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Effect of mobile phase pH on liquid chromatography retention of mepartricin related compounds and impurities as support to the structural investigation by liquid chromatography–mass spectrometry

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ABSTRACT

Mepartricin is a semisynthetic polyene macrolide with antifungal and anti-protozoal activities, and it is widely used for the treatment of benign prostatic hyperplasia. Mepartricin is produced by synthetic methyl esterification of the more toxic partricin, and its activity is due to a complex of related compounds. Among them, the main ones are mepartricin B and mepartricin A which are characterized by the presence of a primary and a secondary amine group, respectively. In this work a previously reported HPLC-UV method was properly modified to make it MS-compatible. The selected conditions entail the use of a C18 reverse phase column, and a mobile phase composed by ammonium formate and acetonitrile, with the addition of heptafluorobutyric acid as modifier. The developed method was applied to the characterization of a mepartricin reference standard and a mepartricin experimental batch. All the UV responding peaks, 30 for the standard and 21 for the experimental batch, were successfully detected by MS, allowing to define their *m/z* values and acquire their fragmentation spectra. For the structural elucidation of isobaric species and, in particular, the identification of toxic partricin-related impurities, the presence of differently ionisable chemical groups was considered, as partricins contain free caboxy-groups, while mepartricins represent their estherified counterparts. A deep study of the effect of mobile phase pH on the chromatographic retention of partricin and mepartricin related compounds was performed in the pH range 2.5–6.5. This study allowed to successfully cluster all the detected species and asses, in the considered batch, the absence of other partricin-related impurities in addition to partricin B and partricin A.

1. Introduction

Polyene macrolides are a versatile and promising family of fungicidal agents belonging to antifungal and anti-protozoal antibiotics [1]. Among them, partricin was first isolated from a strain of *Streptomyces aureofaciens* and showed to be very active against fungi and protozoa. However, partricin has not therapeutic application to date since it shows a relatively poor pathogen/host selective toxicity and a significant hemolytic activity [2]. Nevertheless, recent studies suggest a significant decrease in toxicity because of double bonds trans to cis isomerization, suggesting potential future applications [1].

The partricin methyl ester, namely mepartricin, was first described in by Bruzzese et al. in 1972, and demonstrated to have, respect to partricin, increased activity against yeasts and reduced toxicity and hemolytic activity [2]. As all the member of aromatic heptane macrolide family, mepartricin binds to steroids and, thanks to its capabilities to reduce the effects of estrogen on the prostate gland, it is widely used for the treatment of benign prostatic hyperplasia (BPH). Currently, mepartricin is the active principle of Ipertrofan, which is recommended for the treatment of BPH and chronic nonbacterial prostatitis/chronic pelvic pain syndrome [3,4].

Partricin complex consists in two major components with comparable biological activity, named partricins A and B, and differing for the presence of N-methyl substitution on the aromatic amino group in partricin A. As a result, also mepartricin consists in a mixture of mepartricin A and B, as major components. The structures of (me)

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partricin A and B were first partially established by Tweit et al. [5] in 1977 and completely elucidated few years later [6,7]. The absolute configuration of all the stereogenic centers of mepartricin A and B was only recently defined [3]. Partricin and mepartricin structures are reported in Fig. 1.

HPLC-UV methods for mepartricin detection and quantitation are present in literature, all based on the use of a C18 cloumn and on the presence of EDTA in water as mobile phase component, making them non-MS-compatible [8,9]. Considering one of this method [8] as starting point, a MS-compatible HPLC method suitable for the structural elucidation of mepartricin related compounds and impurities was developed.

The HPLC-UV-ESI-MS/MS method was applied to the characterization of a reference standard sample and an experimental one, resulting from different fermentative and synthetic processes. However, HPLC-MS analysis resulted non exhaustive, especially in the precise identification of toxic partricin-like impurities. Based on the main structural difference between particins (presenting a free carboxy-group) and mepartricins (presenting the carboxy-group as methyl-ester), making these two classes of compounds differently ionizable, a rational investigation of chromatographic behaviors at different mobile phase pH values was carried out as complementary approach for patricin and mepartricin related species structural assignment.

2. Materials and methods

2.1. Materials

Reference standard of mepartricin drug substance and experimental batches of partricin and mepartricin drug substance were provided by Società Prodotti Antibiotici S.p.A. (Milan, Italy).

Ammonium formate, ammonium acetate, formic acid and heptafluorobutyric acid (HFBA) were purchased from Sigma-Aldrich (Milan, Italy). Ammonia (30 %), hydrochloric acid (37 %), dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA) and NN-Dimethylformamide (DMF) were from Carlo Erba Reagenti (Milan, Italy). Water was obtained from a Direct-Q™ system Millipore (Millipore, Milan, Italy). Acetonitrile (ACN) gradient grade was supplied by Sigma-Aldrich (Milan, Italy). All the reagents were of analytical grade.

2.2. Sample preparation

Stock solution of 1.0 mg/mL mepartricin was prepared by dissolving 10 mg of powder in 10 mL of DMF. Then, 1 mL of this solution was diluted to 10 mL with DMSO, to a final concentration of 0.1 mg/mL.

Stock solution of 0.5 mg/mL partricin was prepared by dissolving 10 mg of powder in 20 mL of DMF. Then, 1 mL of this solution was diluted to 25 mL with DMSO to the final concentration of 0.02 mg/mL.

The mixture of mepartricin and partricin was prepared by mixing 1 mL of 1 mg/mL solution of mepartricin in DMF and 0.4 mL of 0.5 mg/mL solution of partricin in DMF, and reaching a final volume of 10 mL with DMSO. The final concentrations were 0.1 mg/mL for mepartricin and 0.02 mg/mL for partricin.

2.3. Chromatographic analysis

Chromatographic separations were performed on an Agilent HPLC series 1200 system, equipped with mobile-phase online degasser, quaternary pump, autosampler, column thermostated compartment and diode array detector. For data acquisition, the ChemStation software version Rev. B.04.01 was used in a Microsoft Windows XP environment (Agilent Technologies, Santa Clara, CA, USA).

