell was opened for 1200 μs, 8 experiments were collected for each spectrum. The instrument was externally calibrated using singly charged arginine clusters resulting in sub-ppm accuracy.

2.8. UV–VIS

The UV/VIS spectra of AQ standards (**1**–**11**) were monitored in methanol in range of 190–700 nm using Shimadzu multipurpose recording spectrophotometer MPS-2000 equipped with graphic printer PR-3.

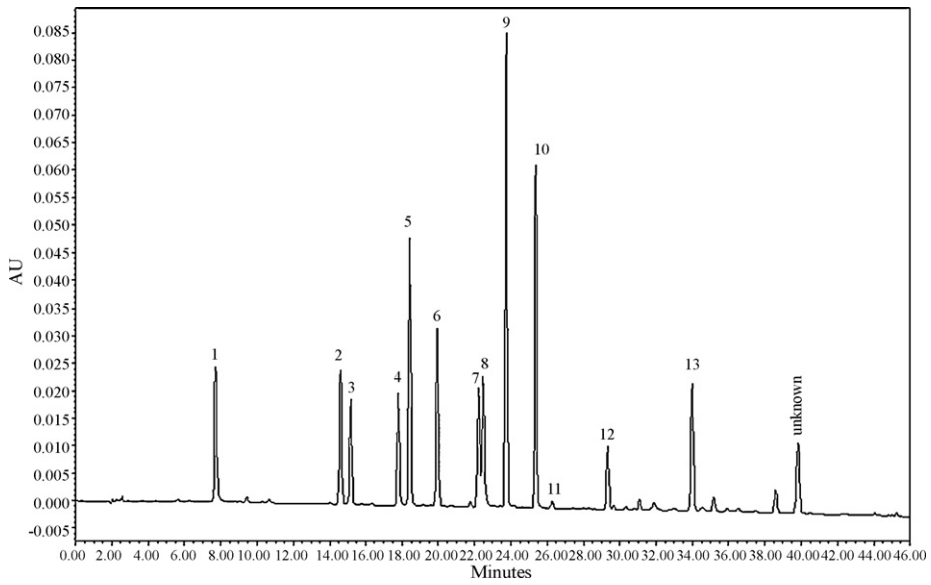

Fig. 1. The chromatogram showing separation of thirteen anthraquinones standards isolated from submerged culture of *G. lavendula*. Chromatographic conditions: column, Kinetex 2.6 μ C18 column (150 mm × 4.6 mm, Phenomenex). Mobile phases: phosphate buffer (50 mM; pH = 2.0)/acetonitrile (9:1, v/v; solvent A) and acetonitrile (solvent B); Gradient elution; 0 min, 20% B; 30 min, 50% B; flow rate, 0.7 ml min^{-1}; injection volume, 3 μl; UV detection at 302 nm; for compounds identification see Table 1.

3. Results

3.1. HPLC method development

Previously published UPLC method for the determination of AQs in fermentation broth of *G. lavendula* [10] used as a mobile phase mixture of 13 mmol trifluoroacetic acid and acetonitrile under isocratic elution. Since this method is not suitable for the standard preparation, the necessary step for bioactivity testing, the procedure was transferred to HPLC condition. The crude extract after separation of the main compounds by column chromatography [10] was analyzed on Gemini 5 μ C18 column using gradient elution. The mobile phases consisted of water (A) and methanol (B), both containing 1% TFA. This separation step yielded thirty-seven fractions; out of them seventeen were found by UV/VIS and FTMS analysis to contain AQs. Unfortunately, detailed FTMS analysis of the most of them revealed mixtures of AQs together with other coeluting impurities and therefore the separation procedure was further developed.

