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Determination of sibiromycin and its natural derivatives using new analytical and structural approaches

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ABSTRACT

A new separation and quantification method using ultra high-performance liquid chromatography (UHPLC) with UV detection was developed for the detection of sibiromycin in fermentation broth of *Streptosporangium sibiricum*. The solid phase extraction method based on cation-exchange was employed to pre-concentrate and purify fermentation broth containing sibiromycin prior to UHPLC analysis. The whole assay was validated and showed a linear range of detector response for the quantification of sibiromycin in a concentration from 3.9 to 250.0 μg mL^{-1}, with correlation coefficient of 0.999 and recoveries ranging from 71.66 ± 3.55% to 74.76 ± 5.18%. Method limit of quantification of the assay was determined as 0.18 μg mL^{-1} and was verified with resulting RSD of 9.6% and accuracy of 97.6%. The developed assay was used to determine the sibiromycin production in 12 different fermentation broths. Moreover, several natural sibiromycin analogues/derivatives were described with pilot characterization using off-line mass spectrometry: the previously described dihydro-sibiromycin (DH-sibiromycin) and tentative bis-glycosyl forms of sibiromycin and its dihydro-analogue.

1. Introduction

In 1969 Gause et al. reported on a new antitumor antibiotic, produced by an actinomycete isolated from Siberian soil and consequently called sibiromycin. According to first morphological and biochemical analyses, the producer organism was assigned to the genus *Streptosporangium* and the species *Streptosporangium sibiricum* sp. nov. was described [1]. Using new molecular-biological methods like comparative 16S rDNA sequence analysis the affiliation of this producer to the genus *Streptosporangium* was questioned [2].

Like anthramycin, tomaymycin, porothramycin, sibanomycin, abbeymycin, chicamycin, neothramycins, etc., sibiromycin belongs to the group of pyrrolo(1,4)benzodiazepine antitumor antibiotics (PBDs). They form covalent adducts with DNA through the exocyclic N2 amino group of guanine in the minor groove of double-stranded DNA, which results in the inhibition of nucleic acid synthesis. Among PBDs mentioned above, sibiromycin shows the highest DNA binding affinity and cytotoxicity in leukemia, plasmacytoma and ovarian cancer cell lines. Despite the potency of sibiromycin, the clinical acceptance of this antitumor antibiotic is very restricted by cardiotoxicity, which is due to the presence of the C9 hydroxyl group in the pyrrolo(1,4)benzodiazepine ring system [for reviews see [3–5], and references therein].

Owning to the cardiotoxicity of sibiromycin, new trends in antibiotics and antitumor reagents design started a revival of the use of so-called "filed away" substances. As a consequence, the sequence of the whole gene cluster, coding for the production of sibiromycin, including gene replacement to demonstrate key steps in the biosynthesis of sibiromycin, was described [6]. This may open the way for acquiring the side-effect free antibiotic and antitumor agent sibiromycin.

For further research attempts – also with a view to the above-mentioned combinatorial biosynthesis of side-effect free antibiotic analogues – an accurate and sensitive routine method for the detection and determination of sibiromycin is required. Moreover, together with a new separation method the revision of sibiromycin structure using modern MS techniques comes to the fore.

The first 1969 report by Gause et al. [1] gave no information about its structure. The purification and partial structure characterization of sibiromycin were published later in 1972 [7]. Sibiromycin was extracted from fermentation broth using chloroform liquid–liquid technique or resin based column extraction and characterized by UV spectroscopy, elemental analysis and infrared

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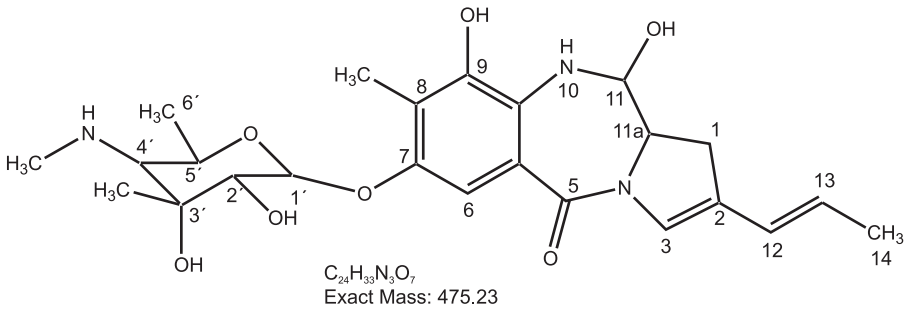

Fig. 1. Structure of sibiromycin molecule as described by Leber et al. [10].

spectroscopy. TLC method (chloroform–methanol 5:1, v/v) coupled with colored reaction with diazo-reactant was employed in routine laboratory determination. The first sibiromycin structure elucidation determined the formula $C_{24}H_{31}N_3O_7$ and molecular weight of 473 by elemental analysis and NMR spectroscopy [8]. Later NMR technique revised the structure, configuration and position of carbohydrate portion (natural product was designed as a C7 rather than C9 glycoside) [9]. The same structure indicated in review article [3] dealt with pyrrolo(1,4)benzodiazepine antitumor antibiotics. The revised sibiromycin structure was published later in articles [9,10] and also by Langlois and Favre [11] who studied the synthesis of antitumor antibiotics of the anthramycin group. In comparison with previous studies [3,8,9] Leber et al. [10] presented a revised sibiromycin formula $C_{24}H_{33}N_3O_7$ with resulting molecular weight of 475 with NMR confirmation by total synthesis of aglycone (see Fig. 1). Two published structures of sibiromycin differ in the presence of double bond (C1–C11a) in pyrrolo(1,4)benzodiazepine skeleton. The analysis of genes involved in biosynthetic pathway of sibiromycin has recently been published [6]. Authors used for sibiromycin determination the HPLC–UV and HPLC–ESI method, but no information about detection limits or other quantification information was available.

The aim of this work was to develop and validate a routine method for sibiromycin determination and quantification using UHPLC method with UV detection. Also a novel pre-concentration method based on solid phase extraction was developed to characterize sibiromycin production in *S. sibiricum* cultivation broths in 12 different media. Further presented method was applied to search all tested media for a potential sibiromycin analogues/derivatives with pilot characterization using mass spectrometry.

