UNIVERSITA' DEGLI STUDI DI PAVIA

UNIVERSITÀ DI PAVIA

Master Biennale di II livello in Discipline Regolatorie "G. Benzi"

1916 2016

FEDERCHIMICA ASCHIMFARMA

Master in Preformulazione, Sviluppo Farmaceutico e Controllo di Medicinali

Master in Tecnologie Farmaceutiche e Attività Regolatorie
Università di Pavia

WORKSHOP

21st OCTOBER, 2016

Modern analytical techniques in Pharmaceutical Industry

Moderne tecniche analitiche nell'Industria Farmaceutica

A tool to support pharmaceutical quality and to ease regulatory process

Aula Magna - Collegio A. VOLTA

Via Ferrata, 17 - Pavia

APPLICATION OF HPLC-MS/MS TO A FERMENTATION PRODUCT

CATERINA TEMPORINI
UNIVERSITÀ DEGLI STUDI DI PAVIA

The Problem:

TEICOPLANIN *GENERIC*

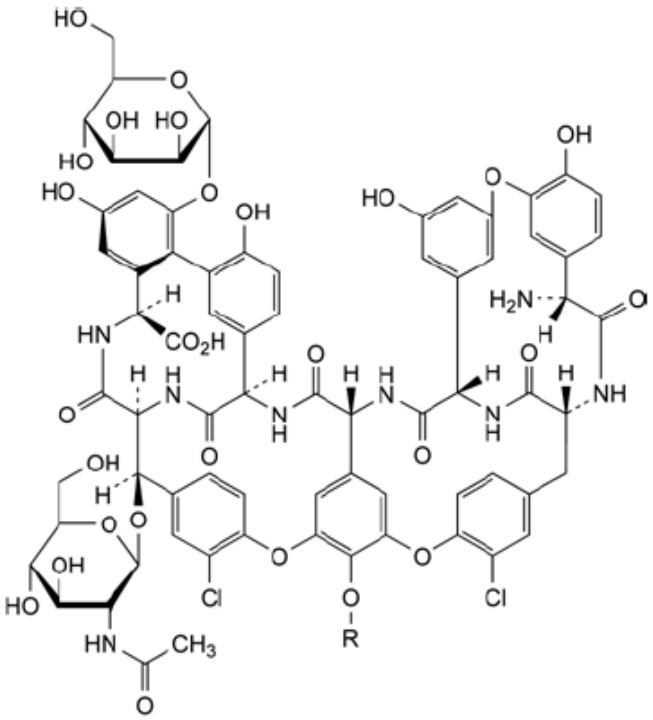
IMPURITY IDENTIFICATION AND QUALIFICATION ACCORDING TO REGULATORY REQUIREMENTS FOR:

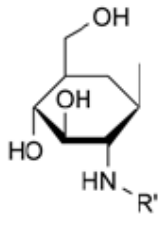
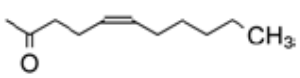
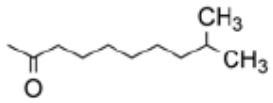
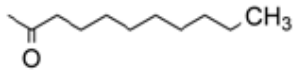
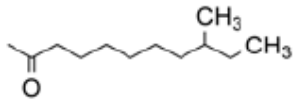
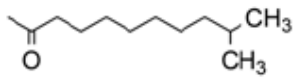
- ✓ Fermentation Products
- ✓ Teicoplanin EP Monograph
- ✓ EMA Guideline Antibiotics
  EMA/CHMP/CVMP/QWP/199250/2009 corr
- ✓ Teicoplanin EMA assessment report
  Procedure no: EMEA/H/A-5(3)/1315

EU Pharmacopoeia Monograph Teicoplanin

01/2009:2358
corrected 6.6

Teicoplaninum



Teicoplanin	R	R'
A_{2-1} $C_{88}H_{95}Cl_2N_9O_{33}$ M. W.: 1878		
A_{2-2} $C_{88}H_{97}Cl_2N_9O_{33}$ M. W.: 1880		
A_{2-3} $C_{88}H_{97}Cl_2N_9O_{33}$ M. W.: 1880		
A_{2-4} $C_{89}H_{99}Cl_2N_9O_{33}$ M. W.: 1894		
A_{2-5} $C_{89}H_{99}Cl_2N_9O_{33}$ M. W.: 1894		
A_{3-1} $C_{72}H_{68}Cl_2N_8O_{28}$ M. W.: 1564	H	

Teicoplanin was first approved for maketing in Italy as TARGOCID, with an IBD of 30 July 1987. Targocid is a complex mixture of products with antibiotic activity, consisting of six closely related subcomponents (A2-1 to A2-5 and A3).

EU Pharmacopoeia Monograph Teicoplanin

Teicoplanin	R	R'
A_{2-1} $C_{88}H_{95}Cl_2N_9O_{33}$ M. W.: 1878		
A_{2-2} $C_{88}H_{97}Cl_2N_9O_{33}$ M. W.: 1880		
A_{2-3} $C_{88}H_{97}Cl_2N_9O_{33}$ M. W.: 1880		
A_{2-4} $C_{89}H_{99}Cl_2N_9O_{33}$ M. W.: 1894		
A_{2-5} $C_{89}H_{99}Cl_2N_9O_{33}$ M. W.: 1894		
A_{3-1} $C_{72}H_{68}Cl_2N_8O_{28}$ M. W.: 1564	H	

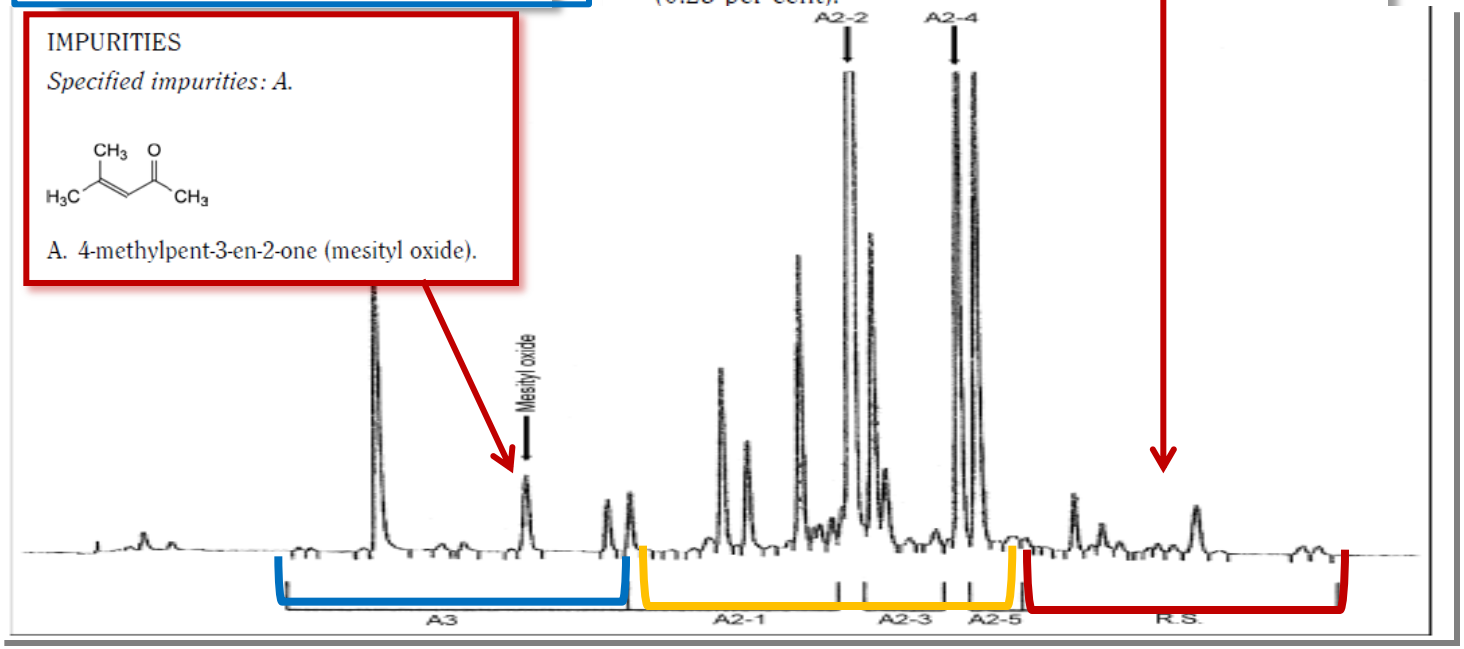
Limits:

- *teicoplanin A_2 group*: minimum 80.0 per cent;
- *teicoplanin A_{2-2}*: 35.0 per cent to 55.0 per cent;
- *teicoplanin A_{2-1} group*: maximum 20.0 per cent;
- *teicoplanin A_{2-3} group*: maximum 20.0 per cent;
- *teicoplanin A_{2-4}*: maximum 20.0 per cent;
- *teicoplanin A_{2-5} group*: maximum 20.0 per cent;
- *teicoplanin A_3 group*: maximum 15.0 per cent;
- *total of impurities other than mesityl oxide with a relative retention more than 1.25*: maximum 5.0 per cent;
- *disregard limit*: the area of the peak due to teicoplanin A_{2-2} in the chromatogram obtained with reference solution (b) (0.25 per cent).

IMPURITIES

Specified impurities: A.

A. 4-methylpent-3-en-2-one (mesityl oxide).



EU Pharmacopoeia
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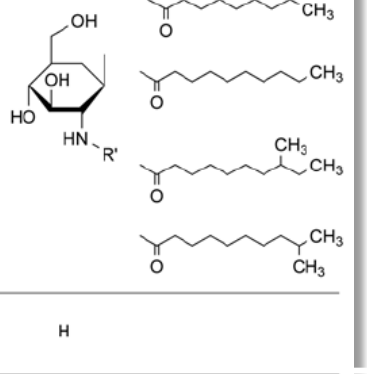
A_{2-1} $C_{88}H_{95}Cl_2N_9O_{33}$ M. W.: 1878		
A_{2-2} $C_{88}H_{97}Cl_2N_9O_{33}$ M. W.: 1880		
A_{2-3} $C_{88}H_{97}Cl_2N_9O_{33}$ M. W.: 1880		
A_{2-4} $C_{89}H_{99}Cl_2N_9O_{33}$ M. W.: 1894		
A_{2-5} $C_{89}H_{99}Cl_2N_9O_{33}$ M. W.: 1894		
A_{3-1} $C_{72}H_{68}Cl_2N_8O_{28}$ M. W.: 1564	H	

Limits:

- *teicoplanin A_2 group*: minimum 80.0 per cent;
- *teicoplanin A_{2-2}*: 35.0 per cent to 55.0 per cent;
- *teicoplanin A_{2-1} group*: maximum 20.0 per cent;
- *teicoplanin A_{2-3} group*: maximum 20.0 per cent;
- *teicoplanin A_{2-4}*: maximum 20.0 per cent;
- *teicoplanin A_{2-5} group*: maximum 20.0 per cent;
- *teicoplanin A_3 group*: maximum 15.0 per cent;
- *total of impurities other than mesityl oxide with a relative retention more than 1.25*: maximum 5.0 per cent;
- *disregard limit*: the area of the peak due to teicoplanin A_{2-2} in the chromatogram obtained with reference solution (b) (0.25 per cent).

