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# Identification of impurities in polymyxin B and colistin bulk sample using liquid chromatography coupled to mass spectrometry

Larissa Van den Bossche, Ann Van Schepdael, Shruti Chopra, Jos Hoogmartens, Erwin Adams*

*Katholieke Universiteit Leuven, Laboratorium voor Farmaceutische Analyse, Faculteit Farmaceutische Wetenschappen, O & N 2, Herestraat 49 (PB 923), B-3000 Leuven, Belgium*

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ABSTRACT

The European Pharmacopoeia (Ph. Eur.) describes liquid chromatography-ultraviolet (LC-UV) methods using $C_{18}$ stationary phases for the analysis of polymyxin B and colistin.

Several unknown impurities were detected in commercial samples of those polypeptide complexes. However, the Ph. Eur. does not specify any related substances for polymyxin B and colistin. Since both methods use non-volatile buffers, the mobile phases were incompatible with mass spectrometry (MS). For the identification of related substances in bulk samples by LC/MS, volatile mobile phase systems were developed. However, the LC/MS methods (with volatile additives) showed inferior chromatographic separation compared to the LC-UV method (with non-volatile additives). Moreover, previously identified impurities by LC/MS could not be assigned in LC-UV methods as the separation in both systems was different.

In this study, known impurities were traced in the LC-UV methods and new impurities present in polymyxin B and colistin bulk samples were characterized. To achieve this, each peak from the non-volatile system was collected separately and reinjected into an LC system with a volatile mobile phase coupled to MS. This way, collected impurity peaks were rechromatographed on a reversed phase column in order to separate the analyte from the buffer salts. Using this method, out of 39 peaks, five novel related substances were characterized in a polymyxin B bulk sample. Fourteen impurities, which were already reported in the literature were traced as good as possible in the LC-UV method. In the case of colistin, a total of 36 peaks were investigated, among which four new compounds. Additionally, 30 known impurities were traced in the LC-UV method.



## 1. Introduction

The polymyxins are polypeptide antibiotics isolated from various strains of *Bacillus polymyxa* and related species. They are known to have a potent bactericidal activity against a broad range of Gram-negative bacteria [1,2]. Like all polymyxins, these polypeptides have a general structure composed of a cyclic heptapeptide and a side-chain consisting of a tripeptide with a fatty acyl residue (FA) on the N-terminus (Table 1) [1,3]. Several related substances of the polymyxin B and E series were already described in literature [1,4–11]. The large variety of components other than amino acids, unusual peptide linkages and also for most of these antibiotics their cyclic nature, makes their structural elucidation in many instances quite complicated [1]. The least toxic members polymyxin B and polymyxin E were introduced into medicine. Polymyxin B contains four major components, differing only in the FA moiety attached to the tripeptide side-chain and cyclic peptide, *i.e.*, 6-methyloctanoic acid in polymyxin $B_1$, 6-methylheptanoic acid in polymyxin $B_2$, octanoic acid in polymyxin $B_3$ and heptanoic acid in polymyxin $B_4$. In commercial samples, polymyxins $B_1$ and $B_2$ are the main constituents while polymyxins $B_3$ and $B_4$ are considered as minor components [4].

Colistin was isolated from *Bacillus polymyxa var. colistinus* in 1950 and was shown to be identical to polymyxin E [12]. Polymyxin E differs from polymyxin B only by the substitution of D-leucine (Leu) for D-phenylalanine (Phe) as one of the amino acids in the cyclic part of the structure. The structures of polymyxins $E_1$, $E_2$, $E_3$ and $E_4$ are illustrated in Table 1(b). Polymyxins $E_1$ and $E_2$ are the two main compounds, which have the same amino acid composition, but differ from each other in the fatty acid. Polymyxins $E_1$ and $E_2$ contain 6-methyloctanoic acid and 6-methylheptanoic acid, respectively, while polymyxin $E_3$ and $E_4$ contain octanoic acid and heptanoic acid, respectively [5].

The lack of novel antibiotics against Gram-negative bacteria and the growing resistance to broad-spectrum antibiotics has reinstated polymyxins as the drugs of last resort to treat serious infections caused by multidrug-resistant (MDR) Gram-

* Corresponding author. Tel.: +32 16 323440; fax: +32 16 323448.
*E-mail addresses:* erwin.adams@pharm.kuleuven.be, Erwin.Adams@pharm.kuleuven.ac.be (E. Adams).

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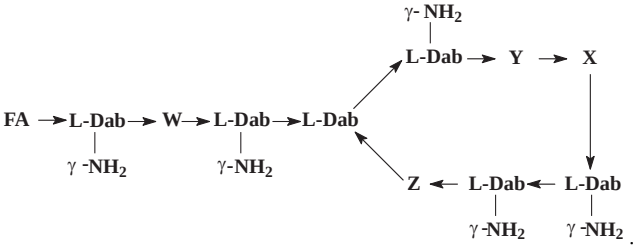
**Table 1**
(a) Chemical structures of known and new polymyxins B with their *m*/*z* ratio of doubly charged ions. Impurities marked with "*" were characterized by Govaerts et al. [9], while those marked with a "#" were new structures of components present in a polymyxin B bulk sample, established in this work; Dab, α,γ-diaminobutyric acid; FA, fatty acyl; Ser, serine; Leu, leucine; Phe, phenylalanine; Thr, threonine; Ile, soleucine; Val, valine; Glu, glutamic acid; Tyr, tyrosine; Ser, serine; Pro, proline; Imp, impurity, %, relative content.
(b) Chemical structures of known and new polymyxins E (colistin) with their *m*/*z* ratio of doubly charged ions. Impurities marked with "*" were characterized by Govaerts et al. [9], while those marked with a "#" were new structures of components present in a polymyxin E (colistin) bulk sample, established in this work; Met, methionine; Norval, norvaline.