2. Experimental

2.1. Chemicals

Formic acid (99.0%), citric acid (99.5%), acetic acid (99.5%) and sodium phosphate dibasic (98.5%) were of ACS reagent grade and were obtained from Sigma (Steinheim, Germany). Ammonium hydroxide (24% aqueous solution, ACS grade), acetonitrile for MS analysis, methanol and absolute ethanol were purchased from Merck (Darmstadt, Germany). Acetonitrile Biosolve used in UHPLC and preparative HPLC were of HPLC grade and were purchased from Chromservis (Prague, Czech Republic).

2.2. Strains and cultivation conditions

Growth and maintenance of *S. sibiricum* (DSM 44039) was performed on agar plates (with agar concentration 15 g L^{-1}) of BG medium (ISP2 medium [12] supplemented with 2 g L^{-1} $CaCO_3$). In order to test sibiromycin production 12 different media were investigated; M1, M2, M3, ISPM4, M6, M7, M8 [13], Gause medium [14], ISP4 medium [15], SM medium [1], SS medium [16] and YEME medium [17]. Spores of *S. sibiricum* were inoculated into BG medium (25 mL) and cultivated for 30 h at 30 °C. 50 mL volumes of tested media in 500 mL Erlenmeyer flasks were inoculated with 5% of inoculation culture and shaken for 72 h at 30 °C. Mycelium suspension was centrifuged (4000 rpm, 4 °C) and supernatant was used for UHPLC and HPLC analysis.

2.3. Sibiromycin standard preparation

Sibiromycin was isolated using a modified protocol described by Brazhnikova et al. [7]. 500 mL of production fermentation broth M8 was extracted three times with equal volume of hexane to eliminate very non-polar compounds from the matrix. Afterwards fermentation broth was extracted three times with equal volume of dichlormethane. The combined organic layers of dichlormethane were evaporated to dryness, resolved in methanol and purified by preparative HPLC.

Purification of sibiromycin was carried out on HPLC system (Waters, MA) equipped with flow controller 600, autosampler 717, and UV detector 486 set at 230 nm. Data were processed with Millennium 32 software (Waters, MA). Phenomenex preparative column Synergy Polar-RP, (250 mm × 15 mm I.D., particle size 4 μm) was used under isocratic elution with mobile phase A:B ratio 30:70 (v/v), where solvent A was 1 mM ammonium formate (pH 4) and solvent B was acetonitrile; flow rate, 4.0 mL min^{-1}; injection volume, 100 μL; total analysis time, 30 min. Sibiromycin fraction was collected in retention window of 20.5–21.8 min. Organic part of mobile phase was evaporated under reduced pressure and aqueous part was then lyophilized to dryness. The purity and identity of the sibiromycin was verified by UHPLC and MS methods.

2.4. Sibiromycin standard stock

1 mg of sibiromycin standard was dissolved in 1 mL of methanol. This solution was further diluted with distilled methanol to the required set of concentrations for method validation performance (calibration curve, precision, and accuracy).

2.5. Sample preparation

Supernatants of *S. sibiricum* culture broths were purified using Waters SPE Oasis MCX 3 mL (60 mg) extraction cartridges. Samples (3 mL) were loaded onto a cartridge pre-conditioned with 1 mL of methanol and 1 mL of water prior to use. Part of the sample matrix was removed by washing with 3 mL of water, 3 mL of methanol and 3 mL of methanol–McIlvaine's buffer pH 7.8 (50:50, v/v). Sibiromycin was eluted with 3 mL of methanol containing 0.24% (v/v) of aqueous ammonium hydroxide. Sibiromycin fraction was evaporated to dryness, and reconstituted in 100 μL of methanol prior to analysis.

2.6. UHPLC conditions

An Acquity UPLC system (Waters, Milford, MA), equipped with 2996 PDA detector operating in the range from 200 to 450 nm was used for sibiromycin analysis under UHPLC conditions. Sibiromycin was quantified by UV detection from extracted chromatograms using wavelength 230 nm. Data were processed with Empower 2 software (Waters).

Sibiromycin was separated on Waters BEH C18 column (50 mm × 2.1 mm I.D., particle size 1.7 μm); mobile phase consisted of solvent A, 0.1% TFA, and solvent B, methanol, linear gradient from 5% to 65% of B in 10 min; flow rate, 0.4 mL min^{-1}; column temperature, 55 °C; data sample rate, 20 pts s^{-1}; filter constant, 0.5; injection volume, 5 μL.

2.7. Method validation

2.7.1. Calibration curve

Calibration curve over the linear range from 3.9 to 250 μg mL^{-1} of sibiromycin was determined. Stock solution of the standard was diluted to required concentrations with methanol. Three sets of calibration standards were prepared, and each point of calibration curve therefore represents arithmetic means of three values.

2.7.2. Instrument limit of quantification (ILOQ)

Lowest point of calibration curves with a precision (RSD) less than 20% and accuracy of 80–120% should be accepted as the ILOQ. Six replicates of samples at a concentration level of 3.9 μg mL^{-1} for sibiromycin standard at which the signal-to-noise ratio was found to be larger than 10 were measured.

2.7.3. Method limit of quantification (MLOQ)

Lowest amount of sibiromycin that can be quantified in a final extracted sample of fermentation broth after SPE should be accepted as the MLOQ. The MLOQ was determined with respect to ILOQ, concentration factor and recovery of target analyte.

2.7.4. Accuracy and precision

To evaluate the precision and accuracy of the assay, quality control samples were prepared at concentration levels of 3.9, 15.6 and 125.0 μg mL^{-1}. Six replicates of quality control samples at each concentration were assayed. Relative standard deviation (RSD) was taken as a measure of precision, and the percentage difference between nominal value and measured amounts was considered a measure of accuracy.

2.7.5. Recovery

The recovery from fermentation broth using SPE was determined at two different concentration levels 5.0 and 10.0 μg mL^{-1}, respectively in four replicates at each level. The medium with the lowest production (YEME, see Table 3) was chosen for the recovery test. Parallel extraction of YEME medium without or with the added sibiromycin standard was performed. The resulting value was obtained as difference of these values and expressed in percentage (%). Media with higher production were not used to avoid final concentration, which could exceed the maximum of calibration curve.