Quali- Quantitative Composition of the “Complex” and related substances are strictly dependent from:

- Microorganism
- Control of the Fermentation process
- Raw material used for fermentation process

Compliance with the Ph. Eur. alone has not been considered sufficient to fulfill the primary condition for a generic application

EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

30 June 2012
EMA/CHMP/CVMP/QWP/199250/2009 corr
Committee for Medicinal Products for Human Use (CHMP)/ Committee for Medicinal Products for Veterinary Use (CVMP)

Guideline on setting specifications for related impurities in antibiotics

5.3. Active substances manufactured by fermentation, family of compounds

Acceptance criteria for related impurities should be set in accordance with the thresholds given below.

Reporting threshold: 0.10%
Identification threshold: 0.15%
Qualification threshold: 0.50%/0.15%

Identification: Informative Methods (i.e. Mass Spectrometry)
Qualification: Impurity profile by comparison with products already on the market or toxicity data (in silico/in vivo)

21 March 2013
EMA/194668/2013

EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

Assessment report

Review under Article 5(3) of Regulation (EC) No 726/2004

Teicoplanin

Procedure no: EMEA/H/A-5(3)/1315

Additional limits and tests proposed for the active substance subcomponents, based on the batch results and proposal for the Ph. Eur. monograph revision

In view of the above discussion and bearing in mind that the composition of individual subcomponents can be impacted by modification of the fermentation conditions, new limits and tests, in addition to those required by the current Ph. Eur. monograph, are proposed to address all teicoplanin like structure peaks. These new tests and limits are given below and these should be forwarded to EDQM for inclusion in the Ph. Eur. monograph.

Teicoplanin A3 group	**4.0 - 12.0%**
Teicoplanin A2 group	**84.0 - 93.0%**
Teicoplanin A2-1 group	**10.0 - 19.0%**
RRT about 0.85 (RS3):	0.5-5.5%
RRT about 0.88 (RS4)	0.5-4.0%
RRT about 0.93 (A2-1)	2.0-7.0%
Teicoplanin A2-2	**37.0% - 50.0%**
Teicoplanin A2-3 group	**5.0 - 11.0%**
RRT about 1.03 (A2-3)	4.0-8.5%
Teicoplanin A2-4	**7.0 - 15.0%**
Teicoplanin A2-5 group	**7.0 - 17.0%**

Qualified Unidentified Impurities

RRT about 1.25 (RS1):	NMT 1.5%
RRT about 1.30 (RS2)	NMT 1.5%
RRT about 1.38	NMT 2.5%
Any non-teicoplanin like impurity	**NMT 0.5%**

Qualification threshold related 0.50%

Full Characterization of a Reference Profile (Targocid)

The Analytical Problems:

- ✓ Identification/Qualification

  Development of a Sensitive ad Informative Analytical method alternative to the HPLC-UV as from Ph. Eur.

- ✓ Quality

  Sensitive and Selective Method for Residual Components from Fermentation and Downstream (i.e. Olygosaccharides)

Analytical Method Transfer from HPLC-UV to HPLC-ESI-MS/MS

Critical Parameters :

- **Buffer Type** (*volatile buffers*)
- **pH** (*same buffering pH range*)
- **Ionic Strenght** (*efficient chromatography but **no** ion suppression in MS*)
- **Sensitivity** (*column downscale*)
- **ESI ionization** for maximum MS signal intensity (*make-up flow*)

Considered Specific Output Data to Adress the Method Choice:

Maintainance of the **RRT** of all the detected peaks.

Peak **Efficiency** and **Sensitivity.**

Analytical Method Transfer from HPLC-UV to HPLC-ESI-MS/MS

Chromatographic HPLC-UV method from Ph. Eur. (Method A):

Column: LiChrospher RP C18, 4x250 mm, 5 µm

Flow rate: 2.3 mL/min

Injection volume: 20 µL - *Diluent*: water

Detection: 254 nm

Column temperature: not controlled

Mobile phases: A) Na_2HPO_4 /NaH_2PO_4 buffer 25 mM pH 6.0:Acetonitrile (90:10)

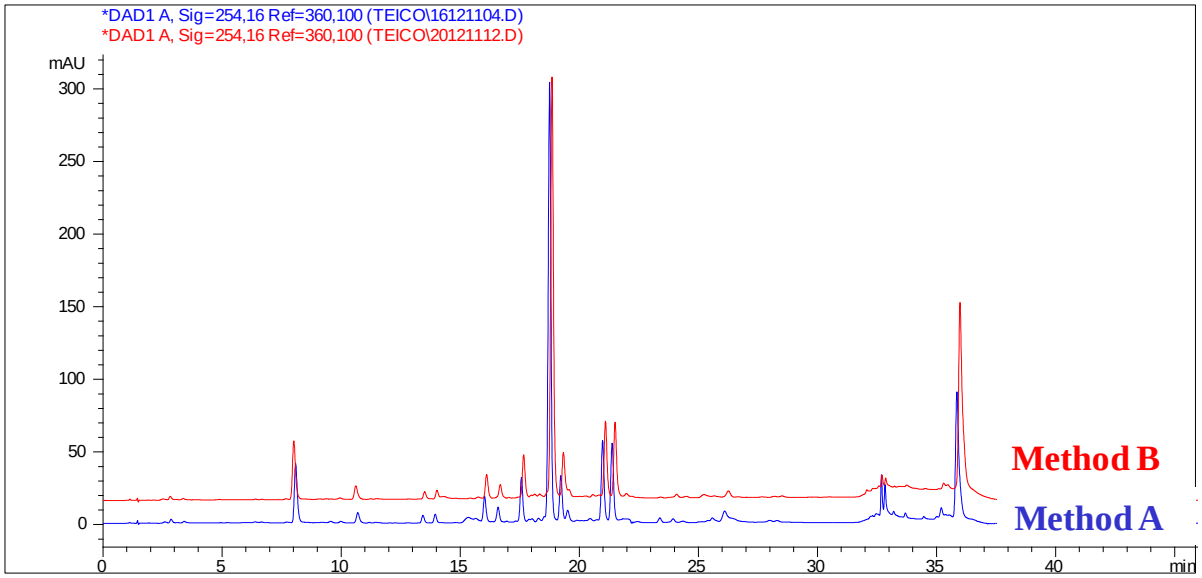
B) Na_2HPO_4 /NaH_2PO_4 buffer 25 mM pH 6.0:Acetonitrile (30:70)

Time	% B
0	0
35	90

1) Substitution of the phosphate buffer with a volatile buffer to allow MS coupling

1) Substitution of the phosphate buffer with a volatile buffer to allow MS coupling

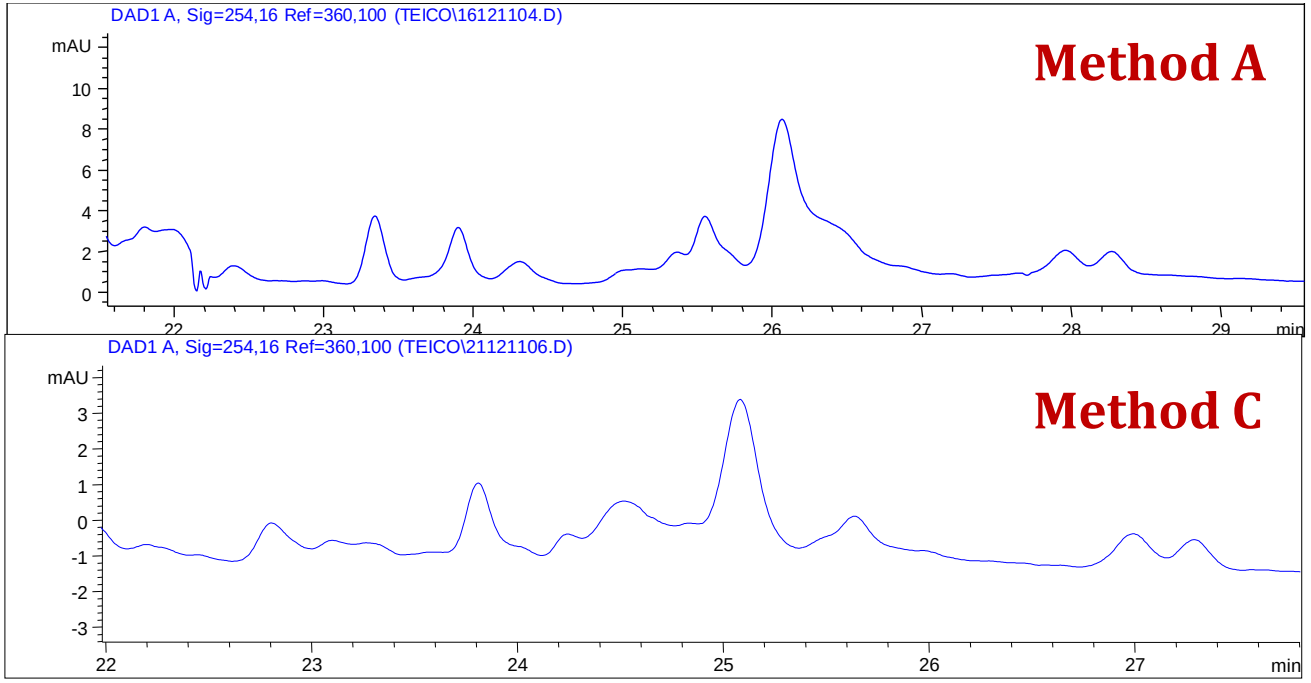
- **Ammonium acetate 25 mM (Method B)**
  - **volatile** buffer widely used in LC-MS
  - **buffering capacity** very close to **pH 6.0** (from pH 3.8 to pH 5.8)
  - same ionic strength (25 mM)



The resulting peaks are similar in **retention time** and **size**, suggesting no effect due to the **nature of the buffer** used in the mobile phase

For LC-MS analysis, the maximum salt concentration in the case of ammonium acetate is 10 mM, as higher concentrations cause an increase in the spray ion current with ion suppression and loose in sensitivity.

- **Ammonium acetate 10 mM (Method C)**
  - volatile buffer widely used in LC-MS
  - buffering capacity very close to pH 6.0 (from pH 3.8 to pH 5.8)
  - **lower ionic strength** (10 mM)



UV profile of impurities with RRT > 1.25

	Rt	RRt	Area	Groups	%	
1	23.296	1.252	7.13823	Impurities	**0.20**	Total Imp
2	23.864	1.283	22.09070		**0.60**	**1.8**
3	24.971	1.342	8.63600		**0.24**	
4	25.508	1.371	9.40412		**0.26**	
5	26.040	1.399	27.45780		**0.75**	
6	27.973	1.503	11.63040		**0.32**	
7	28.293	1.521	11.52140		**0.31**	