For each eluting peak, the rrt value was calculated by the ratio between its retention time (rt) and that of mepartricin B.

2.3.1. HPLC-UV method

The LC-UV (not MS-compatible) method was adjusted starting from the method developed by Shi et al. [8] and entails the use of a Kinetex C18 column (4.6 × 250 mm, 5 μm) from Phenomenex (Torrance, CA, USA) and a mobile phase composed of A) 5 mM EDTA/ACN (67/33, v/v) and B) 5 mM EDTA/ACN (25/75, v/v). After isocratic condition at 4 % B for 2 min, gradient elution from 4 % to 21 % B in 24 min was applied. Flow rate was set at 1.25 mL/min, column temperature at 25 °C and injection volume at 20 μL. UV absorbance was monitored at 378 nm.

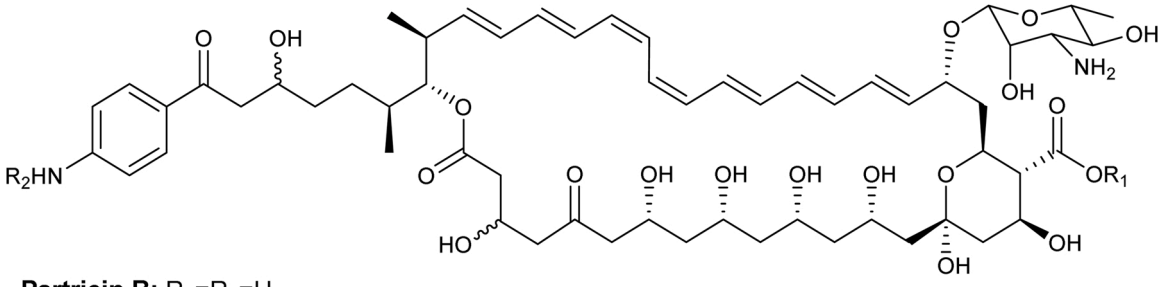
2.3.2. HPLC-UV-ESI-MS/MS method

The MS-compatible method involves the use of the same C18 column (Kinetex C18 column, 4.6 × 250 mm, 5 μm), and mobile phase solutions made up as follows: A) 25 mM ammonium formate /ACN (67/33, v/v) added with 2.5 mM HFBA adjusted at pH 4.85 and B) 25 mM ammonium formate /ACN (25/75, v/v) added with 2.5 mM HFBA adjusted at pH 5.45. After isocratic condition at 5 % B for 2 min, gradient elution from 5 % to 20 % B in 30 min was applied. Flow rate was set at 1.25 mL/min, column temperature at 25 °C and injection volume at 20 μL. UV absorbance was monitored at 378 nm.

MS and MS/MS analysis were performed using a LTQ ion trap mass spectrometer equipped with an electrospray ionization (ESI) ion source and controlled by Xcalibur software 1.4 (Thermo Finnigan, San Jose, CA, USA).

After UV detector, the effluent was splitted in order to reduce the volume of mobile phase delivered to ESI ion source. The split ratio was adjusted to obtain a stable flow rate of around 200 μL/min.

Mass spectra were generated in positive ion mode in the range 500–2000 *m/z*, under the following instrumental conditions: source voltage 5.0 kV, capillary voltage 10 V, capillary temperature 275 °C, sheath gas flow 25 (arbitrary units), auxiliary gas flow 10 (arbitrary units) and tube lens voltage 145 V. MS/MS spectra were obtained by



Partricin B: $R_1=R_2=H$
Partricin A: $R_1=H$, $R_2=CH_3$
Mepartricin B: $R_1=CH_3$, $R_2=H$
Mepartricin A: $R_1=CH_3$, $R_2=CH_3$

Fig. 1. Chemical structures of partricins and mepartricins.

collision induced dissociation (CID) using an isolation width of 5 *m/z* and a normalized collision energy of 35 V.

2.3.3. HPLC-UV analysis at different pH values of mobile phase

The study of chromatographic behavior of partricins and mepartricins at different pH was performed using, as mobile phase, A) ammonium formate adjusted at different pH values (from 2.5 to 6.5) and B) ACN. Elution was performed in isocratic conditions at 35 % B. The flow rate was set at 1.25 mL/min and the column thermostated at 25 °C. Injection volume was set at 20 μL and UV absorbance monitored at 378 nm.

For impurity profiling, mepartricin experimental sample were injected and analysed using the HPLC method described in Section 2.3.2. Each peak was manually collected after UV detection. Collected peaks were analysed using mobile phases adjusted at different pH of component A, as described above. Injection volume was set at 100 μL.

2.3.4. Principal component analysis (PCA)

PCA calculations were performed using SIMCA® software, version 17.

3. Results and discussion

3.1. Development of a HPLC-UV MS-compatible method

UV profile of the mepartricin reference standard, obtained by applying the non-MS compatible method described in Section 2.3.1, is reported in Fig. 2A (black trace) and shows the presence of 27 peaks with percentage area greater than 0.1 %. The major peak, which is assigned to mepartricin B, eluted at 10.55 min, while the one assigned to mepartricin A, eluted at 16.21 min. The analysis with the same method of a partricin batch (Fig. 2A, grey trace) allowed to identify, in mepartricin sample, partricin B and partricin A peaks. Both partricins seem to coelute with other species, making difficult their precise detection and quantification.

Using the same C18 column, EDTA in the mobile phase was exchanged with volatile buffers, 25 mM ammonium acetate (Supplementary, Fig. S1A) or 25 mM ammonium formate (Supplementary, Fig. S1B), adjusting the pH to 4.5. Comparable chromatographic profiles were obtained respect to the starting method, except for an evident shift to higher retention times. A higher number of peaks (28 compared to 25) was observed in the case of formate buffer (Fig. 2B, black trace). The shift in the elution did not affect partricin peaks (Fig. 2B, grey trace), whose retention times remain almost unaltered. The overlay of resulting chromatograms (Fig. 2B) show that, consequently, partricin B eluted in a well-resolved peak at 8.28 min, while partricin A (rt 13.15 min) eluted just between the major peak, mepartricin B, and one of the main component of the sample. The lack of resolution between mepartricin B and partricin A makes the chromatographic method inadequate for the detection and potential quantification of residual patricins. This is a crucial aspect that must be considered in method development due to partricin critical toxicological profile. Moreover, being partricin A and mepartricin B isobaric compounds, MS detection cannot be useful to selectively discriminate between them.