| Polymyxin | Peak | W | X | Y | Z | FA | *m*/*z* | % (rel. content) |
|---|---|---|---|---|---|---|---|---|
| (a) | | | | | | | | |
| $B_1$ | 35 | L-Thr | L-Leu | D-Phe | L-Thr | 6-Methyloctanoyl | 602.4 | 49.9 |
| Ile-$B_1$ | 31 | L-Thr | L-Ile | D-Phe | L-Thr | 6-Methyloctanoyl | 602.4 | 2.4 |
| $B_2$ | 23 | L-Thr | L-Leu | D-Phe | L-Thr | 6-Methylheptanoyl | 595.4 | 23.8 |
| $B_3$ | 25 | L-Thr | L-Leu | D-Phe | L-Thr | Octanoyl | 595.4 | 6.9 |
| $B_4$ | 12 | L-Thr | L-Leu | D-Phe | L-Thr | Heptanoyl | 588.4 | 1.6 |
| $B_5$ | 37 | L-Thr | L-Leu | D-Phe | L-Thr | Nonanoyl | 602.4 | 1.9 |
| $B_6$ | 18 | L-Thr | L-Leu | D-Phe | L-Thr | 3-OH-6-Methyloctanoyl | 610.4 | 2.5 |
| Imp 1* | 1, 7, 8, 9 | Thr | Leu/Ile | Phe | Thr | $C_8H_{15}O_2$ | 603.4 | 0.02, 0.8, 0.2, 0.2 |
| Imp 2* | 11 | Thr | Val | Phe | Thr | $C_8H_{15}O$ | 588.4 | 0.4 |
| Imp 3* | 14 | Thr | Tyr-Leu/Ile or Leu/Ile-Tyr or Glu-Phe | Phe-Glu or | Thr | $C_9H_{17}O$ | 610.4 | 0.13 |
| Imp 4* | 17, 19, 21, 22, 24, 26, 27, 28, 29, 32, 33, 34 | Thr | Leu/Ile | Phe | Thr | $C_8H_{15}O$ | 595.4 | 0.07, 1.3, 0.4, 0.4, 0.5, 0.07, 0.3, 0.06, 0.3, 1.1, 0.4, 0.9 |
| Imp 5* | 20, 21 | Ser | Leu/Ile | Phe | Thr | $C_8H_{15}O$ | 588.4 | 0.5, 0.4 |
| Imp 6* | Not found | Thr | Val | Phe | Thr | $C_9H_{17}O$ | 595.4 | |
| Imp 7* | 32 | Ser | Leu/Ile | Phe | Thr | $C_9H_{17}O$ | 595.4 | 1.1 |
| Imp 8# ($B_1$ isomer) | 2, 4, 6, 29, 30, 33, 34, 36, 38, 39 | Thr | Leu/Ile | Phe | Thr | $C_9H_{17}O$ | 602.5 | 0.1, 0.1, 0.05, 0.3, 0.1, 0.4,0.9, 1.2, 0.2, 0.2 |
| Imp 9# ($B_4$ isomer) | 8, 10, 13, 14 | Thr | Leu/Ile | Phe | Thr | $C_7H_{13}O$ | 588.3 | 0.2, 0.02, 0.4, 0.1 |
| Imp 10# | 5 | Thr | Leu/Ile | Phe | Thr | $C_6H_{11}O$ | 581.4 | 0.1 |
| Imp 11# | 3, 4, 5, 16, 17 | Thr | Tyr-Leu/Ile or Leu/Ile-Tyr or Glu-Phe | Phe-Glu or | Thr | $C_8H_{15}O$ | 603.3 | 0.1, 0.3, 0.1, 0.2, 0.07 |
| Imp 12# | 6 | Thr | Leu/Ile | Phe | Thr | $C_{10}H_{19}O$ | 609.2 | 0.05 |
| Imp 13# | 9 | Thr | Phe-Phe or Met-Tyr or Tyr-Met | | Thr | $C_9H_{17}O$ | 619.4 | 0.2 |
| Imp 14# | 17 | Thr | Leu/Ile-Leu/Ile (~$E_2$ or $E_3$) or Glu-Pro or Pro-Glu | | Thr | $C_8H_{15}O$ | 578.5 | 0.07 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Imp 15# (~$E_1$) (b) | 29 | Thr | Leu/Ile-Leu/Ile | | Thr | $C_9H_{17}O$ | 585.4 | 0.3 |
| $E_1$ | 32 | L-Thr | Leu | D-Leu | L-Thr | 6-Methyloctanoyl ($C_9H_{17}O$) | 585.4 | 36.5 |
| Ile-$E_1$ | 27 | L-Thr | Ile | D-Leu | Thr | 6-Methyloctanoyl ($C_9H_{17}O$) | 585.4 | 1.0 |
| Imp 1* | 1, 6, 8, 23, 25, 28, 29, 33, 36 | Thr | Leu/Ile | Leu | Thr | $C_9H_{17}O$ | 585.3 | 0.25, 0.3, 0.7, 0.1, 0.3, 0.4, 0.2, 0.3, 0.3 |
| Imp 2* | | Thr | Leu/Ile | Leu | Thr | $C_9H_{17}O$ | 585.3 | |
| $E_7$ | 34 | L-Thr | Leu | D-Leu | L-Thr | 7-Methyloctanoyl ($C_9H_{17}O$) | 585.4 | 4.5 |
| $E_2$ | 17 | L-Thr | Leu | D-Leu | L-Thr | 6-Methylheptanoyl ($C_8H_{15}O$) | 578.4 | 41.3 |
| Ile-$E_2$ * | 10, 11, 13, 16, 18, 20, 24, 26 | L-Thr | Ile | D-Leu | Thr | 6-Methylheptanoyl ($C_8H_{15}O$) | 578.4 | 0.1, 0.1, 1.5, 0.4, 2.3, 0.6, 0.1, 0.4 |
| $E_3$ | | L-Thr | Leu | D-Leu | L-Thr | Octanoyl ($C_8H_{15}O$) | 578.4 | |
| Imp 3* | | Thr | Leu/Ile | Leu | Thr | $C_8H_{15}O$ | 578.3 | |
| Imp 4* | | Thr | Leu/Ile | Leu | Thr | $C_8H_{15}O$ | 578.3 | |
| $E_4$ | 7 | L-Thr | Leu | D-Leu | L-Thr | $C_7H_{13}O$ | 571.4 | 2.5 |
| Imp 5* | | Thr | Leu | Leu | Thr | $C_7H_{13}O$ | 571.3 | |
| Val-$E_2$ | 9 | L-Thr | Val | D-Leu | L-Thr | 6-Methylheptanoyl ($C_8H_{15}O$) | 571.4 | 0.1 |
| Imp 6* | | Thr | Val | Leu | Thr | $C_8H_{15}O$ | 571.3 | |
| Imp 7* | | Thr | Val | Leu | Thr | $C_8H_{15}O$ | 571.3 | |
| Val-$E_1$ | 19, 22 | L-Thr | Val | D-Leu | L-Thr | 6-Methyloctanoyl ($C_9H_{17}O$) | 578.4 | 1.8, 0.1 |
| Imp 8* | | Thr | Val | Leu | Thr | $C_9H_{17}O$ | 578.3 | |
| Imp 9* | | Thr | Val | Leu | Thr | $C_9H_{17}O$ | 578.3 | |
| Nva-$E_1$ | | L-Thr | Norval | D-Leu | L-Thr | 6-Methyloctanoyl ($C_9H_{17}O$) | 578.4 | |
| Ile-$E_8$ | Not found | L-Thr | Ile | D-Leu | L-Thr | 7-Methylnonanoyl ($C_{10}H_{19}O$) | 592.4 | |
| Imp 10* | 4, 5, 24 | Thr | Leu/Ile | Leu | Thr | $C_8H_{15}O_2$ | 586.3 | 0.7, 0.2, 0.1 |
| Imp 11* | 9, 12 | Thr | Met | Leu | Thr | $C_8H_{15}O$ | 587.3 | 0.2, 1.2 |
| Imp 12* | 12 | Thr | Leu/Ile | Leu | Thr | $C_9H_{17}O_2$ | 593.3 | 1.2 |
| Imp 13* | 14, 15, 16 | Ser | Leu/Ile | Leu | Thr | $C_8H_{15}O$ | 571.3 | 0.3, 0.3, 0.4 |
| Imp 14* | 23 | Thr | Met | Leu | Thr | $C_9H_{17}O$ | 594.3 | 0.1 |
| Imp 15* | 28 | Ser | Leu/Ile | Leu | Thr | $C_9H_{17}O$ | 578.3 | 0.4 |
| Imp 16* | 30 | Thr | Leu/Ile | Leu | Thr | $C_9H_{15}O$ | 584.5 | 0.4 |
| Imp 17* | 22 | Thr | Leu/Ile | Leu | Ser | $C_9H_{17}O$ | 578.3 | 0.1 |
| Imp 18* | 19, 31 | Most probably formed by loss of water from $E_1$ | | | | | 576.4 | 1.8, 0.3 |
| Imp 19* | 8, 25 | Most probably formed by loss of water from $E_2$ | | | | | 569.5 | 0.7, 0.3 |
| Imp 20# | 1, 2, 25 | Thr | Leu/Ile-Phe or Phe-Leu/Ile | | Thr | $C_8H_{15}O$ | 595.5 | 0.2, 0.1, 0.3 |
| Imp 21# | 3 | Thr | Leu/Ile | Leu | Ser | $C_6H_{11}O$ | 564.3 | 0.4 |
| Imp 22# | 21 | Pro | Leu/Ile | Leu | Thr | $C_8H_{15}O$ | 576.5 | 0.1 |
| Imp 23# | 35 (=$E_1$-Dab) | Thr | Leu/Ile | Leu | Thr | $C_9H_{17}O$ | 535.4 | 0.04 |

