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Identification of impurities in erythromycin by liquid chromatography–mass spectrometric detection

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Abstract

A simple, isocratic liquid chromatographic (LC) method using volatile mobile phase constituents for the identification of related substances in erythromycin samples is described. For method development, evaporative light scattering detection (ELSD) was used. An XTerra™ RP_{18} column was used at 70 °C with a mobile phase consisting of acetonitrile–isopropanol–0.2 M ammonium acetate pH 7.0–water (165:105:50:680). Mass spectral data were acquired on an ion trap mass spectrometer equipped with an electrospray interface operated in the positive ion mode. First, a library was created using MS/MS and MS^n spectra of reference substances available in the laboratory. Using these reference spectra as interpretative templates, eight novel related substances in erythromycin samples were identified: *N*-demethylerythromycin E, erythromycin E *N*-oxide, anhydroerythromycin C, *N*-demethylerythromycin B, anhydro-*N*-demethylerythromycin A, pseudoerythromycin E enol ether, EF lacking the neutral sugar and EA lacking the neutral sugar.

Keywords: Erythromycin; Evaporative light scattering detection

1. Introduction

Erythromycin is a complex macrolide antibiotic consisting mainly of erythromycin A, a 14-membered lactone ring with a 9-keto group, carrying a neutral and an amino sugar [1]. This antibiotic is produced by fermentation of a strain of *Streptomyces erythraeus* (now reclassified as *Saccharopolyspora erythrea*) [2]. Erythromycin is mainly active against Gram-positive bacteria and possesses only limited activity against Gram-negative bacteria [3]. Erythromycin A is the main component, but during fermentation several related substances such as erythromycins B (EB), C (EC), D (ED), E (EE), F (EF), A *N*-oxide (EANO) and *N*-demethylerythromycin A (NdMeEA) are formed in small amounts. The chemical structures are shown in Fig. 1. A characteristic feature is the neutral sugar glycosidically linked to position 3 (cladinose for EA, EB, EE, EF, NdMeEA and EANO and mycarose for EC and ED) and the dimethylamino sugar desosamine linked to position 5 of the 14-membered lactone ring. In addition, degradation products such as pseudoerythromycin A enol ether (PsEAEN), formed in alkaline medium, and anhydroerythromycin A (AEA) and erythromycin A enol ether (EAEN), which are formed in acidic conditions, may also be present [1].

Ample work has been done on the optimization of the separation of EA and its related substances by LC [4–16]. The official non-volatile LC method prescribed by the European Pharmacopoeia [17] and the United States Pharmacopeia [18] is based on work by Paesen et al. [11,13]. With the arrival of new reversed phase stationary phase materials, Govaerts et al. developed a gradient LC method on XTerra™ RP_{18} with volatile mobile phase additives, compatible with MS. Using this volatile method, the structures of four unknown compounds were determined in different commercial samples [19]. Later, Chepkwony et al. developed an isocratic non-volatile method on XTerra™ RP_{18}, which was able to separate more unidentified peaks from the known related substances [20].

The aim of this study is the development of a more selective LC method using volatile mobile phase constituents to characterize by MS more unknown related substances.

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Regulatory agencies insist more and more on the identification of impurities in drug substances. Several erythromycin samples and enriched fractions were screened using the new volatile method developed and the most interesting samples were further examined in order to identify the unknown compounds.

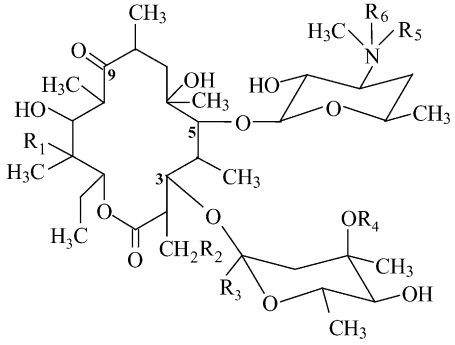
2. Experimental

2.1. Chemicals

Acetonitrile, HPLC grade and ammonium acetate were purchased from Acros Organics (Geel, Belgium), 2-propanol Chromasolv® from Riedel-de Haën (Seelze, Germany) and glacial acetic acid p.a. from VWR (Leuven, Belgium). A Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to further purify glass-distilled water.