Method A

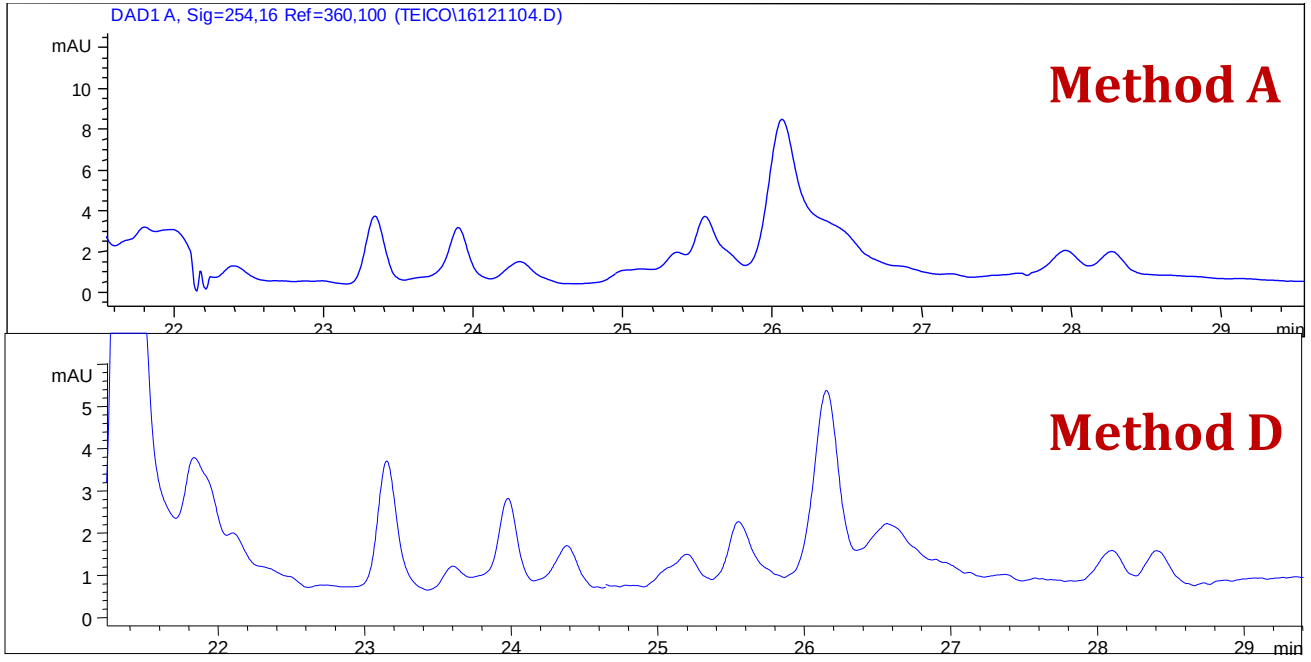
	Rt	RRt	Area	Groups	%	
1	23.809	1.280	16.22060	Impurities	**0.30**	Total Imp
2	24.519	1.318	12.15000		**0.23**	**1.3**
3	25.084	1.349	41.65820		**0.78**	
4	25.638	1.379	11.35720		**0.21**	
5	26.991	1.451	11.95770		**0.22**	
6	27.288	1.467	9.27349		**0.17**	

Method C

The **reduction of** ammonium acetate **molarity** resulted in a significant loss in efficiency.

➢ Ammonium formate 25 mM (Method D)

- volatile buffer widely used in LC-MS
- buffering range between 2.8 and 4.8, which is slightly far from the pH required for Teicoplanin (pH 6.0)
- **Does not cause** significant **ion suppression** also at higher concentrations (25 mM)



UV profile of impurities with RRt > 1.25

	Rt	RRt	Area	Groups	%	
1	23.296	1.252	7.13823	Impurities	**0.20**	Total Imp
2	23.864	1.283	22.09070		**0.60**	**1.8**
3	24.971	1.342	8.63600		**0.24**	
4	25.508	1.371	9.40412		**0.26**	
5	26.040	1.399	27.45780		**0.75**	
6	27.973	1.503	11.63040		**0.32**	
7	28.293	1.521	11.52140		**0.31**	

Method A

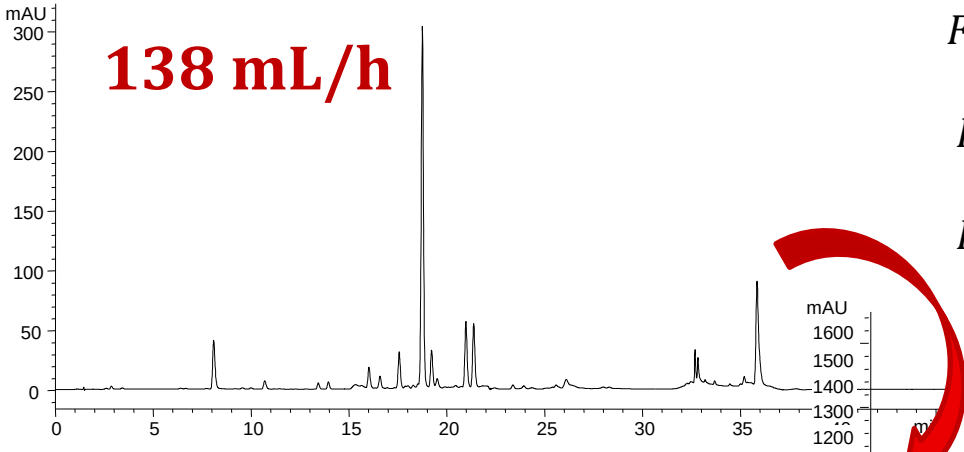
	Rt	RRt	Area	Groups	%	
1	23.977	1.281	16.85930	Impurities	**0.34**	Total Imp
2	24.386	1.303	11.00210		**0.22**	**2.5**
3	25.198	1.346	8.06771		**0.16**	
4	25.551	1.365	14.96930		**0.30**	
5	26.152	1.397	51.92630		**1.05**	
6	27.563	1.419	26.89560		**0.54**	
7	28.100	1.501	8.86832		**0.18**	
8	28.402	1.517	9.22724		**0.19**	

Method D

The same RRT and areas were obtained and the chromatographic profile, mainly in the region for unknown impurities, traced the typical and clear profile of the impurities giving an **unequivocal fingerprint of the unknown impurities**

2) Scale-down the analytical method for LC-ESI-MS

ESI interface does not support a flow rate of 2.3 mL/min due to technical limitations of the ionization source itself.
Scale-down, compared to splitting the flow before ESI, results in an **enhanced sensitivity** and in **saving solvents**.

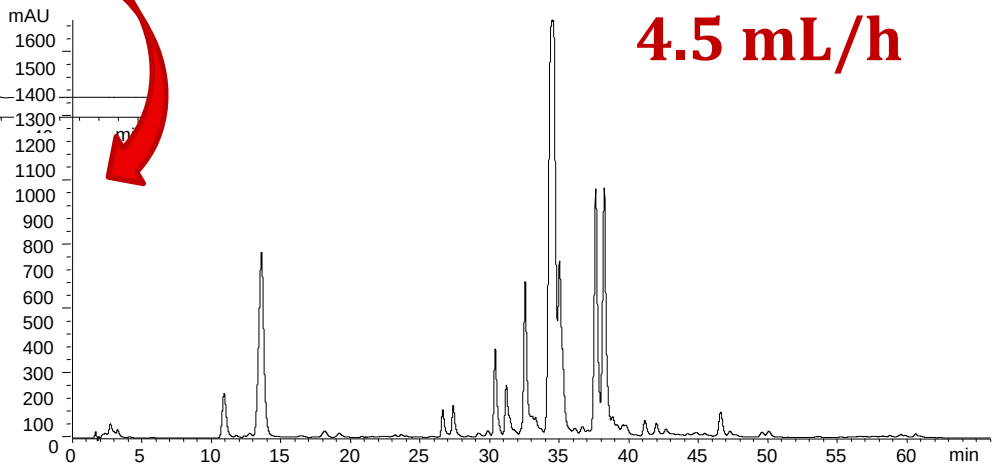


138 mL/h

*From 4.6 to **1 mm column***

*From 2.3 to **0.075 mL/min***

From 300 to 1600 mAU



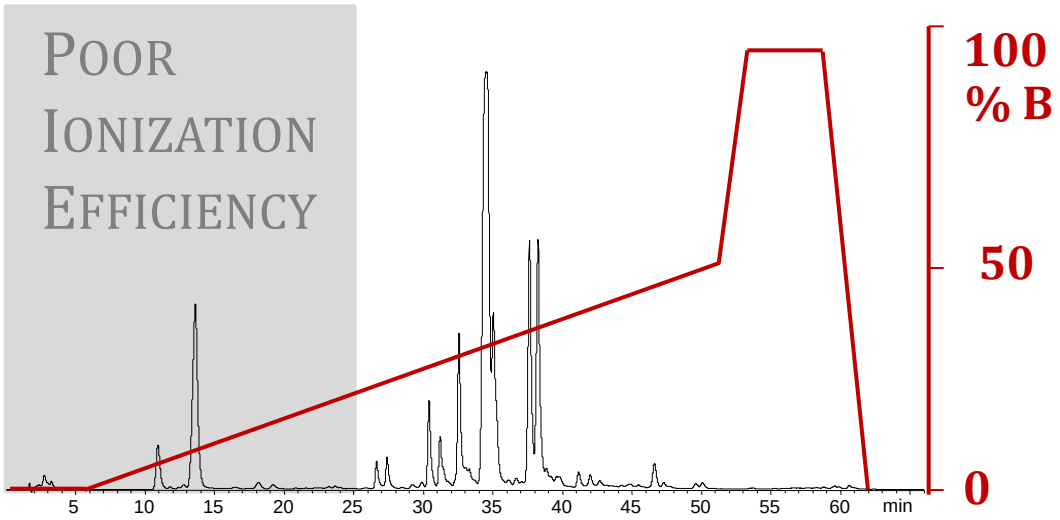
4.5 mL/h

Time	% B
0	0
6	0
50	50
53	90
66	90

Column: Symmetry C18, (1 x 150mm, 3.5 µm)

3) Assisting ESI Ionization

Instrument: LTQ linear ion trap mass spectrometer with ESI source

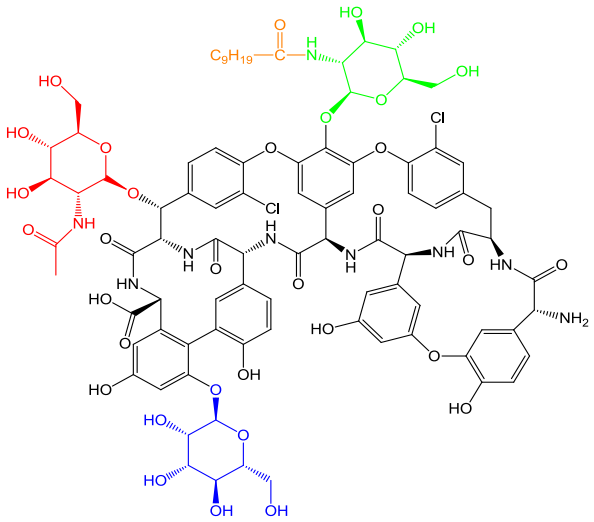


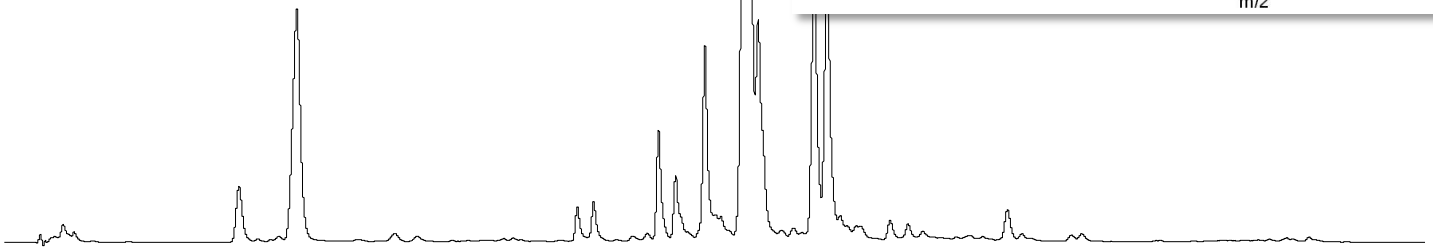
ESI/MS conditions:
Spray voltage 5 kV
Capillary temperature 250.0 ºC
Capillary voltage 18.0 V
Tube lens 115.0 V
Sheat gas flow 25 arbitrary units
Auxiliary gas flow 5 arbitrary units
Mass range 400-2000 Da