Therefore, the chromatographic method was modified by adding an ion-pairing agent, HFBA, to improve resolution. The presence of 2.5 mM HFBA resulted in a further shift to higher retention times for mepartricins, while, in this case, partricins eluted earlier. As a consequence, mepartricin B and partricin A inverted their elution order, resulting in two well-separated peaks (Fig. 2C). Moreover, considering the obtained chromatogram, 30 peaks (3 more than the starting method) were found to have a percentage area greater than 0.1 % (Fig. 3).

The developed chromatographic method was thus selected as MS-compatible method for the analysis of mepartricin samples.

3.2. LC-UV-MS/MS analysis of mepartricin samples

The resulting method was coupled to ESI-MS and first tested in the characterization of the mepartricin reference standard. Zoom of the UV profile is reported in Fig. 3. HFBA is volatile and thus an ESI-MS compatible ion-paring reagent. However, it is known to cause a suppression of MS signal intensity in comparison to the use of ion-pair free mobile phases (e.g. ammonium formate buffer or formic acid added solutions), due to ion-pairing effects in the electrospray [10]. Despite this, all the 30 peaks detected in UV were revealed also in ESI-MS, allowing to determine the *m/z* value of each species and also to underline coelution phenomena. Retention times, relative retention times, percentage areas and *m/z* values of detected peaks are reported in Table 1. When coelutions were assessed, the relative abundance of the compounds was estimated from peak areas in the relative extracted ion chromatograms in MS.

The major peak, mepartricin B (peak 9), was detected as monocharged ion at *m/z* 1127, while the revealed *m/z* for mepartricin A (peak 19) was 1141, in accordance to the presence of an additional methyl group on the aromatic amino group. Partricin B (*m/z* 1113) and partricin A (*m/z* 1127) are still present in methylated mepartricin sample, and correspond to peak 1 and peak 8, respectively. As expected, partricin A and mepartricin B showed the same *m/z* value. They are, in fact, isomers that differ from partricin B for the location of the additional methyl group (on the aromatic amino group for partricin A and on the carboxylic function for mepartricin B).

Many isomers of partricins and mepartricins were detected in the sample: one for partricin B (*m/z* 1113, peak 4), five for partricin A and mepartricin B (*m/z* 1127, peaks 2, 4, 6, 13 and 15), and four for mepartricin A (*m/z* 1141, peaks 11, 16, 24 and 25). Moreover, six species with *m/z* 1155 (peaks 7, 14, 20, 22, 27 and 30), two with *m/z* 1169 (peaks 18 and 23) and one with *m/z* 1183 (peak 29) were detected, indicating the presence of additional methyl groups on mepartricin A structure.

The presence of isomers can be ascribed to the methylation in different positions (as for partricin A and mepartricin B), or to the presence of geometric isomers, formed by *trans* to *cis* isomerization of double bonds.

Tandem MS spectrometry was thus applied to obtain additional structural information. MS/MS fragmentation was achieved by CID. The MS/MS spectrum of mepartricin B is reported in Fig. 4A. As shown, the fragmentation proceeds by the loss of the monosaccharide residue (145 Da). All the other peaks in the spectrum can be assigned to the loss of the hydroxylic groups as water molecules. The increase in the collision energy (from 35 to 50) did not result in an improvement of the fragmentation. Consequently, MS/MS spectra allowed only to localize the structural differences on the saccharide residue or on the macrolidic ring. As an example, in Fig. 4B it is reported the MS/MS spectrum collected for peak 11, with *m/z* 1141. The spectrum shows the loss of methylated monosaccharide residue to generate the same core fragments of mepartricin B (Fig. 4A), allowing to unambiguously identify this species as a mepartricin B derivative methylated on the sugar ring (most likely on the amino group). On the contrary, the methylation on the macrolidic ring, to generate, for example, mepartricin A, results in different core ions in the MS/MS spectra (Fig. 4C).

Based on the MS and MS/MS data, the structure of all the detected species, except for three of them (peaks 3, 17 and 29), were assigned, deducing the structural modifications from the detected *m/z* values and locating the modifications on the saccharidic moiety or on the macrolidic ring based on the fragmentation patterns (Table 1). However, since fragmentation does not involve the molecule skeleton, MS/MS analysis does not permit to locate structural differences inside the macrolidic ring. In Fig. 5C is reported the fragmentation spectrum of partricin A. The comparison of the two, almost identical, spectra (Fig. 4A and C), demonstrates that MS is unable to discriminate among isobaric partricin A and mepartricin B.