negatives. However, polymyxins are nephrotoxic and this may complicate the therapy or even require its discontinuation. During the years, many attempts have been made to characterize the commercially available polymyxin B complex, which is marketed for the topical treatment of cutaneous, otic, external ocular, meningeal and mucosal infections caused by susceptible microorganisms, especially *Pseudomonas aeruginosa* [2,5,13–16]. Since qualification and identification of impurities are critical issues in assessing the safety and quality of a pharmaceutical drug substance, selective and sensitive methods are mandatory.

The Ph. Eur. [17,18] describes selective and sensitive LC-UV methods, based on the LC-UV methods developed by Orwa et al. [19,20], for the separation of related compounds in commercial polymyxin B and polymyxin E samples. However, the Ph. Eur. does not specify any related substances present in these polypeptide complexes.

Despite considerable efforts of isolation and purification, only few structures could be determined [19,20]. In order to identify additional impurities in commercial polymyxin B and polymyxin E bulk samples, an LC/MS method with a volatile mobile phase was developed by Govaerts et al. [9,21]. However, the volatile LC/MS method showed inferior selectivity compared to the non-volatile LC-UV methods, developed by Orwa et al. [19,20]. Furthermore, the elution order differed, which makes the correlation of the peaks with the LC-UV method difficult.

The analysis of proteins and peptides by LC/MS mostly involves the use of trifluoroacetic acid (TFA) as ion-pairing agent despite it is a strong suppressor of the MS signal. Different studies, reporting the effects of using ion-pairing agents and buffers in LC/MS of proteins and peptides, did not yield a single strong candidate that could generally replace TFA. The enhancement in sensitivity observed in some cases with other reagents strongly depended on the analyte and the experimental conditions used, but it did not compensate for the loss in separation and resolution in comparison with TFA [22].

In this study, each peak from the LC-UV system using non-volatile additives was collected and reinjected into an LC/MS system with volatile additives. Because the latter system does not require an ion-pairing agent, TFA was replaced by formic acid, which is known to enhance sensitivity in LC/MS analysis, without giving suppression of the MS signal.

## 2. Experimental

### 2.1. Chemicals

Acetonitrile (HPLC grade S) and formic acid (99% ULC/MS grade) were purchased from Biosolve (Valkenswaard, The Netherlands), anhydrous sodium sulphate from Merck (Darmstadt, Germany) and phosphoric acid solution (85%, m/m) from Acros Organics (Geel, Belgium). A Milli-Q purification system (Millipore, Bedford, MA, USA) was used to further purify demineralized water.

### 2.2. Samples and sample preparation

Polymyxins $B_1$, $B_2$, $E_1$, $E_2$, $B_3$, $B_4$, $B_5$ and $B_6$ reference substances were available in the laboratory. They were prepared by semi-preparative reversed-phase LC [10,23]. All reference substances were dissolved separately in water at a concentration of 0.05 mg/mL. Polymyxin B sulphate and polymyxin E sulphate bulk samples were obtained from Ludeco (Brussels, Belgium) and Asahi Kasei Shiraoi (Hokkaido, Japan), respectively.

### 2.3. LC instrumentation and chromatographic conditions

#### 2.3.1. LC-UV system with non-volatile mobile phase

The methods used for the analysis of polymyxins B and E are those described in the Ph. Eur. [17,18].