ESI/MS/MS conditions:
Isolation width 8 *m/z*
Normalized Collision Energy 35
Activation Q 0.25
Activation time 30 ms
Scan range optimized for each ion

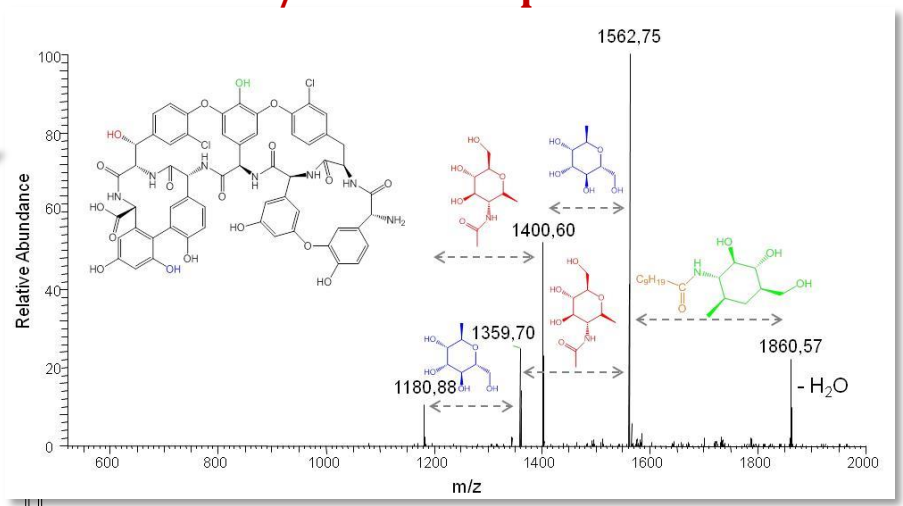
Make-up flow

Identification of Structure of Sub-Components in Teicoplanin API and the Originator



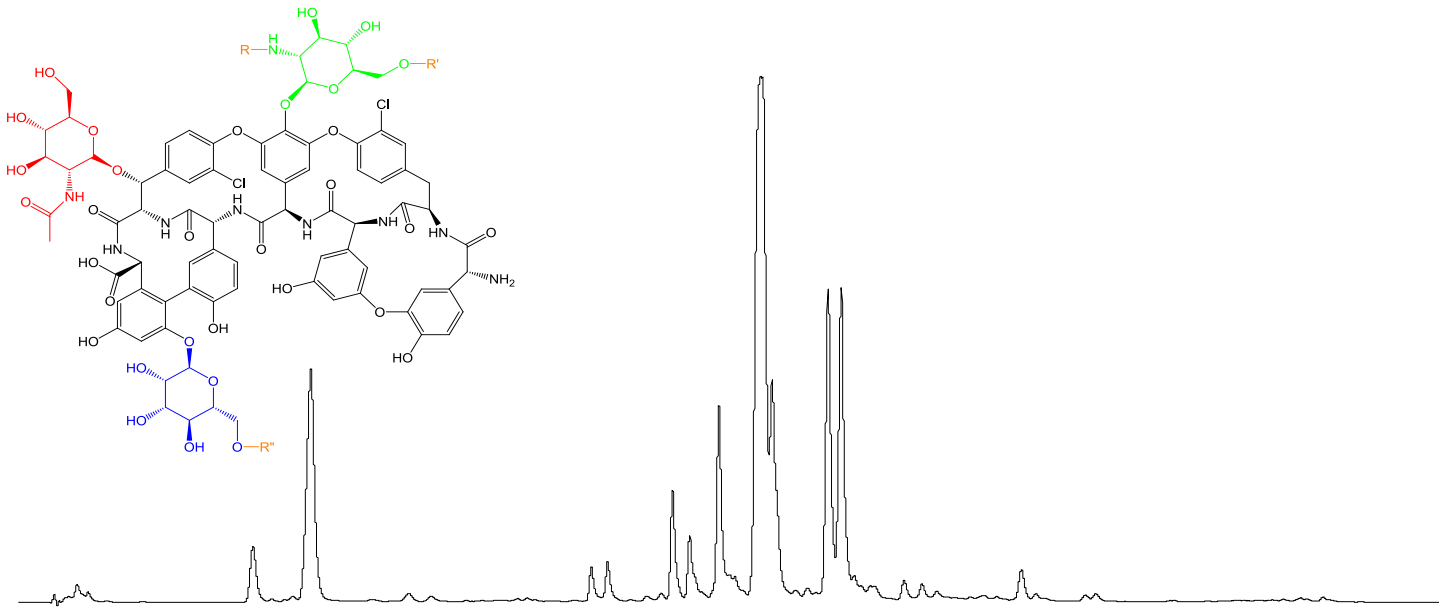


ESI-MS/MS of Teicoplanin A2-2



Structure Elucidation of Qualified Unidentified Impurities

According to the EMA Assesment Report for Teicoplanin (194668/2013) the following impurities have been considered qualified by the originator: RS1 (RRT 1.25), RS2 (RRT 1.30) and the peak at RRT 1.38.



Teicoplanin ($A_{2\text{-}2}$ main peak)	R= OC-C_9H_{19}	R'=H	R"(mannose) = H
RS1 or regio-isomers (RRT 1.25)	R= OC-CH_3	R'=OC-$C_{10}H_{21}$	R" (mannose) = OC-CH_3
RS2 (RRT 1.28)	R= OC-C_8H_{17}	R'=H	R'(mannose) = OC-CH_3
Peak at RRT 1.38	R= OC-C_9H_{19}	R'=H	R'(mannose) = OC-CH_3

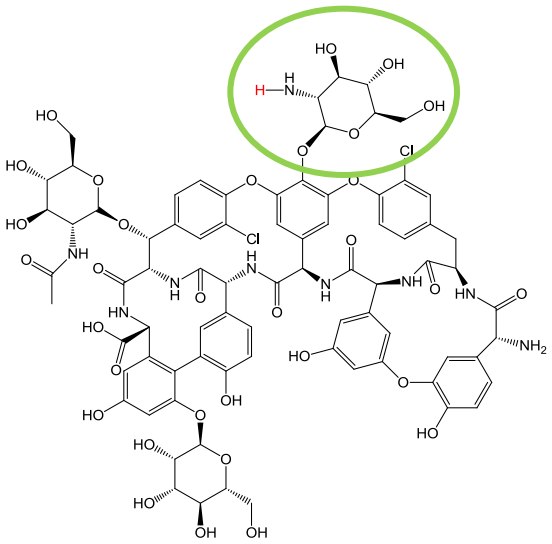
Structure Elucidation of other Unknown Sub-Components

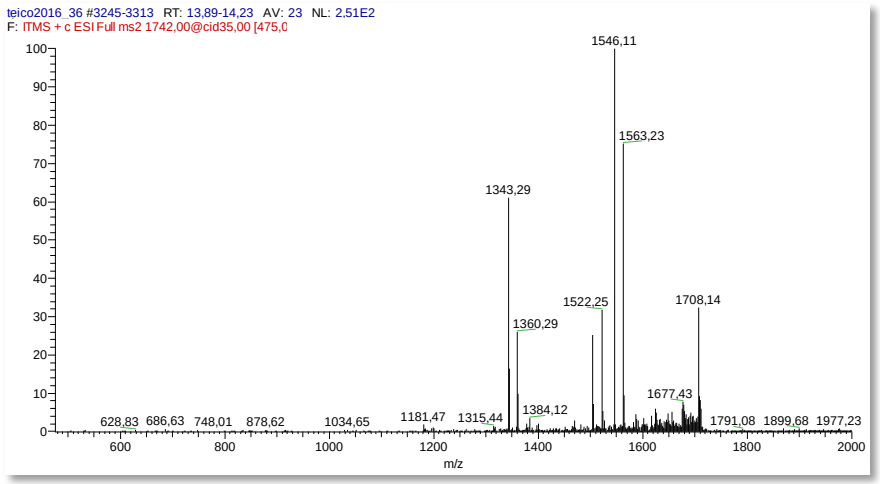
In Targocid other impurities are present in addition to the peaks corresponding to the compound considered part of the Teicoplanin complex API. The same HPLC-MS/MS has been applied to the structure assignment of these sub-components.

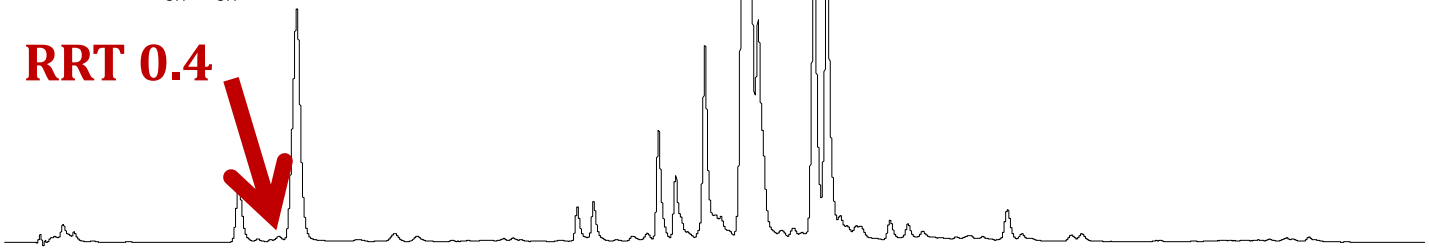
Sub-component	RRT Method Ph. Eur.	RRT Method LC-MS	*m/z* value	
1	0.40	0.39	1742	
2	0.71	0.77	1867	
3	0.74	0.79	1867	
4	0.974	0.96	1893	**A2-1 isomer**
5	0.98	-	1029	
6	1.02	1.01	1692	
7	1.04	-	1733	
8	1.09	1.07	1861	
9	1.11	-	1036	
10	1.17	1.12	1909	**A2-4 isomer**

Structure Elucidation of other Unknown Sub-Components

Subcomponent 1 (m/z 1742)