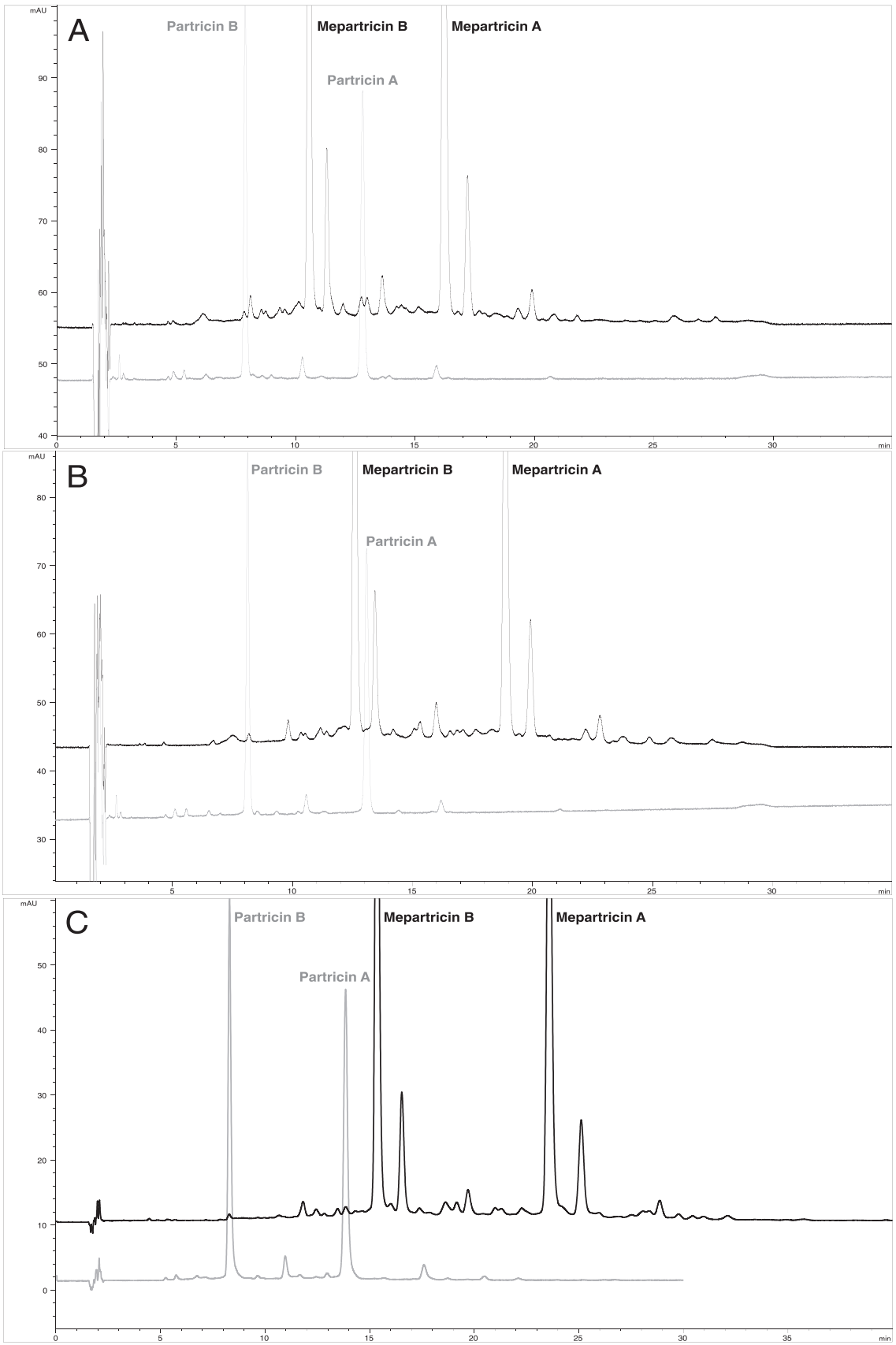


Fig. 2. Chromatographic UV profiles obtained for mepartricin sample (black trace) and partricin sample (grey trace) analysed with different mobile phases: A) 5 mM EDTA/ACN, B) 25 mM ammonium formate pH 4.5/ACN and C) 25 mM ammonium formate/ACN +2.5 mM HFBA. For further details, see Experimental section.

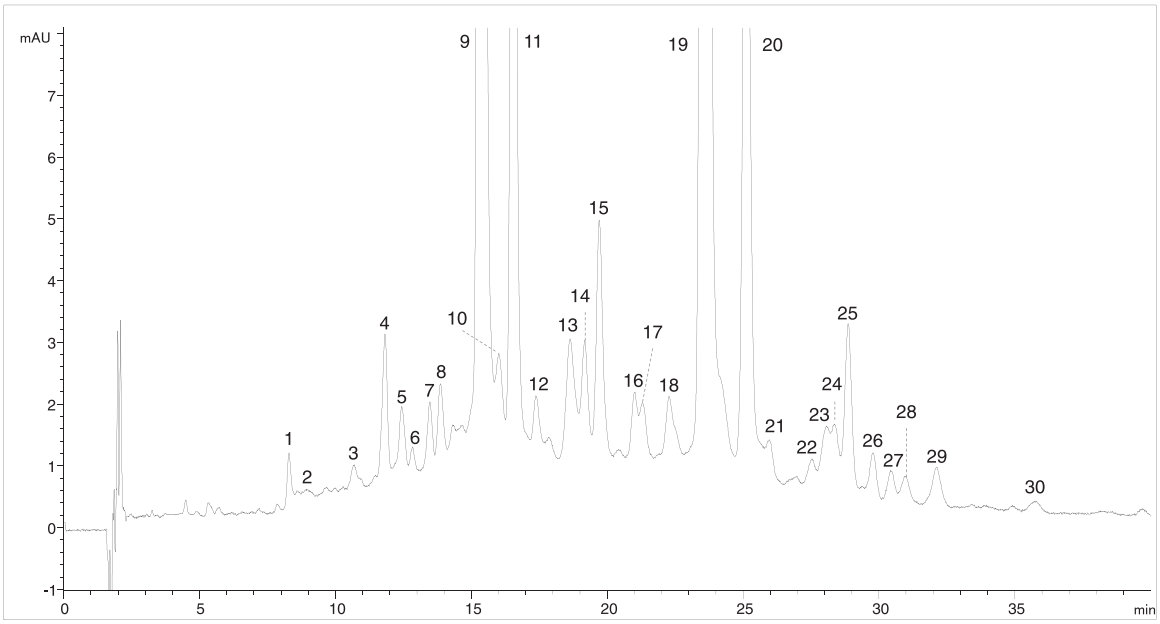


Fig. 3. Chromatographic UV profile obtained for mepartricin reference standard using the developed MS-compatible method. For further details, see Experimental section.

Table 1
Ions detected in mepartricin reference strandard.