The chromatographic behavior of analytes was investigated on several HPLC columns including Gemini C18 and Luna C18 (Phenomenex, Torrance, CA, USA); xTerra Prep RP_{18} (Waters, Milford, MA), and finally a Kinetex C18 column (Phenomenex, Torrance, CA, USA) in mobile phases differing in organic modifier, buffer solution, and pH, i.e. (a) methanol–0.1% acetic acid, (b) methanol–25 mM acetate buffer, (c) methanol–water (both containing 1% TFA), (d) acetonitrile–30 mM phosphate buffer (pH = 3.0), (e) acetonitrile–30 mM phosphate buffer (pH = 2.0), (f) acetonitrile–50 mM phosphate buffer (pH = 2.0), respectively. The Kinetex C18 column was chosen for further experiments because it gave the best separation with respect to peak symmetry, resolution, and satisfactory analysis time using gradient elution with mobile phase system consisting of phosphate buffer (50 mM; pH = 2.0)/acetonitrile (9:1, v/v; solvent A) and acetonitrile (solvent B). The baseline separation was achieved for nearly all components (Fig. 1) with the exception of two closely related compounds **7** and **8**. Fractions containing individual compounds were repeatedly collected, desalted, and used for further studies as a standard compounds.

Table 1
Retention time, *m/z* value, $[M-H]^-$ formula and proposed structure of anthraquinones isolated from the crude extract of *G. lavendula*.

Cmpd	Crude extract content [% peak area]	Rt [min]	*m/z* $[M-H]^-$	Formula $[M-H]^-$	Proposed structure
1	0.10	7.72	329.0303	$C_{16}H_9O_8$	2,4,5,7-Tetrahydroxy AQ–1-carboxylic acid methyl ester
2	1.46	14.60	355.0459	$C_{18}H_{11}O_8$	1,*x*-Diacetyl-2,4,5,7-tetrahydroxy AQ
3	0.23	15.16	371.0408	$C_{18}H_{11}O_9$	1,*x*-Diacetyl-2,4,5,7,8-pentahydroxy AQ
4	35.59	17.79	329.0303	$C_{16}H_9O_8$	1-Acetyl-2,4,5,7,8-pentahydroxy AQ
5	59.23	18.42	313.0354	$C_{16}H_9O_7$	1-Acetyl-2,4,5,7-tetrahydroxy AQ
6	0.48	19.94	369.0616	$C_{19}H_{13}O_8$	1,*x*-Diacetyl-monomethoxy-trihydroxy AQ
7	0.83	22.22	271.0248	$C_{14}H_7O_6$	1,3,6,8-Tetrahydroxy AQ
8	0.02	22.47	287.0197	$C_{14}H_7O_7$	1,3,5,6,8-Pentahydroxy AQ
9	0.48	23.74	641.0572	$C_{32}H_{17}O_{15}$	Heterodimer of cmpds **4** and **5**
10	0.08	25.36	625.0624	$C_{32}H_{17}O_{14}$	Dimer of cmpd **5**
11	0.04	26.27	599.0469	$C_{30}H_{15}O_{14}$	Heterodimer of cmpds **4** and **7**
12	0.02	29.33	341.0667	$C_{18}H_{13}O_7$	1-Acetyl-dimethoxy-dihydroxy AQ
13	0.01	33.99	383.0772	$C_{20}H_{15}O_8$	1,*x*-Diacetyl-dimethoxy-dihydroxy AQ
14	Coeluting with fraction **5** [<0.1][a]	18.42	343.0459	$C_{17}H_{11}O_8$	1-Acetyl-monomethoxy-tetrahydroxy AQ
15	Coeluting with fraction **5** [<0.1][a]	18.42	385.0565	$C_{19}H_{13}O_9$	1,*x*-Diacetyl-monomethoxy-tetrahydroxy AQ
16	Coeluting with fraction **8** [n.d.]	22.47	311.0561	$C_{17}H_{11}O_6$	1-Acetyl-monomethyl-trihydroxy AQ
17	Coeluting with fraction **8** [n.d.]	22.47	327.0510	$C_{17}H_{11}O_7$	1-Acetyl-monomethoxy-trihydroxy AQ
18	Coeluting with fraction **8** [n.d.]	22.47	397.0929	$C_{21}H_{17}O_8$	1,*x*-Diacetyl-trimethoxy-hydroxy AQ

For HPLC conditions see Fig. 1.
[a] Quantity determined from the FTMS ratio of molecular ion intensities.

Table 2
UV–VIS maxima (nm) of eleven anthraquinone standards isolated from crude extract of *G. lavendula* (measured in MeOH).

Compound[a]	λ_{max} (nm)									
7	225	251	261		290	316	450			
5	228	254	262		292	318	455			
1					290		440			
2	220	257			292	310		464		
6		255			295			468		
8	227		262	271		313		467	495	531
4	234		260	275		314			502	537
3	220		260		295	313		468	490	530
10	223		265		294	315		465		
9	223			280	295	314			502	535
11	222			280					500–530	

[a] For compound structures see Table 1.