The equipment used with the non-volatile mobile phase consisted of an UltiMate 3000 pump, an ASI-100 automated sample injector from Dionex (Sunnyvale, CA, USA) and a variable-wavelength TSP Spectra 100 UV-VIS detector set at 215 nm (San Jose, CA, USA). The UV data were acquired with Chromeleon software Version 6.60. The YMC-Pack Pro $C_{18}$ column (5 μm, 250 mm × 4.6 mm i.d.) was obtained from Waters (Milford, MA, USA). The temperature of the column was maintained at 30 °C by immersion in a water bath with a heating circulator (Julabo EM, Seelbach, Germany). The following mobile phase was used for the separation of polymyxin B: 20 volumes of acetonitrile (ACN) and 80 volumes of a solution containing 4.46 g of anhydrous sodium sulphate dissolved in 1 L of water, previously adjusted to pH 2.3 by adding a 10% solution of phosphoric acid. The LC-pump operated at a flow rate of 1.0 mL/min. For polymyxin E the same composition of the mobile phase was used as for polymyxin B, but instead of 20 volumes ACN, 22 volumes of ACN were used. The mobile phase was degassed by sparging with helium. Solutions (0.5 mg/mL) of the bulk samples, dissolved in a mixture of 20 volumes of ACN en 80 volumes of water, were injected (100 μL) into the chromatographic system so that 50 μg was introduced on the column for chromatography.

#### 2.3.2. LC/MS system with volatile mobile phase

The equipment used with the volatile mobile phase consisted of a P680 HPLC pump from Dionex (Sunnyvale, CA, USA), a switching valve (VICI AG International, Schenkon, Switzerland) equipped with a 500 μL loop and a variable-wavelength TSP Spectra 100 UV-VIS detector set at 215 nm (San Jose, SA, USA). ChromPerfect 4.4.23 software (Justice Laboratory Software, Fife, UK) was used to record the signals from the detector. The LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) interface was operated in the positive ion mode by applying a voltage of 4.5 kV to the ESI needle. Mobile phase A consisted of an aqueous solution containing 0.1% formic acid. Mobile phase B consisted of 0.1% formic acid in ACN. The mobile phases were degassed by sparging with helium. The LC pump was operated at a flow rate of 200 μL/min. The YMC-Pack Pro $C_{18}$ column (5 μm, 250 mm × 2.1 mm i.d.) (Waters, Milford, MA, USA) was kept at room temperature.

### 2.4. Electrospray ionization tandem mass spectrometry

The abundant doubly charged ion with $m/z$ 602.4, yielded by direct infusion of a 0.05 μg/μL solution of polymyxin $B_1$, was used to automatically tune the ionization source and MS parameters. A voltage of 4.5 kV applied to the ESI needle resulted in a distinct signal. The temperature of the heated capillary was set at 250 °C. The number of ions stored in the ion trap was regulated by Auto gain control (AGC). Nitrogen supplied by Air Liquide (Liège, Belgium), was used as sheath and auxiliary gas, at a flow rate of 70 arbitrary units (arb) and 15 arb, respectively. Helium was used as damping gas and as collision gas at a pressure of 0.1 Pa. The voltages across the capillary and the octapole lenses were tuned by an automated procedure to maximize the signal for the ion of interest. The capillary voltage was set at 25 V and the tube lens offset voltage at −5 V. Octapole 1 offset voltage, octapole 2 offset voltage and the interoctapole lens voltage were set at −3 V, −6 V and −12 V, respectively. For MS/MS investigation, precursor ions were isolated in the ion trap with an isolation width of 3 u and collisionally activated at a collision energy level (CEL) of 30%. Xcalibur 1.3 software (Thermo

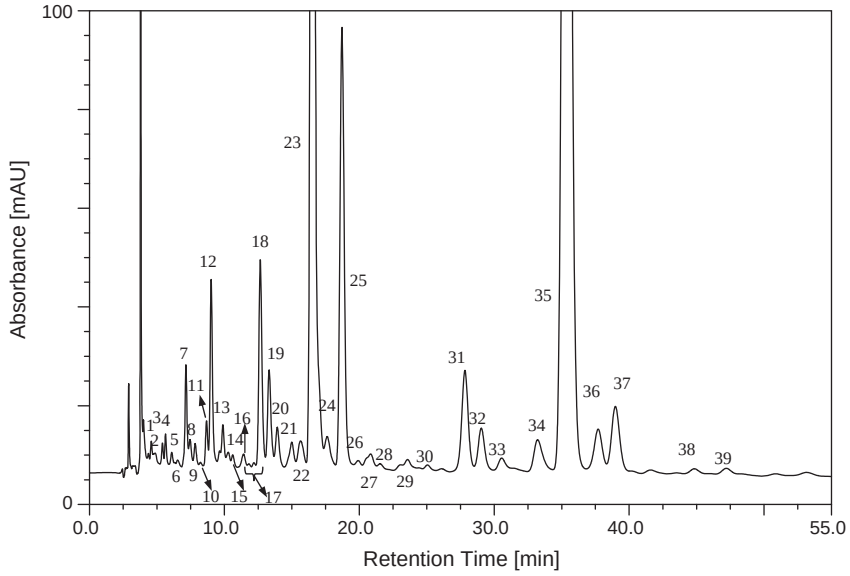



**Fig. 1.** LC-UV chromatogram of a 50 μg commercial polymyxin B sample. The peaks collected for further LC/MS investigation are indicated with a number.

Finnigan) was used for instrument control, data acquisition and processing.

### 2.5. Mass spectrometric investigation of the samples

The polymyxin B or E sulphate samples (50 μg) were introduced on the column in the LC-UV system. Each peak eluted from the non-volatile mobile phase system was collected and reinjected in the volatile mobile phase LC/MS system and analyzed online with the ion trap. Full mass spectra were acquired over the mass range *m*/*z* 500–700. For MS/MS investigation doubly charged polymyxin ions were isolated monoisotopically in the ion trap and collisionally activated at 30% (CEL).

## 3. Results and discussion

The impurity profiling of polymyxins B and E (colistin) is performed. It is worth mentioning that several chromatographic peaks may contain compounds with the same *m*/*z*. Furthermore, one peak may contain several components. The LC-UV chromatograms obtained with the non-volatile mobile phase system for polymyxins B and E are shown in Figs. 1 and 2. Each peak eluted from this system was collected and reinjected into the volatile mobile phase LC/MS system. Because the non-volatile mobile phase of the LC-UV system contains salts, a desalting procedure using a second LC column in combination with mobile phase A was applied. Next, the compound of interest was desorbed from the desalting column with the mixture mobile phase A–mobile phase B (75:25, v/v) which is suitable for LC/MS, and was transferred to the MS. The column was then regenerated with mobile phase A. Full MS spectra of 39 peaks, as indicated on the LC-UV chromatogram (Fig. 1), were obtained. Fragmentation spectra of all 39 peaks of polymyxin B were acquired with 30% CEL using doubly charged precursor ions. In the case of polymyxin E, 36 peaks were investigated by LC/MS (see Fig. 2).