#	UV rt	UV rrt	UV Area (%)	*m/z*	MS rrt	MS Area (%)	Identification	Structure modification (referred to partricin B)	
								Core	Saccharide
1	8.29	0.54	0.24	1113	0.37		Partricin B		
2	8.95	0.58	0.16	1127	0.39			+ CH_3	
3	10.69	0.70	0.18	1212	0.46			Not assigned	
4	11.82	0.77	0.86	1113	0.51	71.05			
				1127	0.52	28.95		+ CH_3	
5	12.44	0.81	0.5	1083	0.55			+ CH_3; - COOH	
6	12.84	0.84	0.12	1125	0.55	26.87		+ CH_3; - 2H	
				1127	0.55	73.13			+ CH_3
7	13.47	0.88	0.31	1155	0.59			+3 CH_3	
8	13.86	0.90	0.38	1127	0.60		Partricin A	+ CH_3	
9	15.35	1.00	36.85	1127	0.66		Mepartricin B	+ CH_3	
10	16.01	1.04	0.4	1171	0.69			+ CH_3	+ COOH
11	16.54	1.08	6.59	1141	0.71			+ CH_3	+ CH_3
12	17.38	1.13	0.29	1109	0.75			+ CH_3	
								– H_2O (not localizable)	
13	18.63	0.79	1.01	1127	0.79			+ CH_3	
14	19.16	0.81	0.63	1155	0.81			+ CH_3	+2 CH_3
15	19.71	0.84	1.46	1127	0.84			+ CH_3	
16	21.02	0.89	0.42	1141	0.89			+ CH_3	+ CH_3
17	21.3	0.90	0.39	1109	0.91			+ CH_3	
								– H_2O (not localizable)	
18	22.27	0.94	0.51	1169	0.93	66.69		Not assigned	
				1123	0.95	33.31		+ 2CH_3	
								– H_2O (not localizable)	
19	23.58	1.00	38.07	1141	1.00	99.65	Mepartricin A	+ 2 CH_3	
				1185	1.03	0.35		+ 2 CH_3	+ COOH
20	25.12	1.07	6.77	1155	1.07			+ 2 CH_3	+ CH_3
21	25.98	1.10	0.24	1123	1.10			+ 2CH_3	
								– H_2O (not localizable)	
22	27.53	1.17	0.19	1155	1.17			+ 2 CH_3	+ CH_3
23	28.07	1.19	0.53	1169	1.19			+ 2 CH_3	+ 2 CH_3
24	28.35	1.20	0.46	1141	1.20			+ 2 CH_3	
25	28.87	1.22	1.19	1141	1.22			+ 2 CH_3	
26	29.79	1.26	0.27	1123	1.25			+ 2CH_3	
								– H_2O (not localizable)	
27	30.42	1.29	0.22	1155	1.28			+ 2 CH_3	+ CH_3
28	30.96	1.31	0.2	1137	1.30			+ 2 CH_3	+ CH_3
								– H_2O (not localizable)	
29	32.14	1.36	0.38	1183	1.34			Not assigned	
30	35.75	1.52	0.12	1155	1.51			+ 3 CH_3	

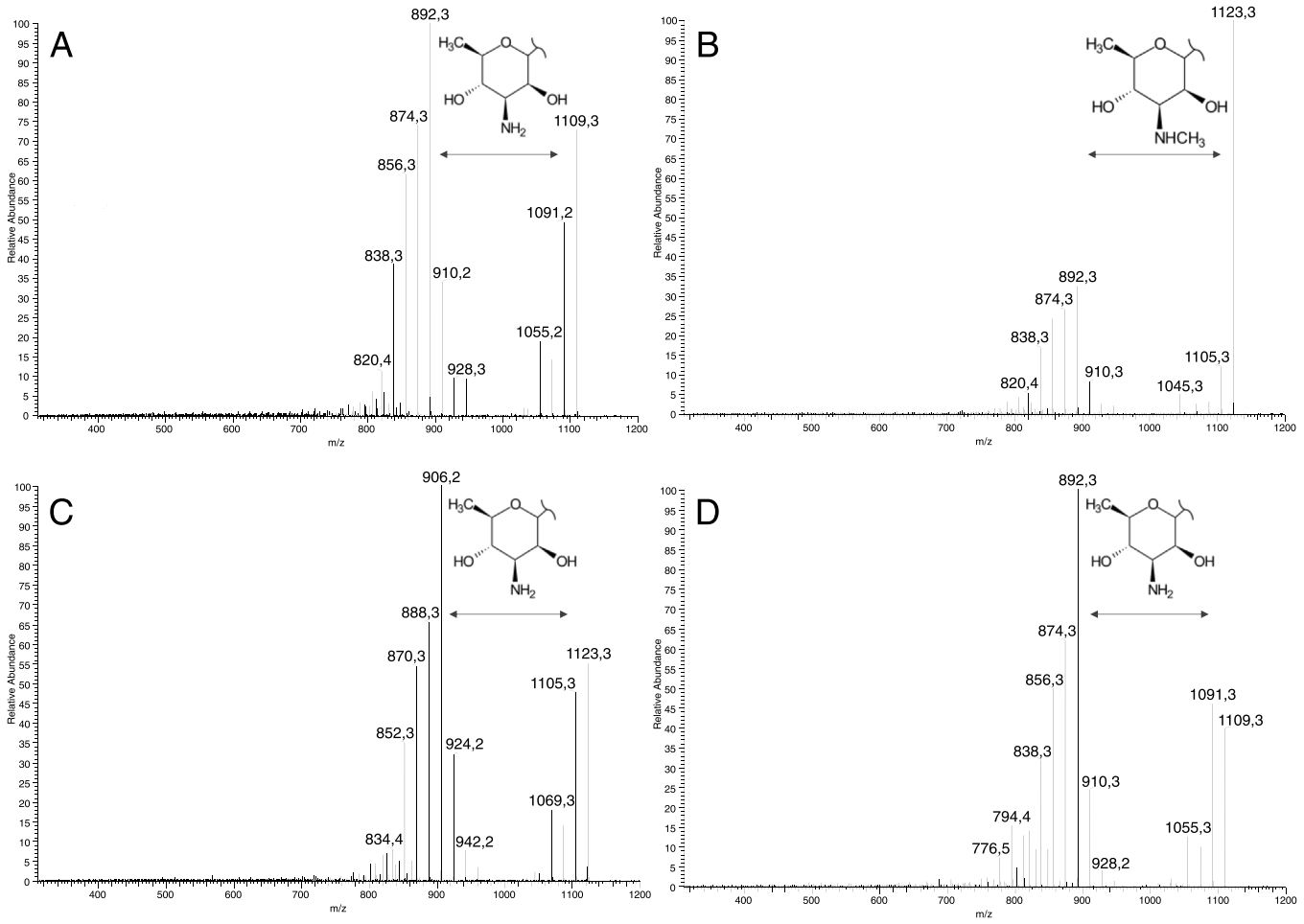


Fig. 4. MS/MS spectra of A) mepartricin B, B) peak 11 (Fig. 3, Table 1), C) mepartricin A and D) partricin A.