The retention characteristics of HPLC fractions collected from analysis on Kinetex C18 column detected at 302 nm are listed in Table 1.

3.2. Structure determination

3.2.1. UV/VIS

UV/VIS spectra of fractions (measured in the range of 190–700 nm) revealed several new candidates showing typical spectral pattern of AQs (Table 2). The distinct shift in absorption spectra to longer wavelength helped us to distinguish between AQs harboring four and five hydroxyl groups. The overlay of UV/VIS spectra of AQs led to the selection of the two most appreciable wavelengths, i.e. 302 and 464 nm for the detection of all identified AQs (cf. Supplementary data Fig. S1). UV/VIS maxima measured in methanol are given in Table 2.

3.2.2. Mass spectrometry analysis

Fractions of AQ compounds were subjected to structure determination by FTMS. Accurate mass measurements with sub-ppm mass accuracy provided us with elemental composition of each isolated compound. Tandem mass spectrometry experiments further helped to ascertain the structure of the compounds. Even though the fragmentation by collision induced dissociation is not very informative it can distinguish between differentially substituted AQs. One such example is shown in Fig. 2 where MS/MS spectra of two isobaric compounds (**1** and **4**) are shown. A comprehensive table summarizes the MS/MS data of thirteen isolated AQs standards. In addition, another five AQs were found by FTMS as coeluting minor impurities in HPLC (Table 3).

3.3. Method application

The analysis of AQs in crude extracts prepared from fermentation broth of *G. lavendula* after 14 days of cultivation is illustrated in Supplementary data Fig. S2. The eluent was monitored at 302 and 464 nm, although they both showed quite similar pattern, detection at wavelength 302 nm was further used for calibration and quantification experiments. Linear calibration graphs with good correlation coefficients were obtained for these AQs standards in the linear range of 1.25–1000 μg ml^{-1} (see Section 2). Using this method the content of main pigments was determined, e.g. **4** – 50.02; **5** – 63.77; and **7** – 0.96 mg l^{-1} of fermentation broth, respectively.

3.4. Post-column desalting

Unfortunately, the composition of the mobile phase offering the best chromatographic resolution (50 mM phosphate buffer) is not compatible with electrospray ionization. When electrosprayed directly the spectra were dominated by adduct peaks and the signal for AQs was missing or strongly suppressed. We therefore tested desalting procedure that can be implemented into an online system. In order to test the effect of the ion pairing agent/pH on the trapping efficiency the same experiment was performed also after neutralization of the phosphate. HPLC fractions (50 μl) containing selected AQs were either neutralized by 50 mM NaOH or diluted to equal volume with water. Next, samples were loaded on the polymeric trap column (bed volume 50 μl) with retentivity similar to C8 (Peptide Macrotrap, MichromBioresources, Auburn, USA). After that the AQs were desalted by 100 μl of water and eluted with 75 μl of 80% methanol in water. In all cases flow-through fractions and purified AQs were collected, dried down and the yield was quantified by HPLC. In both cases the average yield ranged between 80 and 85% (data not shown) which support the possibility of online LC–MS system shown in Supplementary data Fig. S3.

4. Discussion

It is usually difficult to achieve a high sensitivity and baseline separation of AQs in complex sample matrixes because of the great variety of producing species and the wide variations in their levels in crude samples. Previously reported online HPLC–MS methods of AQs analysis [13,24,25] became widely used due to their sensi-