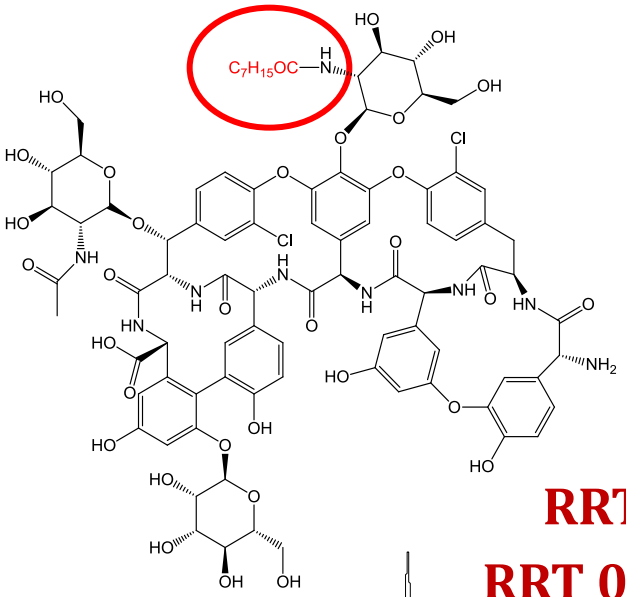


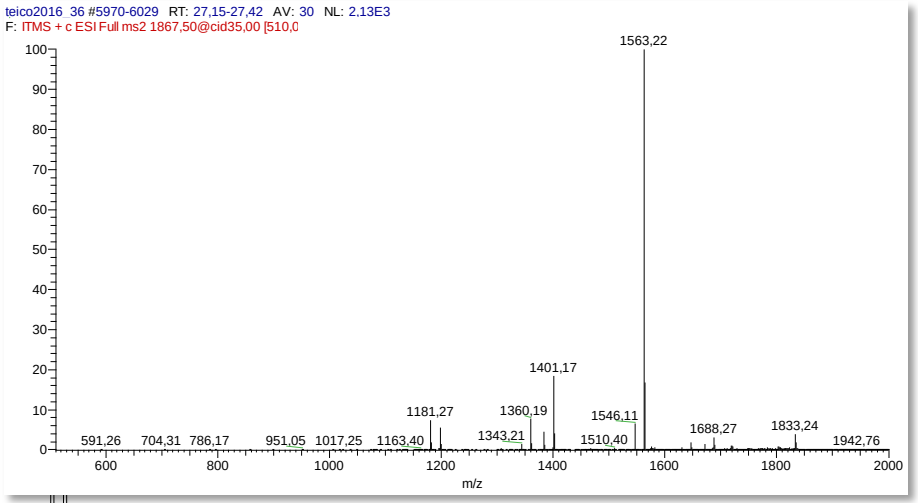


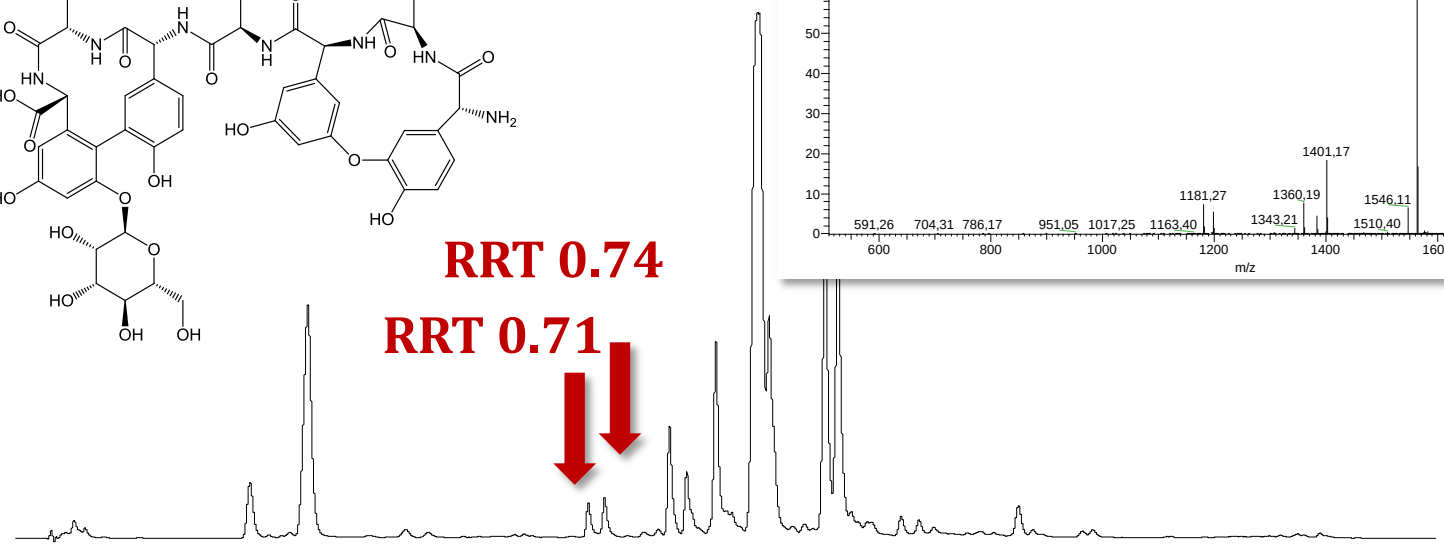
Teicoplanin-like structure belonging to the **A_3 group**, as differs from Teicoplanin main component ($A_{2\text{-}2}$) for the **absence** of the residue **R** on **Glucosamine**

Structure Elucidation of other Unknown Sub-Components

Subcomponents 2 and 3 (m/z 1867)



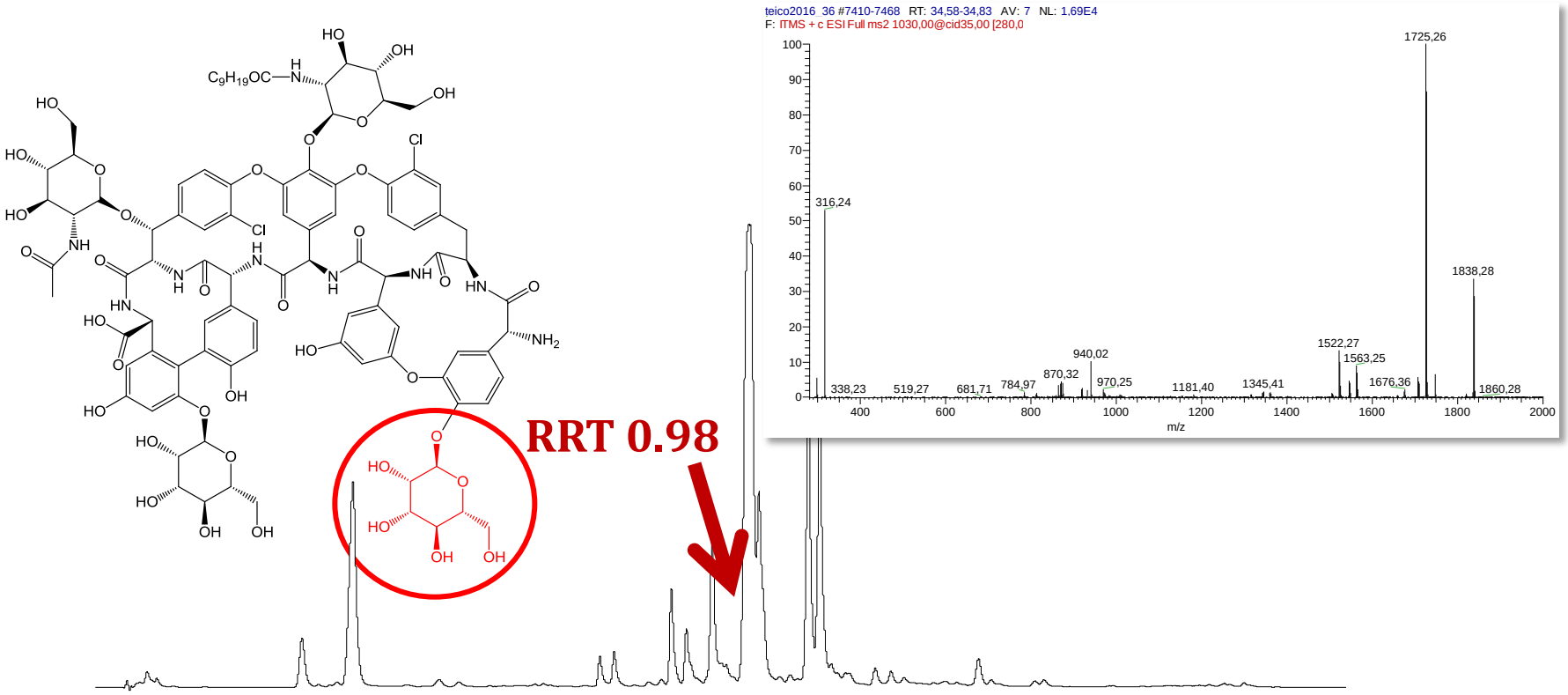




Teicoplanin-like structure hysomers, differs from Teicoplanin main component ($A_{2\text{-}2}$) for the residue R on Glucosamine (**inferior homologues**)

STRUCTURE ELUCIDATION OF OTHER UNKNOWN SUB-COMPONENTS

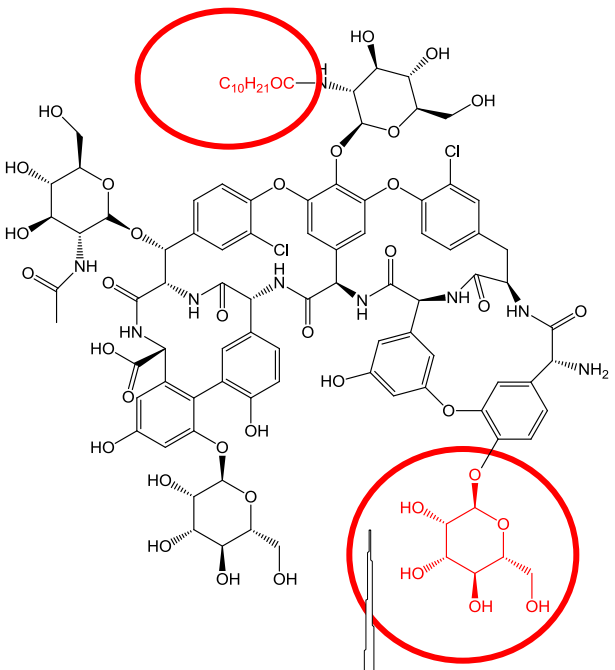
SUBCOMPONENT 5 (m/z 1029 doubly charged)

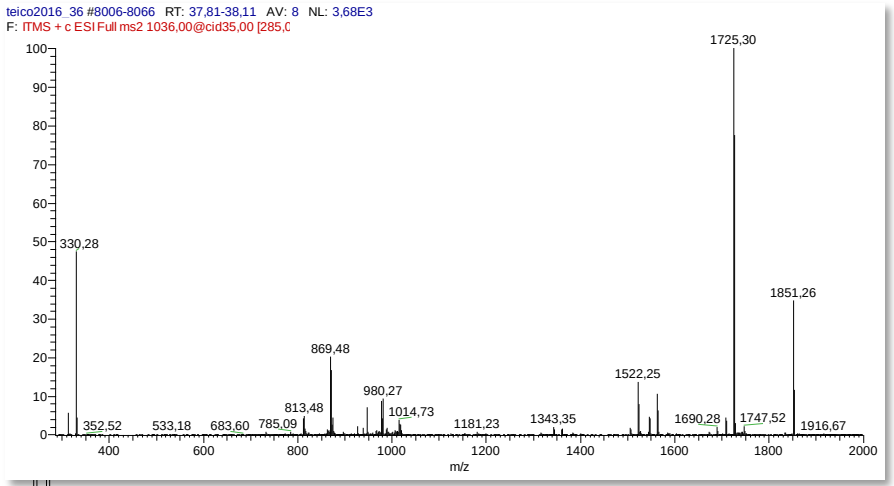


Teicoplanin-like, differs from Teicoplanin main component ($A_{2\text{-}2}$) for **one additional mannose** residue to the most accessible phenolyc group.