2.7.6. Stability

The stability of methanolic standard solution of sibiromycin was investigated for samples stored at room temperature and at −20 °C (conditions usually encountered during actual sample handling and analysis). Samples at concentrations of 15.6 and 125.0 μg mL^{-1} were prepared. One part of prepared aliquots was stored at −20 °C and analyzed after 1, 2, 3, 7 and 14 days. The second part was stored at room temperature and analyzed after 0, 2, 4, 6, 8, 12, and 24 h. All samples were measured in triplicate. The stability of sibiromycin in fermentation broth was determined after 24 h of fermentation broth storage at 10 °C and after one freeze-thaw cycle (duration of the freezing at −20 °C was 24 h). The decrease of sibiromycin concentration was compared with the concentration measured in fermentation broth immediately after the finalization of cultivation.

2.8. Mass spectrometry

MS analysis and MS^n experiments were performed on LCQ^{DECA} ion trap (IT) mass spectrometer (ThermoQuest, San Jose, CA) equipped with a static nanoelectrospray ion source (nanoESI). Spray voltage was held at 1.2 kV, tube lens voltage was 30 V. The heated capillary was kept at 175 °C with a voltage 10 V. Positive-ion full scans were acquired over m/z range 150–2000. The MS^n experiments were done from selected precursor ion with collision energy kept in the 25–40 units range and with the activation time 30 ms for collision-induced dissociation (CID) experiment. All obtained data were interpreted manually with respect to described fragmentation rules.

UHPLC fractionation was performed manually, 50 μL of respective fraction was collected and evaporated to dryness. Hereafter UHPLC fractions were dissolved in 50% acetonitrile with 0.5% acetic acid (v/v) and sonicated for 5 min prior to mass spectrometry analysis. In case of sibiromycin acidic esterification testing, 50% methanol or ethanol with 0.5% acetic acid was used.

3. Results and discussion

As mentioned previously, the major goal of this study was to develop and validate routine UHPLC method for the quantification of sibiromycin production in fermentation broth. Therefore following steps are described in next paragraphs.

3.1. Sibiromycin isolation and purification

Since sibiromycin is not commercially available, its preparation and verification as a primary standard was necessary. Fermentation broth was extracted using liquid–liquid extraction and purified by preparative HPLC (see Section 2.3). Two preparative columns, Luna and Synergi-Polar (Phenomenex) were tested for preparation method development. The Luna column retained sibiromycin and impurities very weakly. Even if the percentage of organic modifier was decreased by less than 30%, sibiromycin interfered with impurities (data not shown). The Synergi-Polar preparative column and isocratic conditions were chosen for sibiromycin purification. Retention time of well separated sibiromycin was 21 min; the total analysis time was 30 min (data not shown). Moreover, a major ratio of organic modifier (70% of acetonitrile) caused a simplification and shortening of the evaporation process, which has to be as rapid as possible to avoid sibiromycin degradation in acid residues of evaporating solvent. Aqueous part of mobile phase was composed of easily evaporated 1 mM ammonium formate (pH 4) causing mild ionic strength. After evaporation of acetonitrile from mobile phase, the residual aqueous fraction was lyophilized.

An amount of 4.5 mg of purified sibiromycin was obtained in this manner and analyzed by UHPLC with PDA detection under final conditions (see below). A spectral purity of 97% and UV maxima of 230 and 310 nm were obtained (data not shown). The preparation of sibiromycin was also analyzed by direct MS analysis and protonated molecule at m/z 476.3 was detected with electrospray ionization. The acquired MS spectra shown in Fig. 2A revealed mainly protonated sibiromycin molecule and its in-source fragments with low intensity (e.g. imine form at m/z 458.3, sibiromycin aglycone at m/z 303.1 and protonated or sodiated molecule of sibiromycin glycosyl