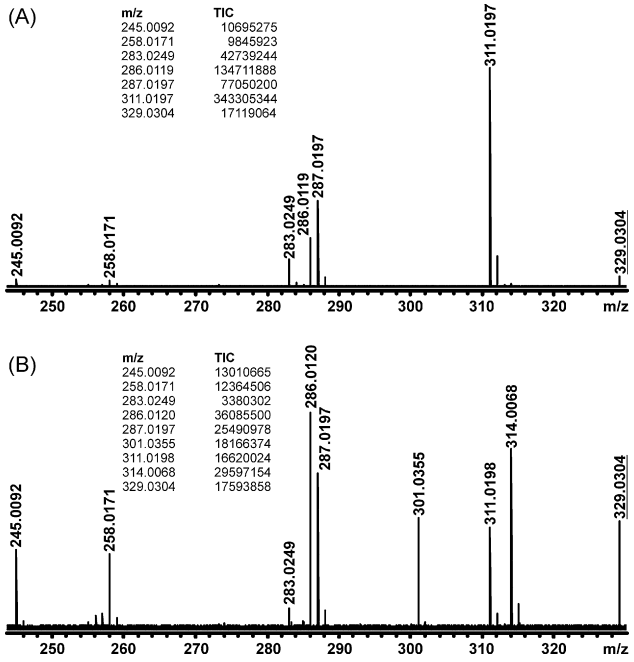

Fig. 2. Comparison of MS/MS fragmentation (using same collision energy) of isobaric anthraquinone standards (*m/z* 329.0304) of (A) 2,4,5,7-tetrahydroxy anthraquinone-1-carboxylic acid methyl ester (compound **1**) and (B) 1-acetyl-2,4,5,7,8-pentahydroxy anthraquinone (compound **4**). Tables shown as insets show TIC (total ion current) values of detected fragment ions.

Table 3
Overview of MS/MS data collected on anthraquinone standards isolated from crude extract of *G. lavendula*. Table lists *m*/*z* of the precursor ion (together with its elemental composition and mass error) selected for MS/MS experiment followed by *m*/*z*, elemental composition, relative intensity, and mass error of detected fragments.