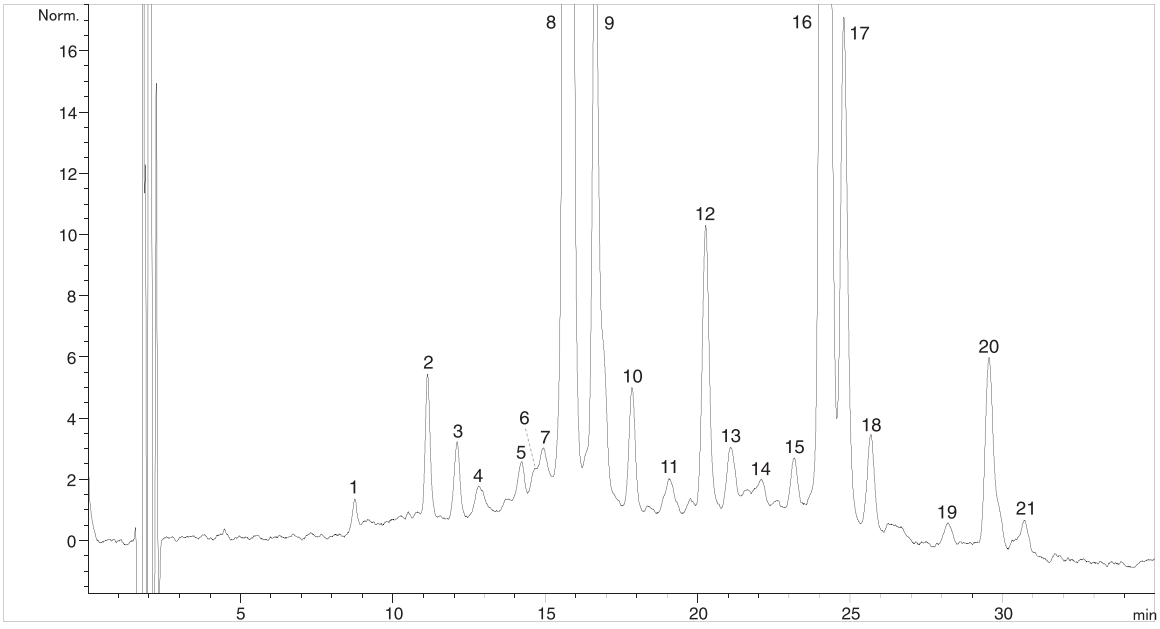


Fig. 5. Chromatographic UV profile obtained for experimental mepartricin sample using the developed MS-compatible method. For further details, see Experimental section.

The same LC-MS approach was applied to the analytical characterization of an experimental mepartricin batch. The resulting UV profile is reported in Fig. 5, showing the presence of 21 peaks with percentage area greater than 0.1 %, nine less than the reference standard.

ESI-MS and MS/MS analysis allowed to assign all the 23 species detected in the sample (Table 2).

Also in this case, most of the detected species are isomers of partricin B (1), partricin A/mepartricin B (3), mepartricin A (4) or methylated derivatives (5). While most of the species were already detected in the reference standard, the principal difference is related to the presence, under peak 2, 9, 10 and 17, of ions with *m/z* 1269, 1283 and 1296 *m/z*. In particular, peak 9 and 17 show to be the most abundant impurities, with a percentage area of 6.85 % and 5.11 %, respectively. The MS/MS spectra (Fig. S2) obtained for the two impurities indicated that they are structurally related species and differ from 14 Da (corresponding to a methylation). Their fragmentation allowed to hypothesize their identification as N-acylurea derivatives of Partricin B and A (Fig. S2**)** and classify them as process impurities, originating during the work-up of the esterification steps.

Considering the collected data, it can be asserted that the application of the developed LC-MS/MS method allows to achieve important information on the impurity profile of mepartricin samples, identifying the structural modifications that characterized each component. However, in most of the cases, the fragmentation data were insufficient to achieve a reliable structural definition due to the presence of many isomers and to the poorly informative CID MS/MS fragmentation spectra. LC-MS/MS method makes impossible to discriminate among isobaric partricins and mepartricins (methylation on the carboxylic group), and isobaric A and B derivatives (methylation on the amino group). In particular, the distinction among partricins and mepartricins is of crucial importance, considering the well-known toxicity of partricins.

3.3. Chromatographic analysis at different mobile phase pH values

Looking to the analyte structures, it can be expected that the methylation of the carboxylic group, instead of the amino group, should significantly affect the chromatographic retention of the compound as a function of the mobile phase pH. Thus, the chromatographic behaviour of the analytes, at different pHs, was investigated and correlated to the methyl group position.

First, the four main species of interest (patricin B, partricin A, mepartricin B and mepartricin A) were considered. A mixture composed by a mepartricin and a partricin batch was submitted to different chromatographic analyses, using mobile phase composed of ACN (component B) and 25 mM ammonium formate buffer adjusted at different pH values (2.5, 3.5, 4.5, 5.5 and 6.5, component A) and in isocratic conditions (35 % of component B). Although the presence of ACN might modify the effective pH of the mobile phase and exerts different effects based on the chemical and physico-chemical properties of each species, this study aimed to point out different chromatographic behaviours at different pH conditions suggesting coherent structure variations which might allow to discriminate partricin and mepartricin related species.

Trends of retention times normalized for retention time at pH 4.5 and peak widths, expressed as ratio between the width at half-height of the considered peak and its retention time, are reported in Fig. 6A and B, respectively. As shown in Fig. 6A, at pH lower than 4.5 all the considered species increase their retention time compared to pH 4.5. This phenomenon affects partricins more than mepartricins. At the same pH, a dramatic peak fronting was observed for all the four considered species, as also suggested by peak width values reported in Fig. 6B (a representative chromatographic trace obtained at pH 2.5 is shown in Fig. S3A).