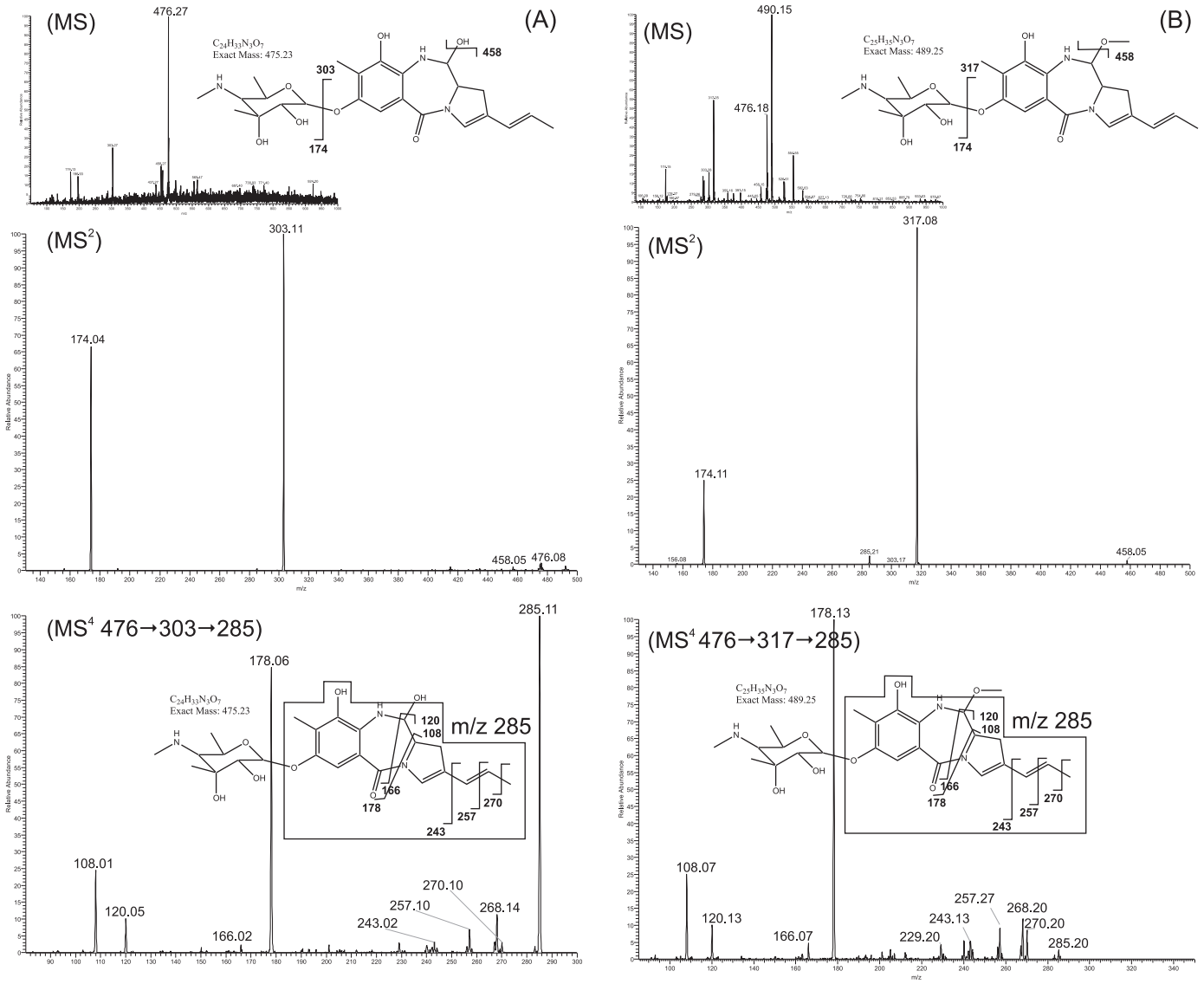

Fig. 2. Mass spectrometry analysis of sibiromycin (A) and its methoxy-product (B). The full MS, MS^2 and MS^4 (476 → 303 → 285) spectra of sibiromycin (*m*/*z* 476.3 of $[M+H]^+$ ion) (A) and the full MS, MS^2 and MS^4 (490 → 317 → 285) spectra of its methoxy-product (*m*/*z* 490.2 of $[M+H]^+$ ion) (B) with proposed fragmentations. In every MS^4 fragmentation scheme the parent signal is marked in sibiromycin structure using indrawn border and labeled with its *m*/*z* value.

moiety at *m*/*z* 174.1 or *m*/*z* 196.1, respectively). This MS data also corroborated a determined spectral purity of 97% by UHPLC. The identity of sibiromycin structure was elucidated by MS^n experiments with manual interpretation. The presented MS^2 spectra in Fig. 2A revealed two major signals, the most abundant at *m*/*z* 303.1 analogous to aglycone of sibiromycin and at *m*/*z* 174.1 corresponding to glycosyl moiety of sibiromycin. Also a fragment of imine form at *m*/*z* 458.3 was detected with very low intensity. The successive CID spectrum of fragment at *m*/*z* 303.1 resulted in one major signal at *m*/*z* 285.1, imine form of sibiromycin aglycone that finally provided MS^4 fragmentation spectrum of sibiromycin aglycone with many signals. Some of them corresponded to 1,4-diazepine ring opening, or consecutive fragmentation of 2-propenyl residue (Fig. 2A).

During the development of sibiromycin purification method, mass spectrometry disclosed that using acidified methanol as solvent resulted in the formation of methoxy-product of sibiromycin providing a protonated molecule at *m*/*z* 490.2. The detailed CID mass spectrometry analysis of sibiromycin prepared in acidified methanolic solvents corroborated its structure shown in Fig. 2B. Briefly, two detected major signals in the MS^2 spectra at *m*/*z* 317.1 and at *m*/*z* 174.1 corroborated the presence of methoxy group at aglycone and not at glycosyl moiety of sibiromycin. The successive CID spectra of fragment at *m*/*z* 317.1 demonstrated similar behavior as CID fragmentation of sibiromycin fragment at *m*/*z* 303.1 (one major signal at *m*/*z* 285.1 corresponding to imine form of sibiromycin aglycone and the same MS^4 fragmentation as was previously described for this fragment of sibiromycin).

We did not validate whether the methoxy-product of sibiromycin was formed during the HPLC preparation under methanolic condition with acidic mobile phase, or arose during evaporating process of collected UHPLC fractions, or was created during electrospray ionization used in mass spectrometry analysis. Nevertheless, a mere dissolution of pure sibiromycin in 50% methanol with 0.5% acetic acid (v/v) using 5 min sonication followed by mass spectrometry analysis provided this methoxy-product. On the other hand, using acidified acetonitrile during mass spectrometry revealed no signal of methoxy-product by mass spectrometry and therefore all other MS experiments were performed in this solvent. Similar sibiromycin behavior, i.e. signal at *m*/*z* 490.2, was described without any discussion by Li et al. as C11 methoxy-product [6]. According to this fact, an additional modifi-

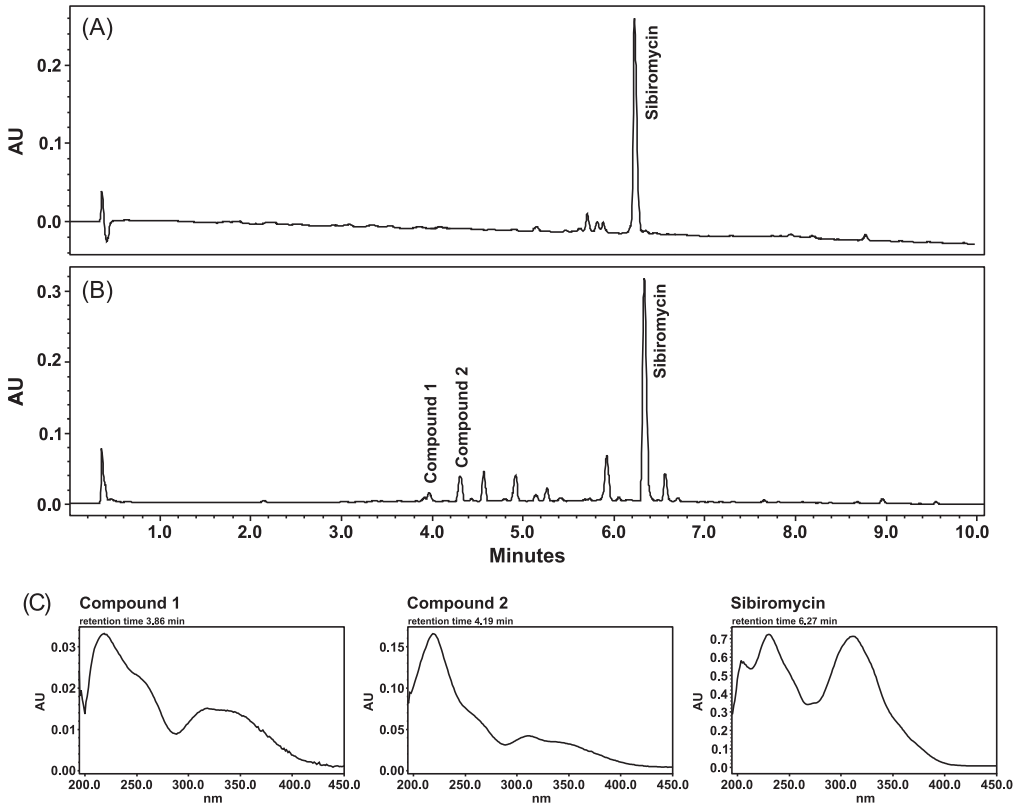

Fig. 3. Comparison of UHPLC chromatograms of *S. sibiricum* cultivated in M7 fermentation medium prepared using LLE (A) or SPE (B) and UV–Vis spectra (C) of compound 1, r.t. 3.86 min, compound 2, r.t. 4.19 min, and sibiromycin, r.t. 6.27 min. Chromatographic conditions: Acquity UPLC BEH C18 column; mobile phase A, 0.1% TFA; B, methanol; flow rate, 0.4 mL min^{-1}; column temperature, 55 °C; and gradient elution (min/%B): 0/5, 10/65; UV, 230 nm; injection volume, 5 μL.