### 3.1. Mass spectrometric results of polymyxin B sulphate and polymyxin E sulphate

Fragmentation patterns for polymyxin B and E were described before by Govaerts et al. [24]. The fragmentation behavior of

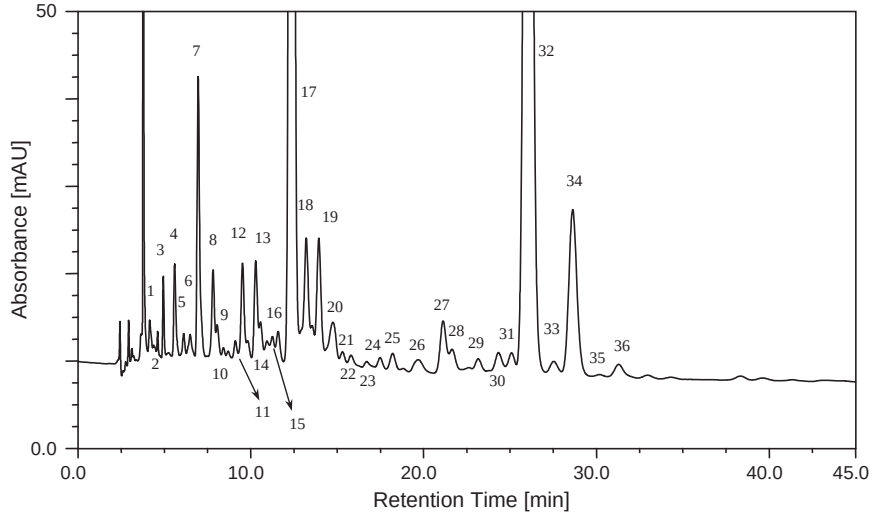



**Fig. 2.** LC-UV chromatogram of a 50 μg commercial polymyxin E or colistin sample. The peaks collected for further LC/MS investigation are indicated with a number.

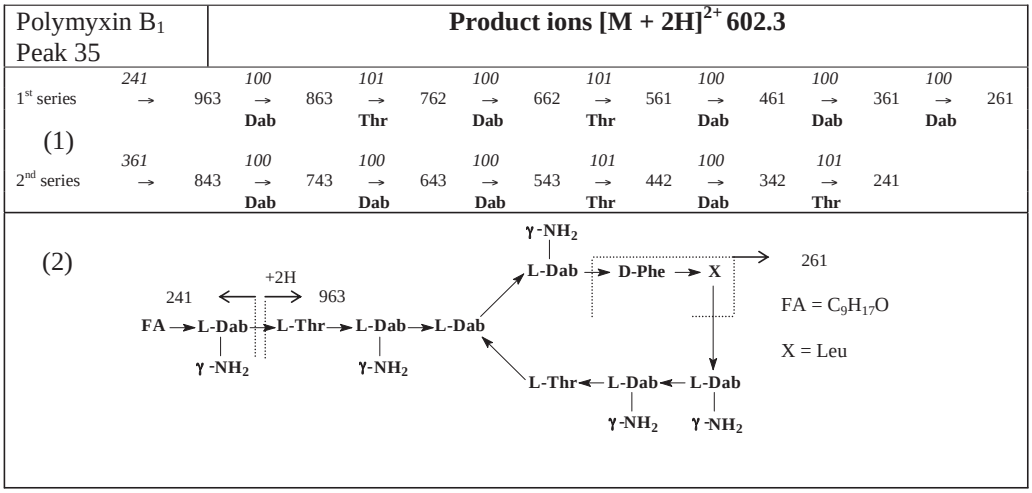



**Fig. 3.** Fragmentation pattern for polymyxin $B_1$. (1) First and second series of product ions acquired for the compound in peak 35 (Fig. 1), the result of isolation and collisional activation at 30% CEL in the ion trap of $[M+2H]^{2+}$ at *m/z* 602.3. The mass differences and the corresponding residues are indicated above and below the arrows. (2) The proposed structure with important ions leading to the characterization of the structure.

polymyxins $B_1$ and $E_1$ is illustrated in Figs. 3 and 4, respectively. MS/MS spectra show singly- and doubly charged product ions formed out of the doubly charged precursors *m/z* 602.3 (polymyxin $B_1$) and 585.3 (polymyxin $E_1$) (data not shown), respectively. Structural information is derived from the singly charged product ions coming from the doubly charged precursors, which may, therefore be observed at higher *m/z* values than the precursor. Two important series of product ions are present in the spectra. The first series of product ions displays a rather high abundance, whereas most product ions belonging to the second series are less intensely present. The first series of product ions is formed by loss of the FA moiety together with the neighbouring α,γ-diaminobutyric acid (Dab) moiety and subsequent losses of amino acids in the linear and circular part. The second series of product ions is yielded by a first loss of three ring amino acids (Dab-X-Y or X-Y-Dab, see Table 1) and subsequent losses of other amino acids. Before starting the structure elucidation of the unknowns, it needs to be mentioned that the mass spectrometric technique used does not distinguish Leu from Ile (isoleucine), and for the FA moieties, only the elemental composition could be defined and not the branching.

### 3.2. Impurity profiling study of polymyxin B

#### 3.2.1. Known polymyxin B related substances

14 polymyxin B compounds were previously described by Orwa et al. [19] and Govaerts et al. [21] (see Table 1(a)). Polymyxins $B_1$, $B_2$, $B_3$, $B_4$, $B_5$ and $B_6$ were identified according to their peak height and $MS^2$ spectra of their reference compounds. Impurities (Imp) 1–7, described by Govaerts et al. using a volatile mobile phase system [21,24], were traced in this non-volatile system by collecting each peak as mentioned in Section 3.2. Table 1(a) illustrates the structures of known and unknown components established in this work.

*3.2.1.1. Peaks 31, 35 and 37 (m/z 602.5).* Mass spectra for the compounds eluted in those peaks are identical to polymyxins $B_1$ and $B_5$. Based on the fragmentation pattern of the reference compounds and peak height, peaks 31, 35 and 37 were assigned as Ile-$B_1$, $B_1$ and $B_5$ respectively.