STRUCTURE ELUCIDATION OF OTHER UNKNOWN SUB-COMPONENTS

SUBCOMPONENT 9 (m/z 1036 doubly charged)

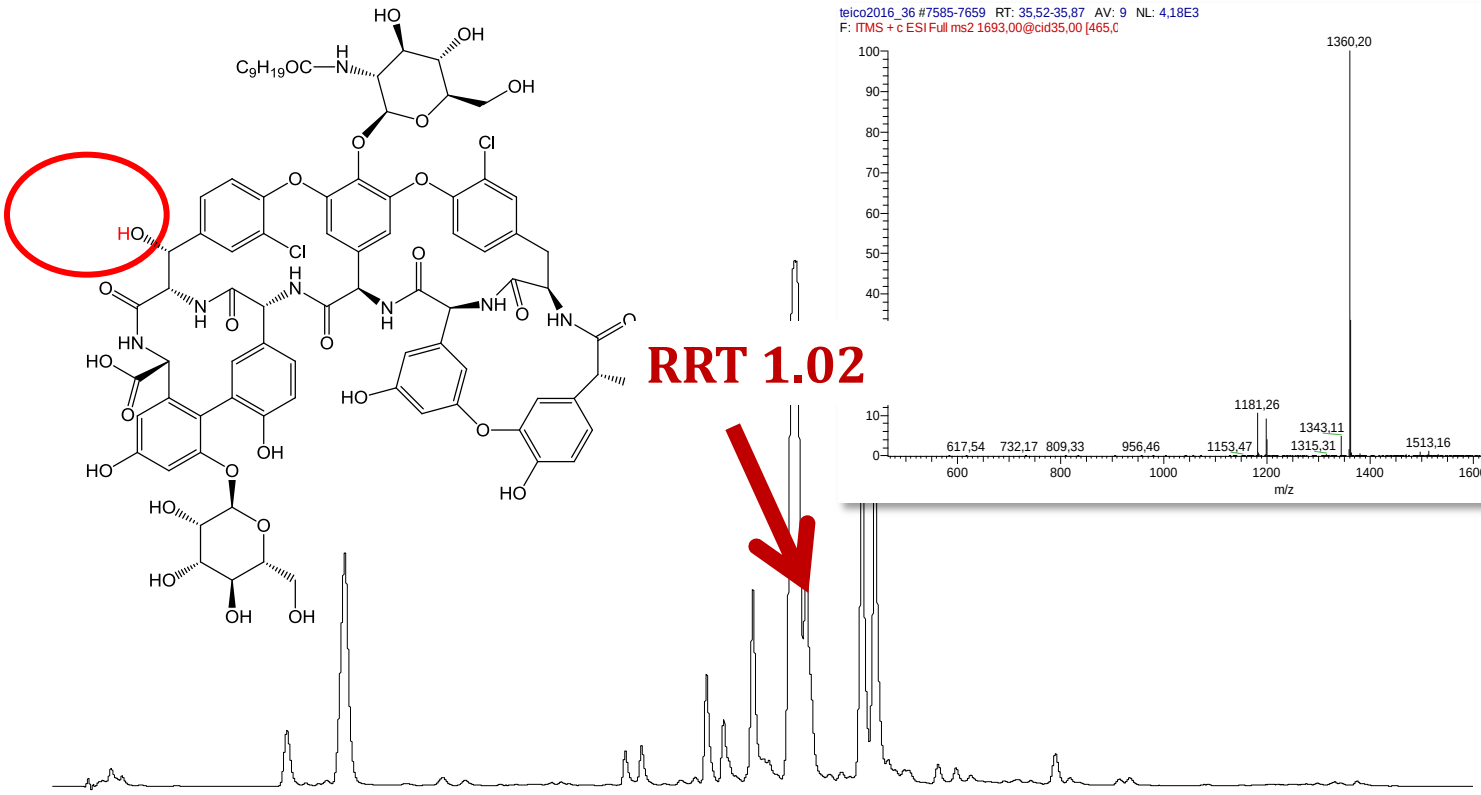




Teicoplanin-like, differs from Subcomponent 5 for the residue R on Glucosamine (**superior homologue**)

Structure Elucidation of other Unknown Sub-Components

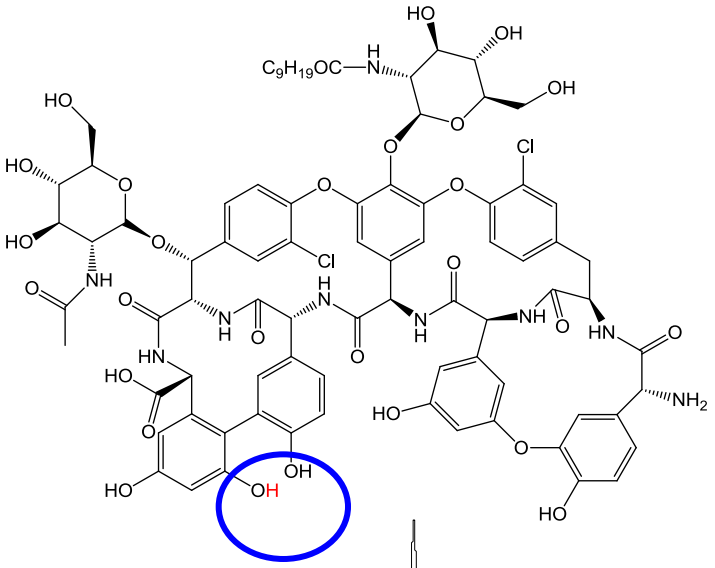
Subcomponent 6 (m/z 1692)

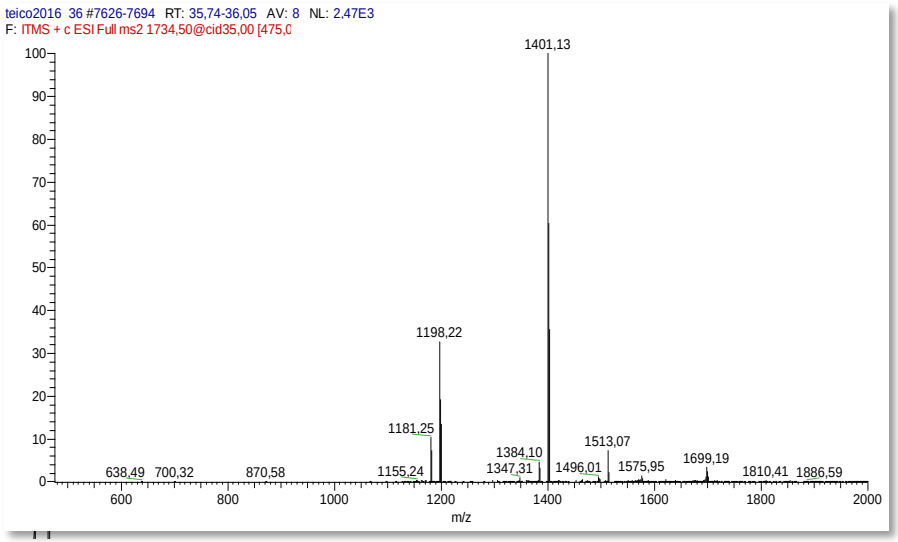


Teicoplanin-like, Teicoplanin main component ($A_{2\text{-}2}$) for the loss of the **N-Acetylglucosammine** residue.

Structure Elucidation of other Unknown Sub-Components

Subcomponent 7 (m/z 1733)



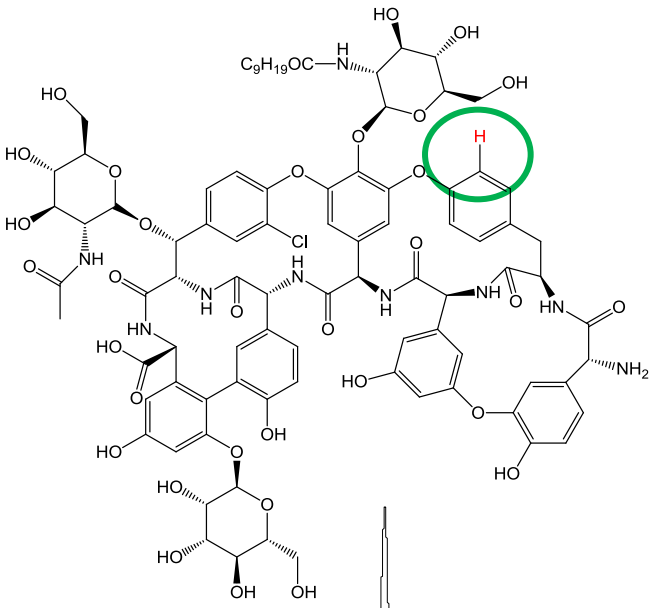


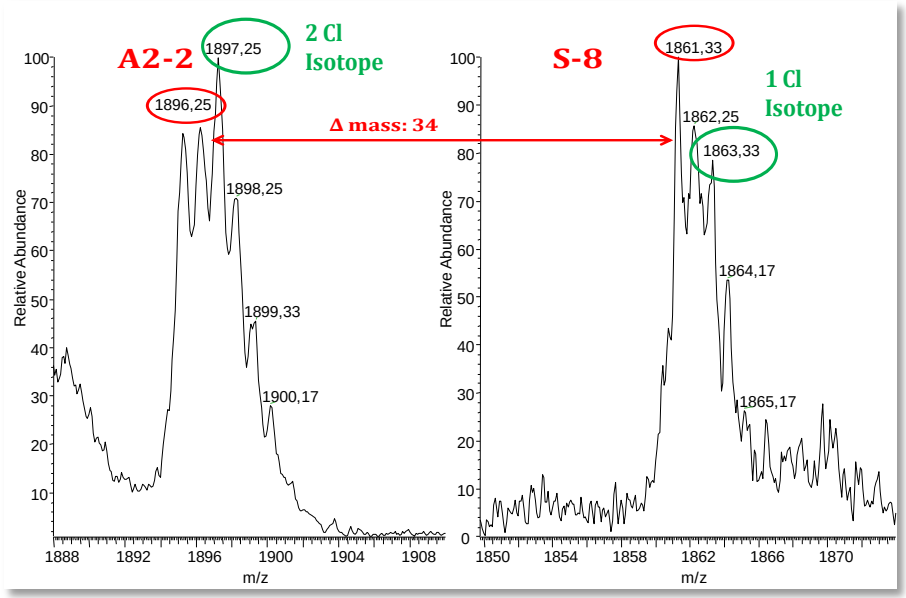
RRT 1.04

Teicoplanin-like, differ from Teicoplanin main component ($A_{2\text{-}2}$) for the loss of the **mannose** residue.

Structure Elucidation of other Unknown Sub-Components

Subcomponent 8 (m/z 1861)