cation experiment using ethanol with acidic traces was performed and similarly ethoxy-product was confirmed by the detection of protonated molecule at m/z 504.3 with corresponding MS^n spectra corroborating its structure (data not shown).

3.2. UHPLC method development

3.2.1. Influence of buffer pH on chromatographic separation

Because sibiromycin stability was previously described by Braznikova et al. [7] in pH range from 3.0 to 10.0, the influence of mobile phase pH on sibiromycin chromatographic separation was tested in this range using 1 mM ammonium formate. It was found that unsuitable pH causes degradation of sibiromycin during UHPLC analysis, with following deformation of its peak. It was observed that only pH range from 3.0 to 4.0 is acceptable (data not shown). When pH is higher than 4.5, degradation process is initiated. When pH of 6.0 is used, the peak shape is deformed and becomes very broad. Using pH 8.5 the sibiromycin peak becomes nearly extinct. Although ammonium formate pH ranging from 3 to 4 was found as convenient for peak shape of individual sibiromycin peaks, other peaks of sibiromycin derivatives were not separated well. Because the bottom limit of formate buffering range is pH 2.75, the 0.1% TFA with similar pH value in mobile phase was finally used and baseline separation of sibiromycin derivatives was observed. Under these conditions stability of sibiromycin during the UHPLC analysis was verified and accurate and precise results were achieved. Our observations and the fact that aqueous solutions of sibiromycin are very labile point to the pH of the mobile phase being a crucial parameter.

3.2.2. Influence of organic modifier content

Using acetonitrile as organic modifier resulted in insufficient separation of *S. sibiricum* metabolites. Methanol as a weaker organic eluent was therefore used in UHPLC mode. The formation of methoxy-product described in Section 3.1 was not observed during UHPLC analysis. Several gradient programs (data not shown) based on a linear increase of the methanol ratio were applied. The resulting gradient program (see Section 2.6) is usable for very rapid analysis of sibiromycin samples and the high throughput qualifies this method for screening and routine analysis.

Analyses under final chromatographic and extraction conditions, shown in Fig. 3B, provide a retention time of sibiromycin 6.27 min and total time of analysis 10 min. The latest published method described HPLC–UV combined with on-line ESI method for sibiromycin determination performed on Zorbax Elipse XDB column with 0.1% TFA/methanol as mobile phase in gradient mode. The retention times of sibiromycin and its equilibrium forms (sibiromycin and its methoxy-product and imine form) ranged between 16 and 20 min [6]. Compared to our results, using UHPLC with methanol as organic solvent and off-line MS with acetonitrile, no formation of methoxy-product was observed and threefold reduction of retention time was achieved.

3.3. UHPLC method validation

The UHPLC method with UV detection was finally characterized by assessing the calibration, instrument and method limit of quantification, accuracy, precision, recovery and stability in accordance with the generally accepted standards [18].

3.3.1. Calibration curve

The sibiromycin calibration curve was prepared over a linear range from 3.9 to 250 μg mL^{-1} at seven concentration levels, 3.9, 7.8, 15.6, 31.2, 62.5, 125.0 and 250.0 μg mL^{-1}. The representative regression equation was $y = 1.18 \times 10^4 x - 1.13 \times 10^4$ with determination coefficient 0.999.

Table 1
Accuracy and precision.

Nominal concentration (μg mL^{-1})	Accuracy (%)	RSD (%)
125.0	96.7 ± 4.14	4.3
15.6	102.5 ± 2.28	2.2
3.9	92.2 ± 5.60	5.7

n = 6 at each concentration level.

3.3.2. ILOQ

ILOQ of sibiromycin was determined as 3.9 μg mL^{-1} and verified with RSD of 5.7% and accuracy of 98.2% (n = 6).

3.3.3. MLOQ

MLOQ of the assay was determined as the lowest amount of an analyte that can be quantified in the fermentation broth. MLOQ was calculated as the final value of 0.18 μg mL^{-1} from the concentration factor (which is 30, because 3 mL of fermentation broth was concentrated to 100 μL), average recovery at this concentration level (70%) and ILOQ (3.9 μg mL^{-1}). Calculated MLOQ was consequently verified with resulting RSD of 9.6% and accuracy of 97.6%.

3.3.4. Accuracy and precision

Accuracy and precision of the assay were determined by analyzing sibiromycin at three concentration levels in six replicates and the results are shown in Table 1. The accuracy ranged from 92.2 to 102.5% with the precision (RSD) from 2.2 to 5.7%. These results indicate that the method is accurate, precise and reproducible.

3.3.5. Recovery

The medium with the lowest production (YEME, see Table 3) was used for the recovery test. Recovery of sibiromycin assay including SPE pre-concentration and UHPLC analysis was 71.66 ± 3.55% and 74.76 ± 5.18% at concentration levels of 5.0 and 10.0 μg mL^{-1}, respectively (Table 2).

3.3.6. Stability

Stability of methanolic standard solution of sibiromycin during the sample storage and processing procedure under conditions described above (see Section 2.7) was evaluated. No significant degradations were found (data not shown). Concentrations of the analytes in question were slightly increased after 14 days of storage at −20 °C caused probably by sample freeze drying.

The sibiromycin detection in the fermentation broth was complicated by its high instability under these conditions (aqueous solution, pH ranging from 7.0 to 7.7). Even rapid freezing of samples does not prevent their rapid degradation process. Using freeze-thaw cycles or storage at 10 °C for 24 h, more than 30% of sibiromycin was degraded (data not shown). Therefore, the sibiromycin determination must be performed immediately after the cultivation was stopped.