*3.2.1.2. Peaks 1, 7, 8 and 9 with m/z 603.4.* The compounds in peaks 1, 7, 8 and 9 were investigated. Instead of a *m/z* 227, these peaks

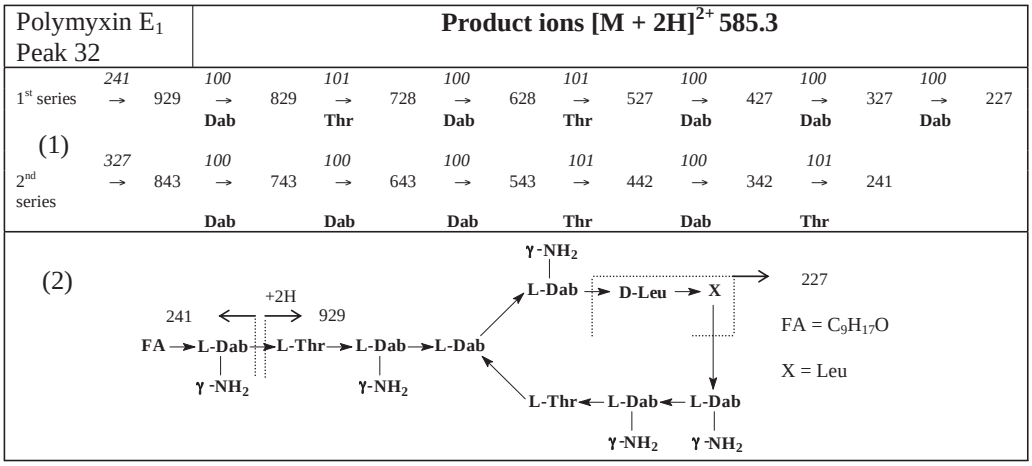



**Fig. 4.** Fragmentation pattern for polymyxin $E_1$ (colistin). (1) First and second series of product ions acquired for the compound in peak 32 (Fig. 2), the result of isolation and collisional activation at 30% CEL in the ion trap of $[M+2H]^{2+}$ at *m/z* 585.3. The mass differences and the corresponding residues are indicated above and below the arrows. (2) The proposed structure with important ions leading to the characterization of the structure.

show a *m/z* 243, corresponding to a FA residue similar to Imp 1 ($C_8H_{15}O_2$), characterized by Govaerts et al. [21].

*3.2.1.3. Peaks 17, 19, 21, 22, 23, 24, 25, 26, 27, 28, 29, 32, 33 and 34 (m/z 595.4).* Mass spectra for the compounds eluted in these peaks are identical to polymyxin $B_2$ and polymyxin $B_3$. The compounds in peak 23 and peak 25 were assigned as polymyxins $B_2$ and $B_3$ based on the comparison with the fragmentation pattern and peak height of polymyxins $B_2$ and $B_3$, respectively. The compound in peak 32 was characterized as Imp 7, where a Ser (serine) replaces the Thr residue in the linear side chain. The other peaks, characterized as Imp 4, are isomers of polymyxin $B_2$ or $B_3$ and are mentioned in Table 1(a).

*3.2.1.4. Peaks 11, 12, 20 and 21 (m/z 588.4).* The compound in peak 12 was established as polymyxin $B_4$. For peaks 20 and 21 the same series of product ions were recorded as for Imp 5, characterized by Govaerts et al. [21]. Peak 11 corresponds to Imp 2.

*3.2.1.5. Peaks 14 and 18 (m/z 610.4).* The compound in peak 14 was characterized as Imp 3. The amino acid combination at positions X and Y differs from that of polymyxin $B_1$. Several amino acid combinations are possible (Table 1(a)). The CID spectrum of peak 18 matches that of polymyxin $B_6$. Polymyxin $B_6$ has a similar sequence as polymyxin $B_1$, except that the FA residue is 3-hydroxy-6-methyloctanoic acid [10].

*3.2.2. New related substances*

The first and second series of product ions of the novel related substances characterized in the polymyxin B sample can be found in the supplementary data.

*3.2.2.1. Peak 5 with m/z 581.4.* The first loss of 199 u, yielding the ion at *m/z* 963 in the first series, corresponds with the FA moiety and the neighboring Dab moiety. Based on the experience with known polymyxins, we propose that the FA residue is probably a branched or linear hexanoic acid ($C_6H_{11}O$). The ion with *m/z* 199 in the second series confirms this. The subsequent mass losses in both series are similar to the mass losses seen for polymyxins $B_1$ and $B_2$. The first series shows a *m/z* 261, which corresponds to the two adjacent amino acids present in the ring of the polypeptide. Because the polymyxin B series is characterized by a Phe at the Y position in the ring, the neighbouring amino acid is a Leu or Ile.

*3.2.2.2. Peaks 3, 4, 5, 16 and 17 with m/z 603.3.* According to the fragmentation pattern described for the polymyxins, the first loss of 227 u, yielding the product ion at *m/z* 979 in the first series, corresponds to the FA residue and the neighboring Dab residue. Based on experience with known polymyxins, we propose that the FA residue has the same mass as the FA residue of polymyxin $B_2$ or $B_3$. The ion at *m/z* 227 in the second series confirms this. The subsequent neutral losses in both series are similar to those seen for polymyxin $B_2$ or $B_3$, except for the first loss in the second series (377 u instead of 361 u). For polymyxin $B_2$ or $B_3$, this loss of 361 u is due to the loss in the ring of Phe and Leu and a Dab residue. The product ion at *m/z* 277 in the first series also confirms that there is a change in the amino acids of the ring. Combinations of two amino acids with mass 277 Da are possible with Tyr (tyrosine) and Leu/Ile or Phe and Glu (glutamic acid). The combination Tyr-Leu/Ile is proposed to be the correct one, since hydroxylation of Phe to Tyr seems plausible [21].

*3.2.2.3. Peak 6 with m/z 609.2.* Out of the product ions and the losses in the series, it was concluded that the sequence of polymyxin $B_1$ or $B_2$ is retained, but the FA moiety differs. The first loss of 255 u, yielding the ion with *m/z* 963 and the product ion with *m/z* 255 in the second series, indicate that the FA residue is probably a branched or linear decanoic acid ($C_{10}H_{19}O$). A loss of 361 u observed in the second series is due to the loss in the ring of Phe and Leu and a Dab residue. For the amino acid at the X position, it needs to be considered that both Leu and Ile are possible.

*3.2.2.4. Peaks 8, 10, 13 and 14 (m/z 588.4).* The subsequent neutral losses for peaks 8, 10, 13 and 14 in both series are similar to those seen for polymyxin $B_4$ (see Table 1(a)). They are proposed to be isomers of polymyxin $B_4$.