Compound	Precursor *m*/*z*	Formula	Δ*m* (ppm)	Fragment ion *m*/*z*	Fragment ion formula	Fragment ion rel. intensity	Fragment ion Δ*m* (ppm)
1	329.0303	$C_{16}H_9O_8$	0.29	314.0068	$C_{15}H_6O_8$	44	0.18
				311.0197	$C_{16}H_7O_7$	51	0.15
				301.0354	$C_{15}H_9O_7$	27	0.35
				287.0197	$C_{14}H_7O_7$	70	0.16
				286.0119	$C_{14}H_6O_7$	100	0.23
				258.0170	$C_{13}H_6O_6$	37	0.30
				245.0091	$C_{12}H_5O_6$	40	0.01
2	355.0459	$C_{18}H_{11}O_8$	0.16	340.0225	$C_{17}H_8O_8$	52	0.34
				337.0354	$C_{18}H_9O_7$	66	0.32
				327.0511	$C_{17}H_{11}O_7$	19	0.51
				313.0354	$C_{16}H_9O_7$	100	0.34
				312.0276	$C_{16}H_8O_7$	48	0.41
				309.0405	$C_{17}H_9O_6$	87	0.38
				298.0120	$C_{15}H_6O_7$	13	0.56
				297.0041	$C_{15}H_5O_7$	16	0.29
				295.0249	$C_{16}H_7O_6$	17	0.53
				285.0405	$C_{15}H_9O_6$	26	0.41
				284.0328	$C_{15}H_8O_6$	15	0.83
3	371.0408	$C_{18}H_{11}O_9$	0.13	339.0147	$C_{17}H_7O_8$	52	0.40
				311.0197	$C_{16}H_7O_7$	100	0.15
				295.0248	$C_{16}H_7O_6$	69	0.19
				267.0300	$C_{15}H_7O_5$	33	0.62
4	329.0303	$C_{16}H_9O_8$	0.29	311.0196	$C_{16}H_7O_7$	100	0.17
				287.0197	$C_{14}H_7O_7$	39	0.16
				286.0119	$C_{14}H_6O_7$	22	0.23
				283.0248	$C_{15}H_7O_6$	13	0.20
5	313.0354	$C_{16}H_9O_7$	0.34	298.0119	$C_{15}H_6O_7$	52	0.22
				295.0248	$C_{16}H_7O_6$	13	0.19
				285.0405	$C_{15}H_9O_6$	43	0.41
				271.0249	$C_{14}H_7O_6$	12	0.58
				270.0170	$C_{14}H_6O_6$	100	0.28
				242.0221	$C_{13}H_6O_5$	11	0.36
6	369.0616	$C_{19}H_{13}O_8$	0.32	354.0382	$C_{18}H_{10}O_8$	11	0.50
				351.0511	$C_{19}H_{11}O_7$	23	0.47
				336.0275	$C_{18}H_8O_7$	100	0.08
				327.0511	$C_{17}H_{11}O_7$	33	0.51
				326.0432	$C_{17}H_{10}O_7$	30	0.26
				323.0561	$C_{18}H_{11}O_6$	12	0.24
				312.0276	$C_{16}H_8O_7$	21	0.41
				311.0198	$C_{16}H_7O_7$	14	0.47
				309.0405	$C_{17}H_9O_6$	11	0.38
				308.0327	$C_{17}H_8O_6$	16	0.44
				285.0405	$C_{15}H_9O_6$	11	0.41
				283.0249	$C_{15}H_7O_6$	18	0.55
7	271.0248	$C_{14}H_7O_6$	0.21	243.0298	$C_{13}H_7O_5$	100	0.14
				229.0142	$C_{12}H_5O_5$	30	0.03
				227.0350	$C_{13}H_7O_4$	78	0.34
				215.0349	$C_{12}H_7O_4$	27	0.11
				199.0401	$C_{12}H_7O_3$	37	0.43
8	287.0197	$C_{14}H_7O_7$	0.16	259.0248	$C_{13}H_7O_6$	100	0.22
				243.0299	$C_{13}H_7O_5$	20	0.27
				231.0299	$C_{12}H_7O_5$	20	0.29
				215.0350	$C_{12}H_7O_4$	30	0.36
9	641.0572	$C_{32}H_{17}O_{15}$	0.10	623.0465	$C_{32}H_{15}O_{14}$	46	0.13
				605.0358	$C_{32}H_{13}O_{13}$	100	0.39
				595.0516	$C_{31}H_{15}O_{13}$	92	0.12
				587.0254	$C_{32}H_{11}O_{12}$	30	0.14
				579.0567	$C_{31}H_{15}O_{12}$	14	0.11
				577.0411	$C_{31}H_{13}O_{12}$	26	0.04
				563.0257	$C_{30}H_{11}O_{12}$	18	0.38
				561.0463	$C_{31}H_{13}O_{11}$	13	0.15
				553.0415	$C_{29}H_{13}O_{12}$	10	0.68
				551.0619	$C_{30}H_{15}O_{11}$	17	0.08
				537.0463	$C_{29}H_{13}O_{11}$	16	0.16
				353.0303	$C_{18}H_9O_8$	13	0.27
				337.0354	$C_{18}H_9O_7$	11	0.32
				287.0198	$C_{14}H_7O_7$	75	0.51
				245.0091	$C_{12}H_5O_6$	10	0.01
10	625.0624	$C_{32}H_{17}O_{14}$	0.28	607.0511	$C_{32}H_{15}O_{13}$	41	0.94
				589.0413	$C_{32}H_{13}O_{12}$	13	0.30
				579.0563	$C_{31}H_{15}O_{12}$	100	0.80

Table 3 (*Continued*).