3.4. SPE procedure development and comparison with LLE

A sample clean-up and pre-concentration is an important and crucial point for any routine analysis. For sibiromycin sample preparation a new solid-phase extraction (SPE) method was employed. The final pH of fermentation broth after cultivation usually ranges from 7.0 to 7.7. Sibiromycin exists at this pH in ionic form and it is not adsorbed onto universal C18 cartridges. SPE cartridges based on polymeric sorbent with cation exchange ligands (Mixed-mode Cation-eXchange; MCX), which are selective for basic compounds were finally used. Similar approach was previously applied using sulfonic acid resin SDV-3 [7]. The extraction protocol (see Section 2.5) was optimized with respect to maximum recovery of the extraction method.

Table 2
Recovery of LLE and SPE methods.

Extraction	Nominal concentration (μg mL^{-1})	Recovery (%)
SPE	5.0	71.66 ± 3.55
	10.0	74.75 ± 5.18
LLE	5.0	31.67 ± 21.07
	10.0	35.34 ± 21.66

n = 4 at each concentration level.

SPE proved to be not only less time consuming, with easier sample handling and with less solvent consumption, but also especially more efficient than classical liquid–liquid extraction (LLE). The comparative experiment with 3 mL of fermentation broth extracted by both newly developed SPE and earlier developed LLE [7] was accomplished. While the sibiromycin recovery of final SPE procedure was approximately 70% with RSD of 1.8%, recovery of LLE was only 35% with RSD of 21%, for more details see Table 2.

Moreover, UHPLC analysis of the sample pre-concentrated by SPE showed the presence of several sibiromycin derivatives, which have not yet been described. These compounds are more polar than sibiromycin and they are not extractable with dichlormethane described in original LLE method [7] and the latest one [6]. Fig. 3 compares UHPLC analysis of sibiromycin extract prepared by LLE (A) and SPE (B), respectively. It was observed, that only SPE technique provided peaks marked as compounds 1 and 2 having similar UV–Vis spectra as sibiromycin (maxima at 230 and 310 nm). The absorption maximum at 230 nm was influenced by matrix background absorption at 217 nm. It was supposed that these peaks may represent sibiromycin derivatives and they were therefore characterized by MS.

3.5. Production of sibiromycin and its potential derivatives

Up to day, no systematic studies to optimize the production of sibiromycin (composition of the production medium, influence of pH, temperature, cultivation duration, etc.) were provided. Because of this information lack, we used a simple test to show the presence of bioactive sibiromycin in different cultivation media. The production of sibiromycin during the whole cultivation time was examined using sensitivity test of the *Kocuria rhizophila* ATCC 9341 microorganism, an accredited standard for biological assay of antibiotics [19]. For further analytical experiments we used the fermentation broth after a cultivation time of 70–72 h exhibiting the highest sibiromycin production. No correlation between biomass and sibiromycin production was found.

The influence of media composition on sibiromycin production was established by testing 12 ordinarily used cultivation media for actinomycetes. Also the screening of sibiromycin potential derivatives based on their similar UV–Vis behavior was performed. Table 3 summarizes the production of sibiromycin, r.t. 6.40 min, and two newly found compounds (compound 1, r.t. 3.85 min, and compound 2, r.t. 4.19 min) in 12 tested cultivation media. All these determined structures demonstrated two absorption maxima at 230 nm and 310 nm to allow their quantification. Since standards of the compounds 1 and 2 were not available, for their quantification the calibration curve of sibiromycin was used. The media M7 and M8 showed the highest sibiromycin production with almost four times higher value of sibiromycin concentration in fermentation medium than cultivation in Gause medium that was used in the several original papers on the first sibiromycin characterization [1]. The M7 medium also demonstrated the highest amount of compound 2 with a similar rate of biosynthesis as sibiromycin. On the other hand, the determined concentrations of compound 1 were low or not detectable. To characterize all these structures the SPE extracts of *S. sibiricum* cultivated in M3, M7 and M8 were prepared and MS analysis of collected UHPLC fractions was performed.