From pH 4.5 to pH 6.5, different trends were registered for the considered species both in retention time shift (causing the inversion in the elution order between partricin A and mepartricin B) and peak distortion. Even if outside the optimal buffering range of ammonium formate, repeatable retention times were observed in the explored pH range (RSD from triplicate injections at pH 6.5 $\leq$ 0.26 % for partricins and $\leq$ 0.39 % for mepartricins). For partricin peaks, the retention time decreases and the efficiency remains almost unaffected, while for mepartricins higher retention times and dramatic peak tailing were observed (Fig. 6, Fig. S3B and C). These additional interactions can be related to the ionization, at these pH values, of the underivatized silanol groups on the stationary phase, which are thus available for ion-exchange interactions with protonated species. At these pH values the only protonated group in partricins and mepartricins is expected to be the aliphatic amino group on the monosaccharidic ring. It can be hypothesized that in partricins the free and dissociated carboxylic group can interact by an intramolecular bond with the protonated amine,

Table 2
Ions detected in mepartricin experimental sample.

#	UV rt	UV rrt	UV Area (%)	*m/z*	MS rrt	MS Area (%)	Identification	Structure modification (referred to partricin B)	
								Core	Saccharide
1	8.51	0.54	0.26	1113	0.54		Partricin B		
2	11.14	0.71	0.95	1269	0.72		Acylurea derivate		
3	12.11	0.77	0.62	1113	0.78				
4	12.83	0.81	0.43	1083	0.81			+ CH_3; - COOH	
5	14.22	0.90	0.29	1125	0.89			+ CH_3; - 2H	
6	14.33	0.91	0.15	1127	0.91		Partricin A	+ CH_3	
7	14.95	0.95	0.35	1127	0.94			+ CH_3	
8	15.75	1.00	40.07	1127	1.00		Mepartricin B	+ CH_3	
9	16.64	1.06	6.85	1283	1.06	79.87	Acylurea derivate	+ CH_3	+ CH_3
				1141	1.08	20.13			
10	17.85	1.13	1.06	1283	1.14		Acylurea derivate		
11	19.06	1.21	0.47	1127	1.22			+ CH_3	
12	20.26	1.29	2.85	1127	1.29			+ CH_3	
13	21.09	1.34	0.60	1141	1.34			+ CH_3	+ CH_3
14	22.09	1.40	0.29	1141	1.40			+ 2 CH_3	
15	23.17	1.47	0.45	1155	1.48			+ 2 CH_3	+ CH_3
16	24.16	1.53	34.98	1141	1.54		Mepartricin A	+ 2 CH_3	
17	24.80	1.57	5.11	1297	1.58		Acylurea derivate		
18	25.69	1.63	0.92	1155	1.63			+ 2 CH_3	+ CH_3
19	28.22	1.79	0.23	1155	1.80			+ 2 CH_3	+ CH_3
20	29.56	1.88	2.39	1141	1.88	74.24		+ 2 CH_3	+ CH_3
				1155	1.89	25.76		+ 2 CH_3	
21	30.73	1.95	0.44	1155	1.94			+ 3 CH_3	

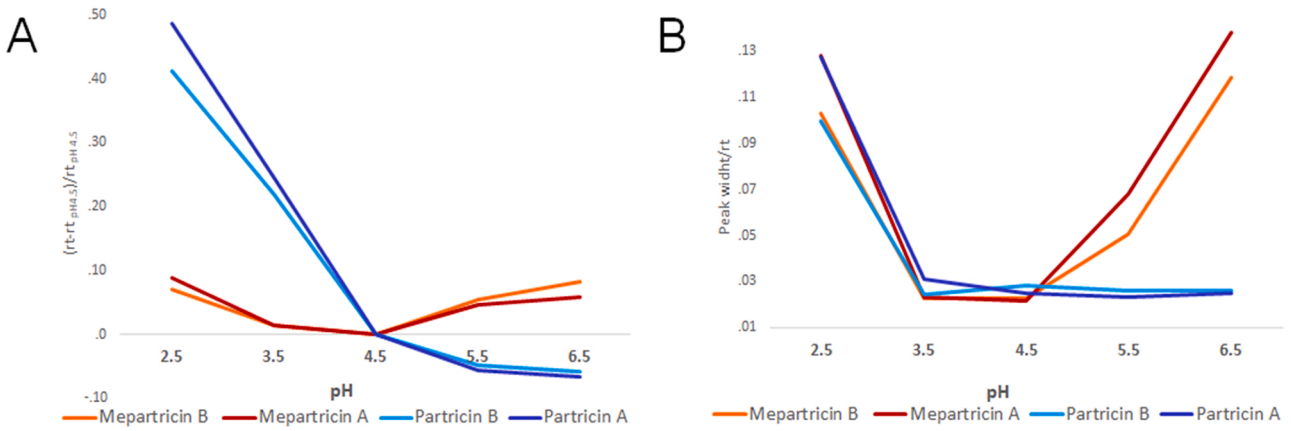


Fig. 6. Trends in the chromatographic parameters of partricins and mepartricins analysed at different pH of the mobile phases (the reported pH is referred to the component A of the mobile phase). A) Retention times, reported as difference between the retention time at the considered pH and the retention time for the same species at pH 4.5, divided for retention time at pH 4.5; B) Peak width, reported as ratio between the width at half-height of the considered peak and its retention time.

avoiding its undesired interaction with the stationary phase and the subsequent increase in retention times and peak tailing phenomena.

On the light of these results, an experimental study was set-up to investigate if the different chromatographic behaviour observed for partricin A and B and mepartricin A and B might be useful to classify the species revealed in mepartricin samples as partricin- or mepartricin-like derivatives.

To properly describe the chromatographic behaviour of all the detected compounds, each peak was collected and individually injected and analysed. A higher volume of a mepartricin experimental sample was thus injected in the HPLC-UV system and analysed using the developed method (see Paragraph 2.3.2). During the elution, 21 fractions, corresponding to the 21 detected peaks, were manually collected. Each fraction was then subjected to different analysis in isocratic conditions, at different pH of mobile phase (component A).