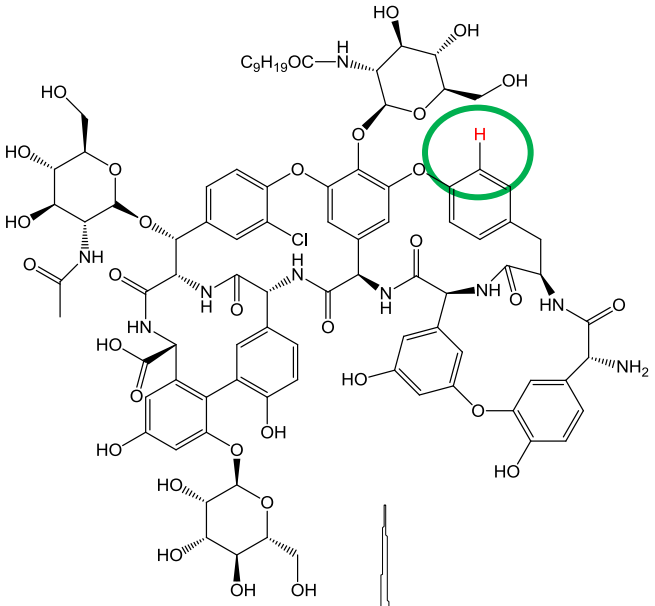


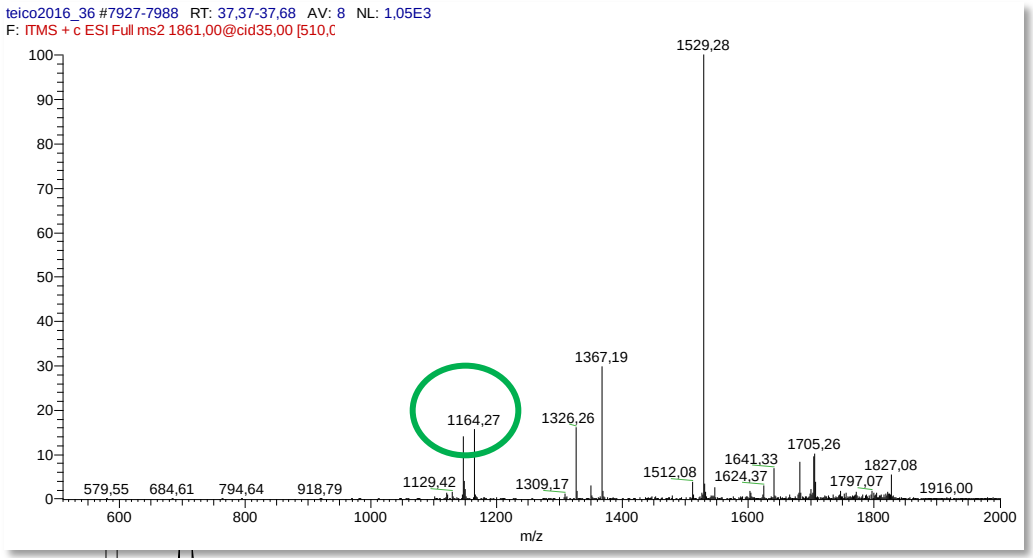


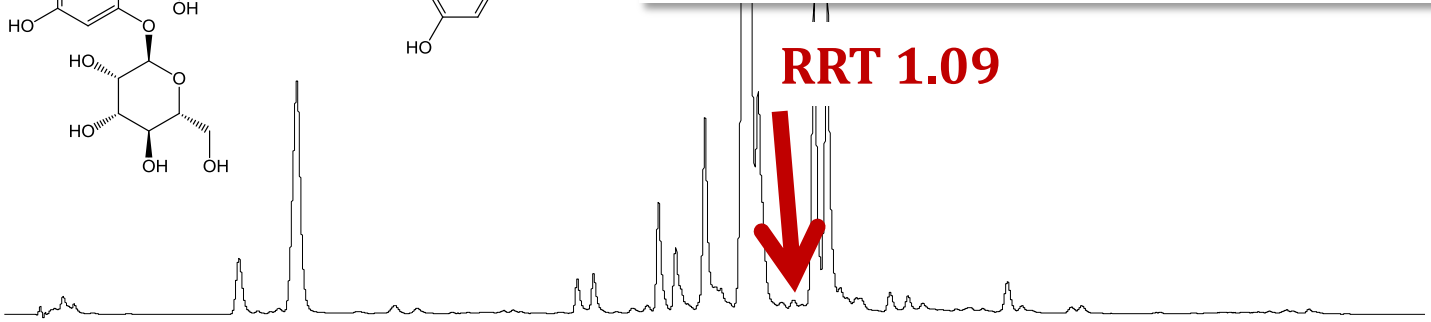
Teicoplanin-like, differ from Teicoplanin main component ($A_{2\text{-}2}$) for the loss of **one Cl atom in the core** .

Structure Elucidation of other Unknown Sub-Components

Subcomponent 8 (m/z 1861)







Teicoplanin-like, differ from Teicoplanin main component ($A_{2\text{-}2}$) for the loss of **one Cl atom in the core** .

Full Characterization of a Reference Profile (Targocid)

The Analytical Problems:

- ✓ Identification/Qualification
  Development of a Sensitive ad Informative Analytical method alternative to the HPLC-UV as from Ph. Eur.

- ✓ Quality
  Sensitive and Selective Method for Residual Components from Fermentation and Downstream (i.e. Olygosaccharides)

LOW AND HIGH MW POLYSACCHARIDIC IMPURITIES DETERMINATION IN TEICOPLANIN

PARAMETERS TO CONSIDER:

- Chromatographic **Selectivity** for Polar Compounds (*Hydroplilic Interaction Liquid Chromatography HILIC*).

- Detection **Specificity** and **Sensitivity** (ESI-MS/MS *Neutral Loss monitoring*)

Development of an HILIC-ESI-MS/MS Method for Resisual Low and High MW Polysaccharide Determination

Column: **Amide 80**, (2 x 150mm, 3mm), Tosoh Bioscience

Flow rate: 0.2 mL/min

Injection volume: 10 µL

Elution conditions:

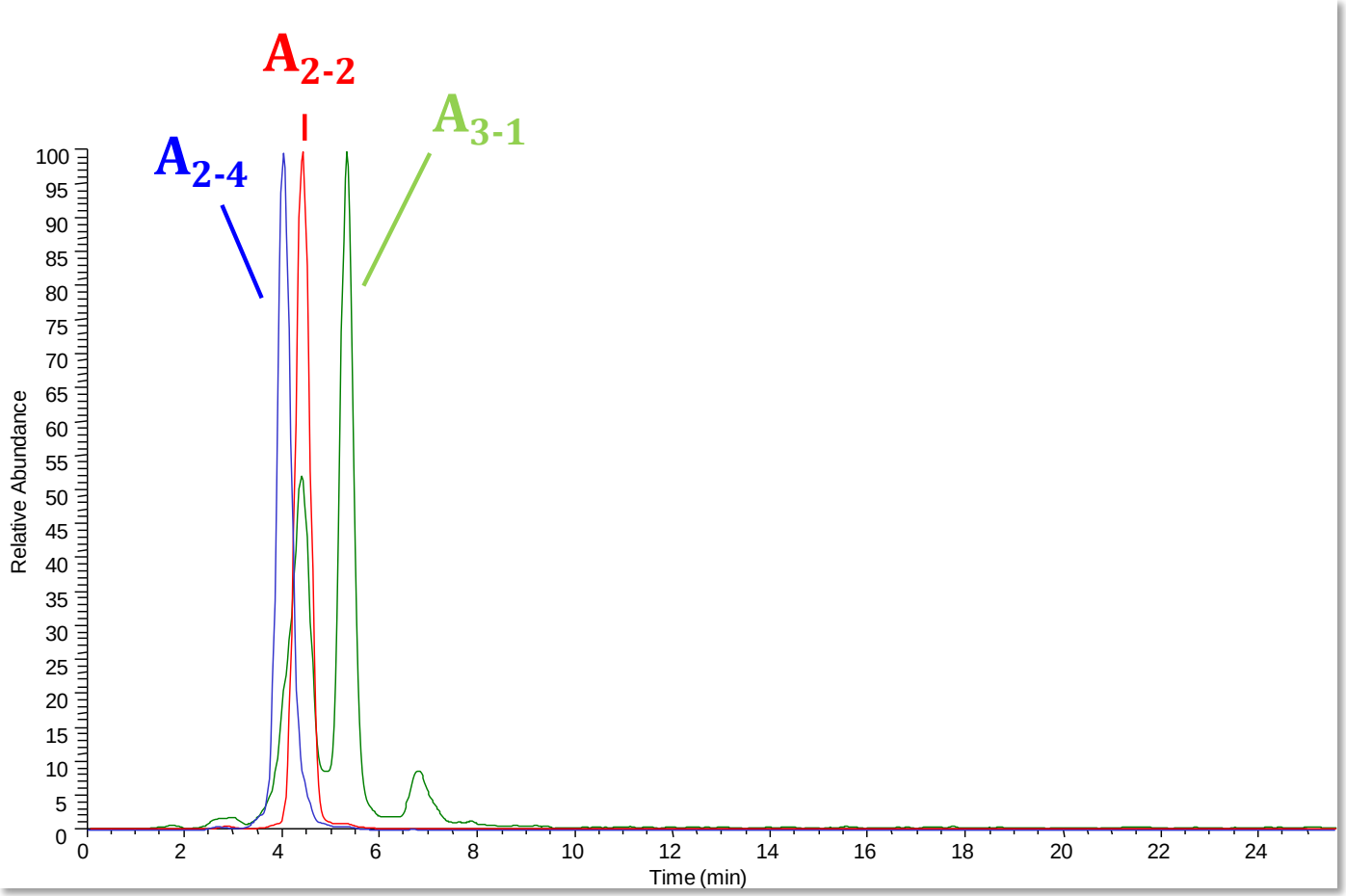
0 min.	70% ACN + 0.05% TFA	30% H2O + 0.05% TFA
30 min.	50% ACN + 0.05% TFA	50% H2O + 0.05% TFA

Detection: ESI-MS/MS

Diluent: 70% of acetonitrile and 30% of water

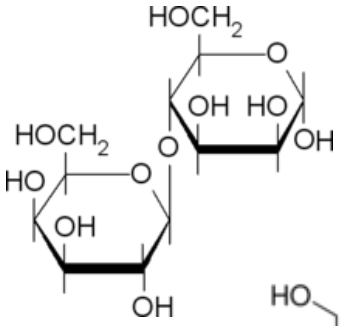
Definition of "Complex" Region

Subcomponents of the Complex are resolved and their eution order is OPPOSITE than in RP.

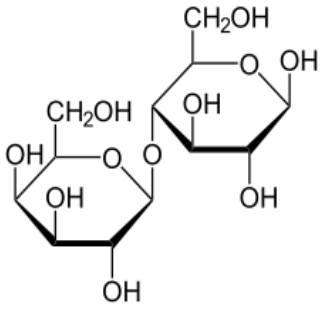


Selection of Appropriate Standards: Low and High MW Polysaccharides

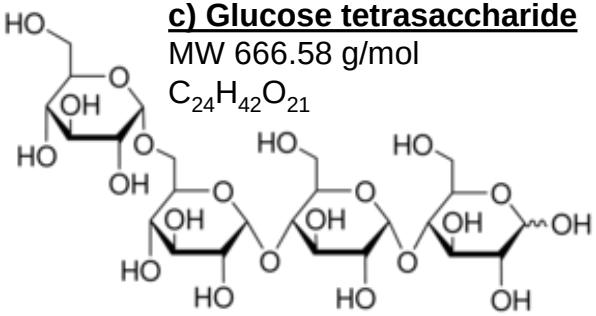
a) 4-O-β Galactopyranose D-Mannopyranose
MW 342.3 g/mol
$C_{12}H_{22}O_{11}$



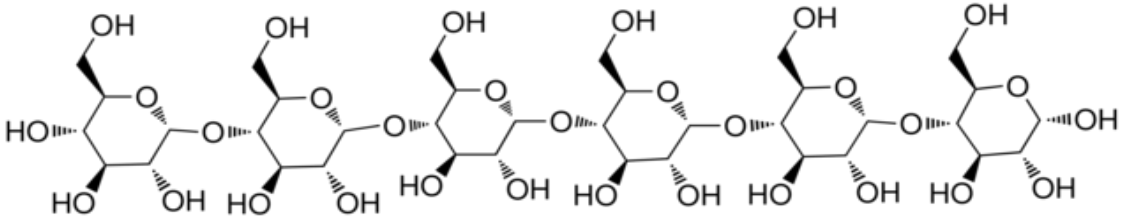
b) Lactose (β-D-Galactopyranosyl-D-Glucose)
MW 342.3 g/mol
$C_{12}H_{22}O_{11}$



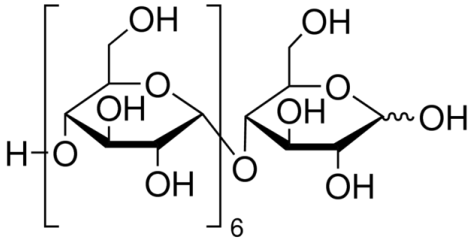
c) Glucose tetrasaccharide
MW 666.58 g/mol
$C_{24}H_{42}O_{21}$