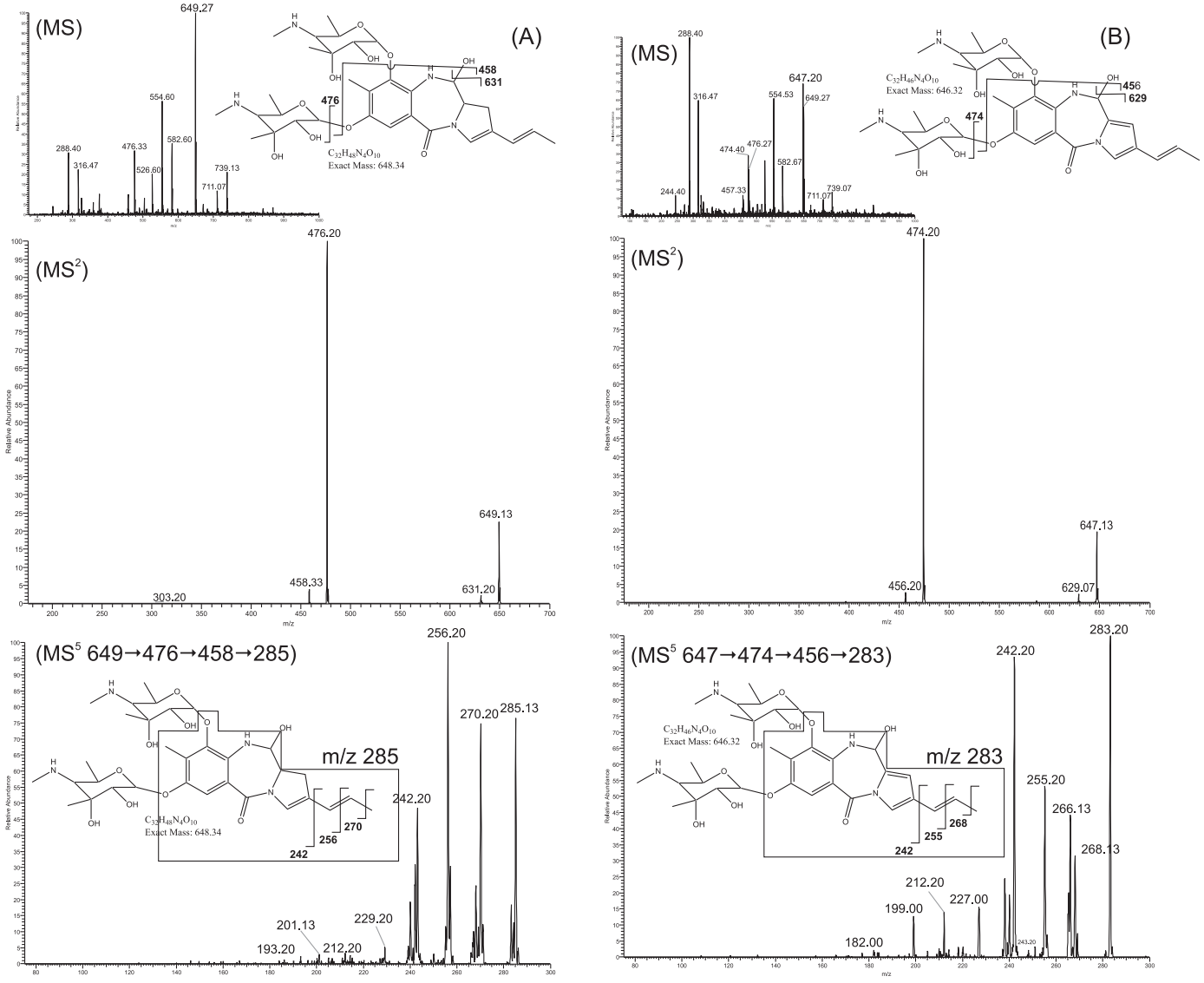

Fig. 4. Mass spectrometry analysis of compound 1 (A) and compound 2 (B). The full MS, MS^2 and MS^5 (649 → 476 → 458 → 285) spectra of compound 1 (*m*/*z* 649.3 of $[M+H]^+$ ion) (A) and the MS, MS^2 and MS^5 (647 → 474 → 456 → 283) spectra of its dehydro-derivative compound 2 (*m*/*z* 647.2 of $[M+H]^+$ ion) (B) with proposed fragmentations. In every MS^5 fragmentation scheme the parent signal is marked in sibiromycin structure using indrawn border and labeled with its *m*/*z* value.

Table 3
The production of sibiromycin and its derivatives determined in different cultivation media.

Cultivation medium	Determined concentration in fermentation broth (μg mL^{-1})		
	Compound 1	Compound 2	Sibiromycin
M1	nd	nd	0.9 ± 0.04
M2	nd	nd	2.6 ± 0.08
M3	0.3 ± 0.02	0.9 ± 0.06	2.9 ± 0.11
M7	0.2 ± 0.01	2.6 ± 0.11	4.2 ± 0.14
M8	0.2 ± 0.02	0.3 ± 0.18	3.9 ± 0.12
SM	nd	nd	1.0 ± 0.11
SS	0.3 ± 0.02	0.6 ± 0.02	2.9 ± 0.09
Gause	0.2 ± 0.03	0.2 ± 0.01	0.8 ± 0.05
YEME	nd	nd	0.2 ± 0.01
ISP4	0.2 ± 0.03	0.3 ± 0.02	1.5 ± 0.07
ISPM4	nd	nd	1.1 ± 0.10
BG	0.2 ± 0.02	0.6 ± 0.04	1.8 ± 0.11

Legend: results are presented as average of triplicate ± standard deviation; nd, not determined.

Considering a higher amount of compound 2 in all tested media, the MS spectra of this metabolite were initially acquired. The preparation of all three selected media of this compound 2 provided a protonated molecule at *m*/*z* 649.3 by electrospray ionization. The acquired MS spectra, shown in Fig. 4A, revealed mainly protonated molecule and its in-source fragments with lower intensity (e.g. fragment of sibiromycin at *m*/*z* 476.3) except background signals. The assumed structure was elucidated by MS^n experiments with manual interpretation. The MS^2 spectra contained a major fragment at *m*/*z* 476.3 analogous to protonated molecule of sibiromycin and low intensity of two imine forms at *m*/*z* 631.2 and at *m*/*z* 458.3. But detailed CID analysis of this major fragment at *m*/*z* 476.3 revealed different fragmentation patterns compared to sibiromycin molecule. The successive CID spectra revealed only a fragment of imine form at *m*/*z* 458.3 that provided in MS^4 experiment only major signal at *m*/*z* 285.2 analogous to the imine form of sibiromycin aglycone. Although both signals gave the same value of *m*/*z*, the obtained fragmentation MS^5 spectrum (649 → 476 → 458 → 285) significantly differed from MS^4 spectrum (476 → 301 → 285), and revealed only consecutive loss of three CH_2 groups from 2-propenyl residue. No fragmentation correspond-