In addition to pH 4.5, one lower pH value (3.0) and two higher pH values (5.5 and 6.0) were considered, since the behaviour at basic pH resulted more informative compared to acidic one. Extreme pH values (2.5 and 6.5) were discarded, since they showed to induce drastic peak distortions, that could compromise peak detention in the case of low abundant species. Retention times were registered for all the species at the four-tested pH values (Table S1). Due to the low abundance and the high retention times, peak 19 and peak 21 were not detected in all the pH conditions, and were thus discarded from the study.

Retentions times of the considered 19 peaks at the four different pH values were considered and elaborated by principal component analysis (PCA). Since variables show a significant asymmetry, they were mathematically converted in their logarithm and scaled to unit variance. The first component explains 98.6 % of total data variance (Fig. 7) and, on this component, all the four variables show the same significance and the same direction. The second component add 1.29 % of explained variance and, on this, variables have different significance. From the loading plot (Fig. 7) it can be observed that on the second component, variable pH 3.0 has a high positive value, variable pH 4.5 approximates zero, while variables pH 5.5 and 6.0 have high negative values.

The score plot in the plane of the two first principal components (Fig. 8) clearly shows a differentiation of Partricin A and B (due to the high positive scores in the second component) and of the acyl-urea derivatives (due to the negative scores in the second component). Mepartricin A and B are instead differentiated on the first principal component, with all the other species aligned on it.

The PCA analysis suggested the absence, except for partricin B and partricin A, of partricin-like impurities, and allowed to achieve further information on sample components. As an example, peak 3 (indicated as A in Fig. 8) was suspected to be an isomer of partricin B, having the same mass and the same macrolidic core mass (as demonstrated by its MS/MS spectrum in Fig. S4). However, peak 3 clearly showed a mepartricin-like behaviour, revealing its nature as mepartricin B demethylated (on the core) derivative.

A peculiar behaviour was also highlighted for the four components identified as acylurea derivatives (Fig. 7). Their clustering is mainly based on their retention time trends at pH above 4.5. In fact, while for all the mepartricin-like components the retention time increased from pH 4.5–5.5 and remained almost unaffected above pH 5.5, in the case of acylurea derivatives the influence of pH on retention was greater and evident also moving from pH 5.5–6. This behaviour can be ascribed to

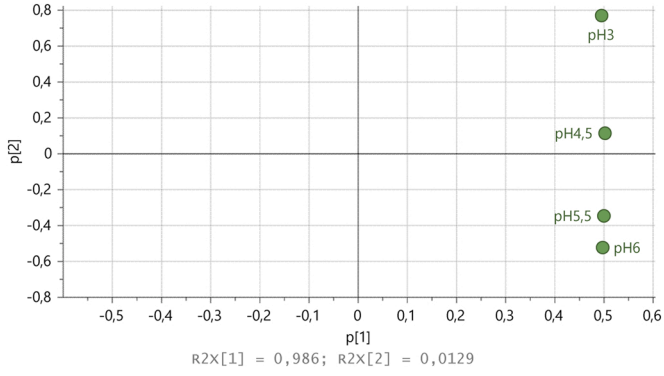


Fig. 7. Loading plot showing the influence of each variable (indicated as pH 3.0, 4.5, 5.5 and 6) on the first two principal components in PCA.

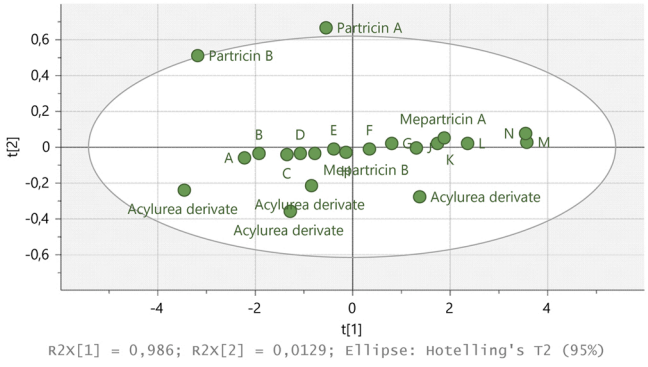


Fig. 8. Score plot in the plane of the first two principal components derived from PCA analysis of chromatographic retention times (Table S1). Unidentified species are indicated by their letters.

the presence of an additional aliphatic amino group in the molecular structure (Fig. S2) which, being ionized at pH 5.5–6, can form ion-exchange interaction with the underivatized silanol groups of the stationary phase.

Peak width data of all the collected peaks were also considered. However, their elaboration did not permit to achieve further significant information, probably due to the small area of many peaks that, also considering broadening and tailing effect, made the integration variability too significant. Nevertheless, the analysis of chromatographic retention times alone allowed to exclude the presence in the sample of partricin-like impurities and to unambiguously classify all the detected specie as related compounds of mepartricins.

4. Conclusions

In this work, a MS-compatible HPLC method was developed for the analysis of mepartricin drug substance and its related compounds and impurities. The developed LC-UV-MS/MS method was applied to the characterization of, first, a reference standard sample and, then, an experimental batch of mepartricin, allowing to detect 34 and 23 species, respectively. Interestingly, despite the lower number of detected compounds, four impurities different from those observed in the reference standard were detected in the experimental sample. The interpretation of MS and MS/MS spectra permitted to confirm all the detected species as structurally related substances. However, the presence of many isomers and the low informative fragmentation patterns made difficult to assign putative structures and, most important, to identify partricin derivatives, whose presence influences the toxicological profile of the batch. The chromatographic behavior of partricins and mepartricins at different pH of the mobile phases was thus investigated, highlighting differences useful to cluster the related compounds. Effectively, the analysis, at different pHs, of all the collected peaks from the experimental batch enabled to confirm the absence of partricin-like impurities, except for partricin B and partricin A.

The developed method and the obtained results represent a useful tool in the characterization of produced mepartricin samples, allowing to properly address fermentation processes and to assess the quality of the obtained product.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2022.114971.

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