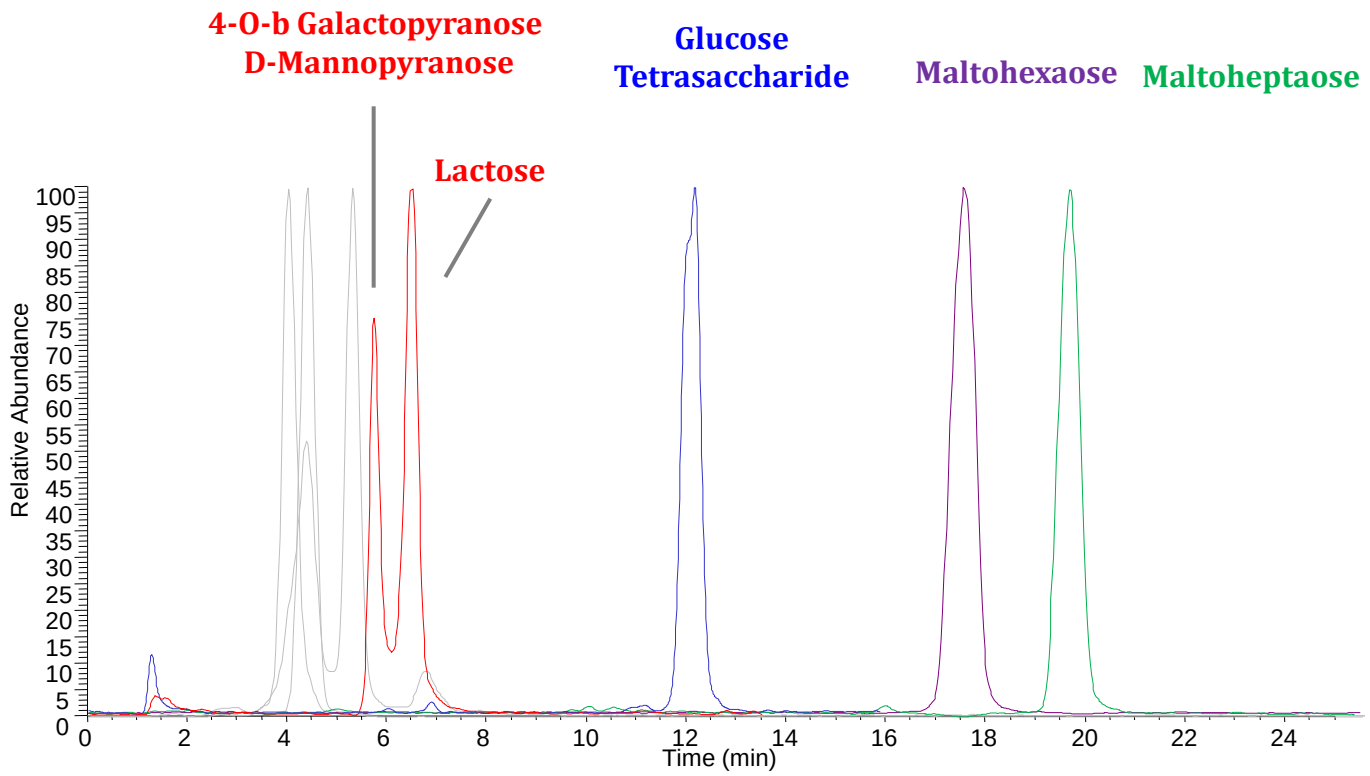
d) Maltohexaose
MW 990.86 g/mol
$C_{36}H_{62}O_{31}$



e) Maltoheptaose
MW 1153.00 g/mol
C42H72O36



OLYGOSACCHARIDES SEPARATION



✓ Olygosaccharides differing for one sugar unit are completely resolved (ΔRT minimun 2 min)

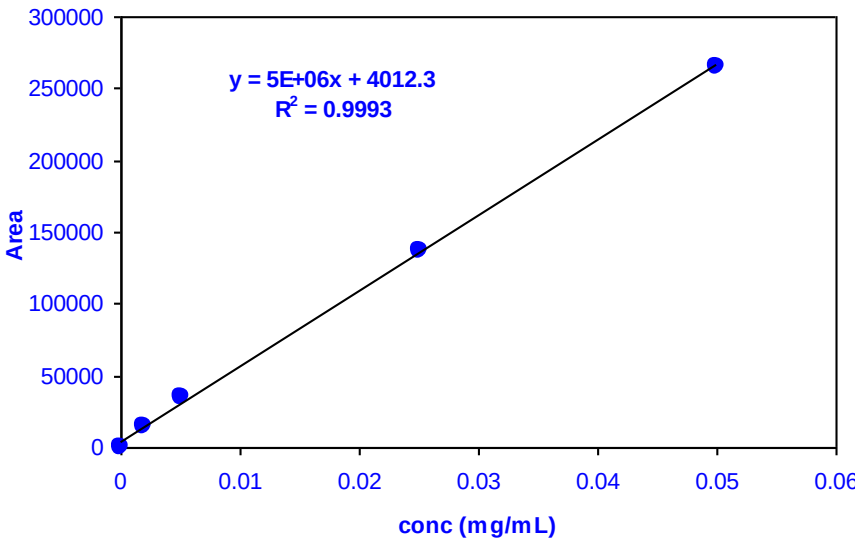
✓ Selectivity for isomers

LOD AND LOQ DETERMINATION

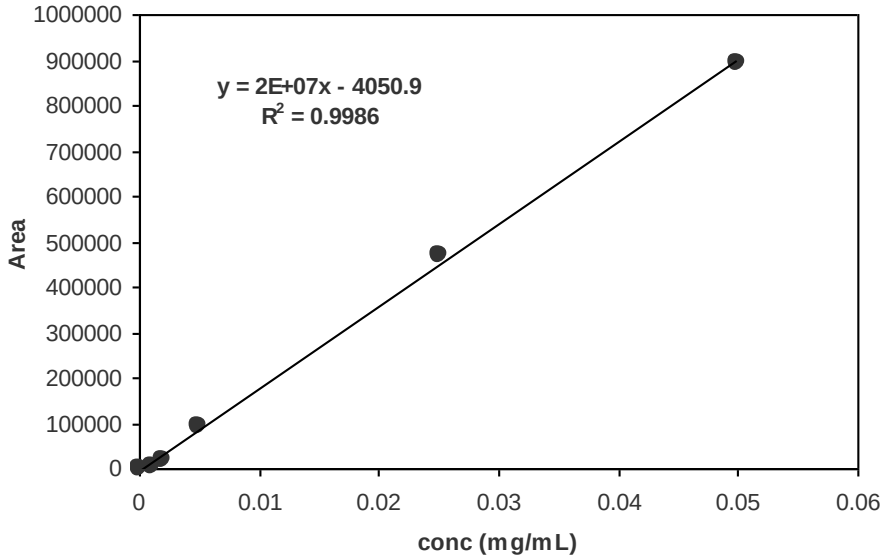
Different concentration levels of the standards were considered to assess sensitivity and linearity of the method.
The concentrations were selected as referred to 2 mg/mL Teicoplanin:

- 0.05 mg/mL (corresponding to **2.5%** of Teicoplanin concentration, or 25000 ppm);
- 0.025 mg/mL (corresponding to **1.25%** of Teicoplanin concentration, or 12500 ppm);
- 0.005 mg/mL (corresponding to **0.25%** of Teicoplanin concentration, or 2500 ppm);
- 0.002 mg/mL (corresponding to **0.1%** of Teicoplanin concentration, or 1000 ppm); ← **LOQ**
- 0.001 mg/mL (corresponding to **0.05%** of Teicoplanin concentration, or 500 ppm). ← **LOD**

4-O-β GALACTOPYRANOSE D-MANNOPYRANOSE

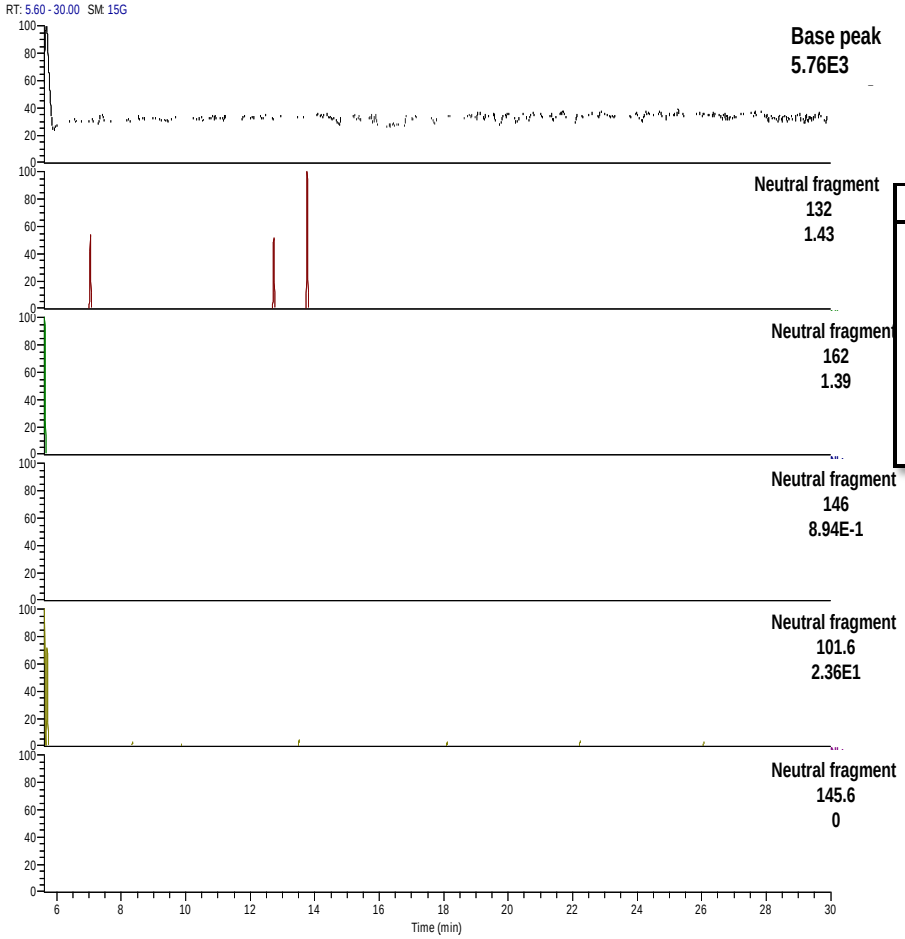


MALTOHEPTAOSE



RESIDUAL OLYGOSACCHARIDES ANALYSIS IN TARGOCID

To determine the presence of carbohydrates impurities, the characteristic **Neutral Loss** of different classes of sugars were followed in the MS/MS spectra generated in data dependent mode.



Carbohydrate	Composition	Neutral loss	
		+ 1	+ 2
Penthose	$C_5O_5H_{10}$	132	66
Hexose	$C_6O_6H_{12}$	162	81
Deoxy-Hexose	$C_6O_5H_{12}$	146	73
N-acetylhexose	$C_8O_6NH_{15}$	203.2	101.6
Sialic acid	$C_{11}O_9NH_{19}$	291.3	145.6

✓No residual polysaccharides were detected in the Reference Sample and in the API

QUALIFICATION OF THE API

The ANALYTICAL Solutions

Identification/Qualification

✓ Developed a Sensitive ad Informative Analytical method alternative to the HPLC-UV as from Ph. Eur.

Quality

✓ Developed a Sensitive and Selective Method for Residual Olygosaccharides.

Grazie per l’attenzione