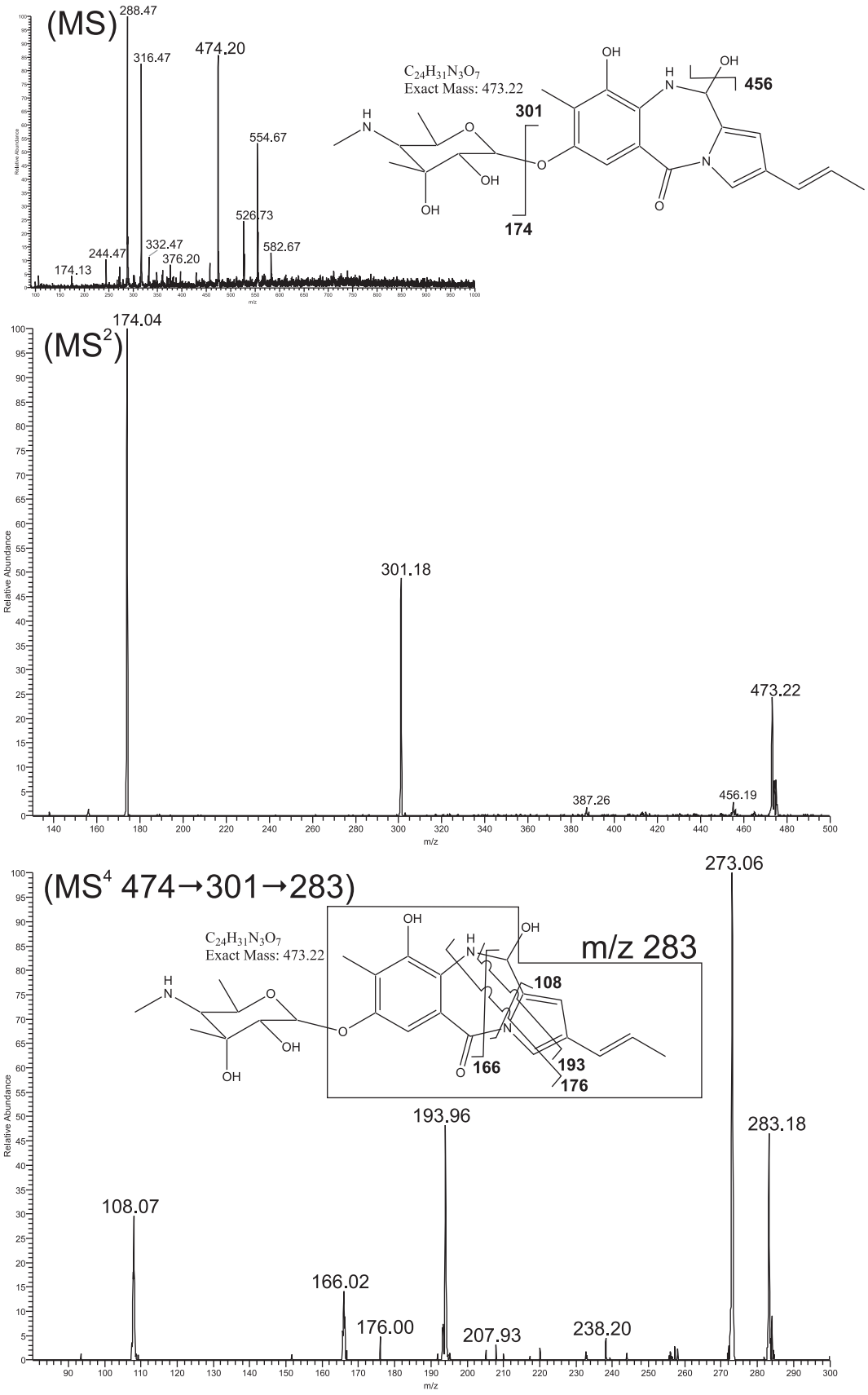

Fig. 5. Mass spectrometry analysis of dehydro-sibiromycin. The full MS, MS^2 and MS^4 (474 → 301 → 283) spectra of sibiromycin dehydro-derivative (m/z 474.2 of $[M+H]^+$ ion) and its proposed fragmentation schemes. In the MS^4 fragmentation scheme the parent signal is marked in sibiromycin structure using indrawn border and labeled with its m/z value.

ing to opening of 1,4-diazepine ring was observed in contrast to sibiromycin or its methoxy-product. Since the significant difference of C11 methoxysibiromycin and compound 2 successive CID spectra of m/z 285 signal has been acquired, we propose a novel sibiromycin analogue with additional glycosyl moiety at C9 hydroxyl of sibiromycin aglycone. Because the obtained amount of compound 2 was not sufficient for NMR analysis, only a speculative and tentative character of proposed bis-glycoside structure with glycosyl moiety at C7 and C9 positions and the corresponded fragmentation scheme in Fig. 4A are presented.

The prepared UHPLC fraction of compound 1 yielded a very surprising result on acquiring MS spectrum, i.e. signal of protonated molecule at m/z 647.1 and analogous fragmentation to compound 2 with shift of 2 mass units. These data indicated the bis-glycoside form similarly to compound 2 and dehydro-derivative of sibiromycin aglycone (data shown in Fig. 4B). Unfortunately the position of additional double bond in aglycone skeleton based on CID spectra could not be distinguished, because only the fragmentation of 2-propenyl residue was observed but not any ring opening. These findings reopen the question of sibiromycin structure elucidation mentioned above. The first sibiromycin structure elucidation using elemental analysis, with resulting formula of $C_{24}H_{31}N_3O_7$ and molecular weight of 473, was performed by Mesentsev et al. [8] using NMR spectroscopy. Later the sibiromycin formula was revised to $C_{24}H_{33}N_3O_7$, with resulting molecular weight of 475 and dihydropyrrole moiety in aglycone skeleton (C1–C11a). It has been proved by total synthesis of sibiromycin aglycone and comparison of the ^1H and ^{13}C NMR spectra. A facile oxidative aromatization of the dihydropyrrole moiety under acidic conditions was suggested [10]. However, in our case no drastic conditions such as 6 N HCl used previously to dehydrate the carbinolamine were employed. Therefore the detailed analysis of UHPLC chromatogram together with MS analysis of collected fractions was performed to find the presumable form of sibiromycin with dehydropyrrole moiety. Indeed this structure was found in a shoulder of sibiromycin UHPLC peak with signal of protonated molecule at m/z 474.1 (data shown in Fig. 5). The CID fragmentation of this signal was analogous to sibiromycin, with a shift of 2 mass units until MS^4 experiment ($474 \rightarrow 301 \rightarrow 283$). The fragmentation of signal at m/z 283 is very similar as m/z 285 of sibiromycin but no fragment corresponding to m/z 120 (probably selective 1,4-diazepine ring opening behind C11) or to m/z 270, 257, 243 (proposed as consecutive fragmentation of 2-propenyl residue) was observed. On the other hand, a new fragment at m/z 193 (probable opening of both 1,4-diazepine and pyrrole rings) was detected (Fig. 5). Though we do not have evidence that the position of double bond was found, we proposed its presence between C1 and C11a as was described previously in original sibiromycin structure elucidation using NMR approach [8]. Based on our data, we proposed a natural dehydro-analogue of sibiromycin named DH-sibiromycin.

4. Conclusions

This study developed and validated a routine UHPLC method with reasonable values of LOQ for the quantification of sibiromycin production in fermentation broth. To solve this problem pure sibiromycin standard was prepared and verified, mobile phase pH, organic modifier content and gradient profile were studied, and a routine SPE procedure to simplify the complex sample matrix was employed. During this step the comparison with formerly developed liquid–liquid extraction was performed and showed qualitative and quantitative differences, e.g. loss of other products with UV–Vis behavior similar to sibiromycin by LLE technique. Optimized procedures were further validated and then applied to characterize the sibiromycin production in 12 different cultivation media. All UHPLC peaks providing UV–Vis spectra similar to sibiromycin were collected and their pilot characterization using MS was performed. Based on this MS analysis only tentative structures of novel bis-glycoside sibiromycin derivatives were proposed and the existence of DH-sibiromycin was described as a natural product of sibiromycin biosynthesis.

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