Compound	Precursor m/zFormula Δm (ppm)	Fragment ion m/z	Fragment ion formula	Fragment ion rel. intensity	Fragment ion Δm (ppm)
		581.0362	$C_{30}H_{13}O_{13}$	37	0.29
		563.0257	$C_{30}H_{11}O_{12}$	26	0.38
		555.0571	$C_{29}H_{15}O_{12}$	11	0.61
	599.0469	537.0464	$C_{29}H_{13}O_{11}$	12	0.35
11	$C_{30}H_{15}O_{14}$	513.0464	$C_{27}H_{13}O_{11}$	15	0.36
	0.53	509.0515	$C_{28}H_{13}O_{10}$	21	0.39
		311.0198	$C_{16}H_7O_7$	14	0.47
		287.0198	$C_{14}H_7O_7$	100	0.51
		245.0092	$C_{12}H_5O_6$	16	0.39
		326.0432	$C_{17}H_{10}O_7$	11	0.26
		323.0561	$C_{18}H_{11}O_6$	11	0.24
	341.0667	313.0718	$C_{17}H_{13}O_6$	15	0.44
12	$C_{18}H_{13}O_7$	299.0561	$C_{16}H_{11}O_6$	56	0.26
	0.37	298.0483	$C_{16}H_{10}O_6$	100	0.32
		283.0249	$C_{15}H_7O_6$	34	0.55
	343.0459	328.0224	$C_{16}H_8O_8$	100	0.05
13	$C_{17}H_{11}O_8$	311.0197	$C_{16}H_7O_7$	16	0.15
	0.16	300.0276	$C_{15}H_8O_7$	59	0.42
		339.0147	$C_{17}H_7O_8$	100	0.40
	385.0565	311.0197	$C_{16}H_7O_7$	64	0.15
14	$C_{19}H_{13}O_9$	295.0249	$C_{16}H_7O_6$	72	0.53
	0.28	267.0299	$C_{15}H_7O_5$	23	0.25
		283.0613	$C_{16}H_{11}O_5$	56	0.66
		283.0248	$C_{15}H_7O_6$	19	0.20
		269.0456	$C_{15}H_9O_5$	23	0.47
	311.0561	267.0663	$C_{16}H_{11}O_4$	34	0.36
15	$C_{17}H_{11}O_6$	240.0428	$C_{14}H_8O_4$	100	0.23
	0.25	239.0714	$C_{15}H_{11}O_3$	20	0.45
		225.0557	$C_{14}H_9O_3$	23	0.21
		224.0479	$C_{14}H_8O_3$	22	0.30
		353.0667	$C_{19}H_{13}O_7$	40	0.36
		352.0589	$C_{19}H_{12}O_7$	50	0.42
		351.0511	$C_{19}H_{11}O_7$	57	0.47
	383.0772	341.0667	$C_{18}H_{13}O_7$	13	0.37
16	$C_{20}H_{15}O_8$	336.0275	$C_{18}H_8O_7$	69	0.08
	0.20	325.0354	$C_{17}H_9O_7$	16	0.33
		323.0562	$C_{18}H_{11}O_6$	10	0.55
		309.0405	$C_{17}H_9O_6$	100	0.38
		312.0275	$C_{16}H_8O_7$	21	0.08
	327.051	309.0404	$C_{17}H_9O_6$	10	0.05
17	$C_{17}H_{11}O_7$	299.0561	$C_{16}H_{11}O_6$	24	0.26
	0.20	284.0327	$C_{15}H_8O_6$	100	0.48
		379.0824	$C_{21}H_{15}O_7$	36	0.49
	397.0929	355.0823	$C_{19}H_{15}O_7$	100	0.24
18	$C_{21}H_{17}O_8$	354.0746	$C_{19}H_{14}O_7$	19	0.58
	0.34	340.0225	$C_{17}H_8O_8$	13	0.34
		287.0198	$C_{14}H_7O_7$	26	0.51

tivity. The compatibility with MS is achieved by an application of low concentrations of free acids in the mobile phase, e.g. formic, acetic or trifluoroacetic acid. The most effective procedure for the simultaneous determination and identification of both known and unknown anthraquinones present in cell cultures or fermentation broth is HPLC–DAD–MS [26,27]. Obtained data can be used as a guide for further isolation and determination of selected fractions/compounds.

In our study, several gradient mobile phases utilizing different acids and buffers were examined in order to obtain the best separation of AQs standards isolated from the crude extract of fungus *G. lavendula*. A mobile phase containing 1% TFA gave only partial separation as determined by FTMS. The best separation was achieved using mobile phase containing 50 mM phosphate buffer. Unfortunately, the composition of this mobile phase is not suitable for direct online HPLC–MS analysis. Nevertheless, the results of the tested desalting procedure are very promising and could be used as a base for direct coupling with MS via online post-column desalting. This may greatly speed-up the analysis in screening methods.

The presented HPLC chromatogram provides a fingerprint of the anthraquinone profile produced by submerged culture of *G. lavendula* after 14 days of cultivation. Two predominant anthraquinones (**4** and **5**) were found in the crude extract which is in agreement with previously published data [10]. In addition, ten new anthraquinones were detected and their structure was determined by a combination of UV/VIS and FTMS data.

These results show that presented analytical method is suitable tool for the efficient detection, identification, and quantification of anthraquinones, e.g. in submerged cultivation of the fungus *G. lavendula* or other strains belonging to *Geosmithia* family in response to different treatments (e.g. for screening or monitoring accumulation under different conditions). Standard compounds prepared by the described procedure were used for biological activities testing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.08.009.

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