*3.2.2.5. Peak 9 with m/z 619.4.* The doubly charged ion with *m/z* 619 fragments to the product ion *m/z* 997 by means of a loss of 241. This loss shows that the FA part has the elemental composition of $C_9H_{17}O$. The last ion with mass 241 u in the second series confirms this. The first loss of mass 395 u in the second series indicates that at the Phe-X position in the ring other amino acids are present than previously reported for the polymyxins B in the literature. Combinations of two amino acids with mass 295 u are possible with Phe and Phe or Met (methionine) and Tyr. Based on the fragmentation pattern defined for the polymyxins and the feature that all polymyxins B in literature have a D-Phe at position Y, it was deduced that the variable amino acid X in the cyclic part is Phe instead of Leu. So, the combination of two amino acids in the ring structure was established as Phe–Phe.

*3.2.2.6. Peak 17 with m/z 578.5.* The first loss of 227 u, yielding the product ion with *m/z* 929 in the first series and the ion with *m/z* 227 in the second series, indicate that the FA moiety has the elemental composition of $C_8H_{15}O$. Both series have all product ions in common, which makes the structure elucidation even more complex. The product ion with *m/z* 227 in the second series corresponds with FA-Dab$^+$, but is also indicative in the first series for the two amino acids in the ring at the X-Y position. Combinations of two amino acids with mass 227 u are possible with Leu/Ile and Ile/Leu or Glu and Pro. The combination Leu-Leu/Ile is proposed to be the correct one, since a characteristic of all colistins previously identified is the amino acid D-Leu at the Y position. Probably the FA moiety of peak 17 corresponds with the FA moiety of polymyxin $E_2$. In the group of colistins with *m/z* 578.3, three compounds (polymyxin $E_2$ and Ile-polymyxin $E_2$ and $E_3$) with similar first and second series of product ions were already described by Govaerts et al. [9].

*3.2.2.7. Peak 29 with m/z 585.4.* The mass spectrum for the compound eluted in peak 29 is similar to polymyxin $E_1$ (colistin A analogue). Identical product ions are formed. Polymyxin E differs from the polymyxin B series as having a different amino acid residue at the Y position in the ring of the polypeptide. Polymyxin E series are characterised with a Leu at the Y position instead of a Phe. It needs to be considered that Ile is also possible instead of Leu.

*3.2.2.8. Peaks 2, 4, 6, 29, 30, 33, 34, 36, 38 and 39 (m/z 602.5).* These peaks are isomers of polymyxin $B_1$ or $B_5$ and are illustrated in Table 1(a). The branching of the FA, which cannot be defined, can differ. It needs to be considered that Ile is also possible instead of Leu.

*3.3. Impurity profiling study of polymyxin E or colistin*

*3.3.1. Known polymyxin E related substances*

The structure of colistin differs from polymyxin B by the substitution of D-Leu for D-Phe as one of the amino acid residues in the cyclic part of the structure.

The fragmentation behavior of polymyxin E, previously described by Govaerts et al. [24], is similar to polymyxin B as discussed in Section 3.2.

Besides (isoleucine-)polymyxins $E_1$ ((Ile-)$E_1$), (Ile-)$E_2$, $E_3$, $E_4$, $E_7$, Ile-$E_8$, norvaline-polymyxin $E_1$ (Nva-$E_1$), valine-polymyxin $E_1$ (Val-$E_1$) and Val-$E_2$, 17 other minor components were described in the literature. As shown in Table 1(b): Nva-$E_1$ [4], Ile-$E_1$ [11], Val-$E_1$ [11] differ from polymyxin $E_1$ in the amino acid Leu at position X, which is replaced by Nval, Ile or Val, respectively. Similarly, Ile-$E_2$ [11] and Val-$E_2$ [4,11] differ from polymyxin $E_2$ in the amino acid Leu at position X, which is replaced by Ile and Val, respectively. Polymyxin $E_7$ has the same amino acid composition as $E_1$, but differs in the fatty acid moiety, which is 7-methyloctanoic acid instead of 6-methyloctanoic acid. The 19 minor components were identified more recently by Govaerts et al. [9] and are illustrated in Table 1(b). The structures of the impurities differ from the known structures by a different FA residue, or a different amino acid at positions X, W or Z. The possible structures are shown in Table 1(b).

*3.3.1.1. Peaks 1, 6, 8, 23, 25, 27, 28, 29, 32, 33, 34 and 36 with m/z 585.3.* $[M+2H]^{2+}$ CID spectra for the compounds eluted in the peaks 1, 6, 8, 23, 25, 27, 28, 29, 33, 34 and 36 are identical to polymyxin $E_1$ eluted in peak 32. Based on the peak heights and retention times reported by Orwa et al. [23], peak 27 and peak 34 were assigned as Ile-$E_1$ and polymyxin $E_7$, respectively. The other peaks are isomers of polymyxin $E_1$.

*3.3.1.2. Peaks 10, 11, 13, 16, 17, 18, 19, 20, 22, 24, 26 and 28 with m/z 578.3.* The compounds eluted in peaks 10, 11, 13, 16, 18, 20, 24, 26 and 28 show identical product ions, but different retention times compared to polymyxin $E_2$ (peak 17). It is possible that they differ in branching of the FA residue or that at position X, Leu in the ring structure is replaced by Ile. The compound eluted in peaks 19 and 22 was identified as Val-$E_1$, though an isomeric FA residue is possible. CID investigation of the compound eluted in peak 28 yielded identical product ions as Imp 15 (see Table 1(b)). This variant was already synthesized by Kline et al. [25], but these authors mentioned that the compound was not readily observed in native unenriched fractions. Afterwards, Govaerts et al. [9] reported that this compound could be present in an unenriched bulk sample. The compound in peak 22 was characterized as Imp 17. The compound is similar to the compound eluted in peak 28, except that a Ser replaces the Thr (threonine) in the ring instead of the Thr in the side-chain.

*3.3.1.3. Peaks 7, 9, 14, 15 and 16 with m/z 571.3.* The compound in peak 9 shows a similar fragmentation pattern as described for Val-$E_2$, but a different branching is possible. The structure Val-$E_2$ was already isolated and characterized by Elverdam et al. [4]. The compound in peaks 14, 15 and 16 was established as Imp 13 (see Table 1(b)). A Ser replaces the Thr in the linear part of the structure. The compound in peak 7 shows a fragmentation pattern similar to polymyxin $E_4$, but a different branching is possible.

*3.3.1.4. Peaks 4, 5 and 24 with m/z 586.3.* The fragmentation pattern of this compound was similar to Imp 10. The FA residue is a hydroxylated branched or linear octanoic acid ($C_8H_{15}O_2$).

*3.3.1.5. Peaks 9 and 12 with m/z 587.4.* The compounds in peaks 9 and 12 showed a similar fragmentation pattern as Imp 11. The amino acid combination in the ring at positions X and Y is a Met-Leu. Moreover, an isomeric FA residue is possible.

*3.3.1.6. Peak 12 with m/z 593.3.* The compound eluted in peak 12 shows the same *m/z* value and identical first and second series product ions as Imp 12. The FA residue for the compound in peak 12 is a $C_9H_{17}O_2$.

*3.3.1.7. Peak 23 with m/z 594.3.* The compound in peak 23 was characterized as Imp 14 (Table 1(b)). The first series of product ions for the compound in peak 23 is identical to the first series of product ions for peak 9 and 12 with *m/z* 587.3 (see Section 3.3.1.5). This means that a Met is present at position X in the ring.

*3.3.1.8. Peak 30 with m/z 584.5.* The compound in peak 30 was characterized as Imp 16. It was assumed that this compound has a FA residue like in polymyxin $E_1$ but with a double bound somewhere in the alkyl chain ($C_9H_{15}O$).

*3.3.1.9. Peaks 19 and 31 (m/z 576.4) and 8 and 25 (m/z 569.5).* The compounds with *m/z* 576.4 (peaks 19 and 31) and *m/z* 569.5 (peaks 8 and 25) were characterized as Imp 18 [9] and Imp 19 [9], respectively. These compounds are formed by a loss of water from either polymyxin $E_1$ (*m/z* 585.3) or polymyxin $E_2$ (*m/z* 578.3). The part of the molecule which loses water could not be defined, due to the low abundance of the product ions in the MS/MS spectra.

*3.3.2. New related substances*

The first and second series of product ions of the novel related substances characterized in the polymyxin E (colistin) sample can be found in the supplementary data.

*3.3.2.1. Peaks 1, 2 and 25 with m/z 595.5.* Mass spectra for the compounds eluted in these peaks are identical to polymyxin $B_2$ and polymyxin $B_3$. Identical product ions are formed. Since only the combination of the amino acids at positions X and Y could be determined and not the exact order, the combination Leu/Ile-Phe (polymyxin E analogue) is also possible instead of Phe-Leu/Ile (polymyxin B analogue).

*3.3.2.2. Peak 3 with m/z 564.3.* The first loss of mass 199 yielding the product ion with *m/z* 929 in the first series corresponds with the FA moiety and the neighbouring Dab moiety. Based on the experience with known polymyxins we propose that the FA moiety has the elemental composition $C_6H_{11}O$. The ion with *m/z* 199 in the second series confirms this. The *m/z* values of the second series are 28 u lower than the corresponding *m/z* values of the second series of polymyxin $E_2$. The subsequent mass losses in the first series are identical to the mass losses seen for polymyxin $E_2$. Combinations of two amino acids with mass 277 u are possible with Leu/Ile and Leu/Ile or Glu and Pro. The combination Leu-Leu/Ile is proposed to be the correct one, since a characteristic of all colistins previously identified is the amino acid D-Leu at the Y position.

*3.3.2.3. Peak 21 with m/z 576.5.* The product ions of the first and second series are similar, which makes the structure elucidation more complex. The first loss of 227 u yielding the product ion at *m/z* 925 in the first series and the ion at *m/z* 227 in the second series indicate that the FA residue has the same mass as the FA residue of polymyxin $E_2$ ($C_8H_{15}O$). The product ion at *m/z* 227 in the second series was formed from *m/z* 324 by loss of 97 u. The loss of 97 u corresponds to the loss of a Pro (proline) residue. It was concluded that the sequence of this unknown polymyxin is similar to polymyxin $E_2$, except for the Thr in the linear part, which is replaced by Pro. The presence in the first series of the product ions at *m/z* 825 and 728 confirms this.

*3.3.2.4. Peak 35 with m/z 535.4.* The first and second series of product ions in the $[M+2H]^{2+}$ CID spectrum matches with the first and second series of product ions acquired for polymyxin $E_1$. The first series starts at *m/z* 829 instead of *m/z* 929 (polymyxin $E_1$) by a loss of 241 u corresponding to a FA residue similar to the FA residue of polymyxin $E_1$ ($C_9H_{17}O$). The second series starts at *m/z* 743 instead of *m/z* 843 (polymyxin $E_1$), resulting in a structure that is 100 Da

lower in mass than $E_1$. This difference of 100 Da corresponds to the loss of a Dab residue. Different fragmentation pathways of the first and second series of product ions were investigated. Since the first loss of 100 u is due to one of the ring Dab residues, the ring opening can occur at 3 different sites in the ring. The exact Dab could not be specified. It can be concluded that the compound eluted in peak 35 lacks one Dab in the ring compared to polymyxin $E_1$.

## 4. Conclusions

Until now, the Ph. Eur. monographs of polymyxin B and colistin do not report any impurities. Moreover, previously characterized impurities by LC/MS using volatile additives [9,21] could not be assigned in the more selective LC-UV methods [19,20].

In this study, the impurities previously identified by LC/MS were traced in the LC-UV methods and new impurities present in polymyxins B and E samples were identified.

The low-resolution MS technique provided suggestions about the sequence of the peptides.

The limited number of structural possibilities for the polymyxin B complex enabled partial characterization of five novel related substances. Ten additional peaks in the complex sample were assigned as isomers of the main compound polymyxin $B_1$. Twelve peaks were assigned as isomers of polymyxin $B_2$ and four peaks were isomeric to polymyxin $B_4$. Furthermore, one peak was related to the polymyxin E series. This confirms the fact that many impurities in polymyxin B are isomeric in nature.

During the investigation of the polymyxin E complex, four novel related substances were characterized. Nine additional peaks in the polymyxin E complex were assigned as isomers of polymyxin $E_1$ and eight peaks were related to polymyxin $E_2$.

Most of the impurities are due to modifications at positions W, X, Y and Z in the polypeptide structure. The isomeric fatty acids are often the source of structural variation. A drawback of the applied low-energy CID technique is the inability to distinguish the isomeric amino acids Leu and Ile, which both occur in polymyxins. It is noted that further determination of the FA moiety and the spatial configuration (D or L) of the amino acids still needs to be performed by NMR, since the MS technique only allows the determination of the mass of the FA part, but not the branching of the carbon chain.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.11.044.

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