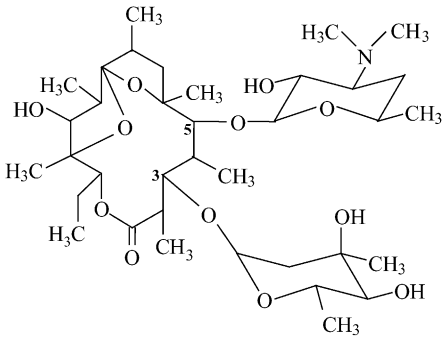
2.2. Samples and sample preparation

A pure standard of EA, obtained by open column chromatography followed by subsequent crystallization and secondary standards of EE [21], EF [22], EANO [23], NdMeEA [24], PsEAEN [25,26], PsEAHK [25], AEA [26] and EAEN [27], which were prepared according to procedures found in literature, were available in the laboratory. Reference substances EB and EC were obtained from the European Pharmacopoeia Laboratory (Strasbourg, France). An impure sample, known to contain ED and mainly EA, as verified by thin-layer chromatography, was also available [28]. All these compounds were used as reference substances. An erythromycin sample containing the impurities EANO, EF, NdMeEA, EC, EE, AEA and EB, was used as house standard at a concentration of 4 mg/mL. Several fractions, obtained from the purification of crude erythromycin samples, were available in the laboratory.

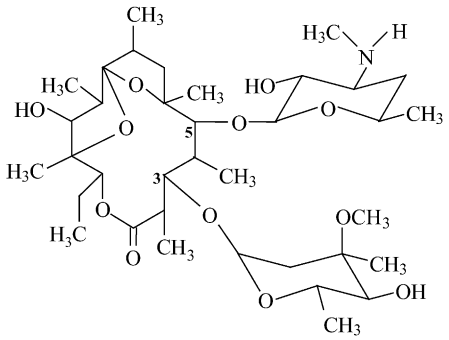


	m/z $[M+H]^+$	R_1	R_2	R_3	R_4	R_5	R_6
Erythromycin A	734	OH	H	H	CH_3	CH_3	-
Erythromycin B	718	H	H	H	CH_3	CH_3	-
Erythromycin C	720	OH	H	H	H	CH_3	-
Erythromycin D	704	H	H	H	H	CH_3	-
Erythromycin E	748	OH	-O-		CH_3	CH_3	-
Erythromycin F	750	OH	OH	H	CH_3	CH_3	-
N-demethylerythromycin A	720	OH	H	H	CH_3	H	-
Erythromycin A N-oxide	750	OH	H	H	CH_3	CH_3	O
N-demethylerythromycin E (IMP1)	734	OH	-O-		CH_3	H	-
Erythromycin E N-oxide (IMP2)	764	OH	-O-		CH_3	CH_3	O
N-demethylerythromycin B (IMP4)	704	H	H	H	CH_3	H	-

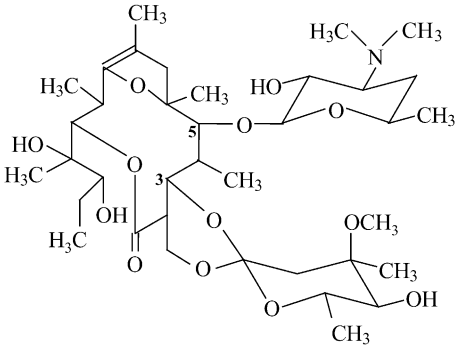
Fig. 1. Chemical structure of erythromycin A, its related substances and proposed structures for the novel related substances IMP1, IMP2, IMP3, IMP4, IMP5 and IMP6.



Anhydroerythromycin C (IMP 3)



Anhydro-N-demethylerythromycin A (IMP 5)



Pseudoerythromycin E enol ether (IMP 6)

Fig. 1. (*Continued*).

All samples were dissolved in a 30:70 mixture of acetonitrile–ammonium acetate solution pH 7.0. Reference substances were dissolved at a concentration of 0.02 mg/mL. The commercial sample containing ED was dissolved at a concentration of 0.1 mg/mL. All the erythromycin fractions investigated were dissolved at a concentration of 4 mg/mL.

2.3. LC instrumentation and chromatographic conditions

The LC/ELSD apparatus consisted of a Varian 9012 solvent delivery system, a 9095 Varian autosampler (Walnut Creek, CA, USA) equipped with a 100 μL loop and an ELSD 2000 (Alltech Associates, Deerfield, IL, USA) operated in 'Impactor Off' mode with the drift tube temperature at 106 °C and the nitrogen gas at a flow rate of 2.5 L/min as nebulizing gas. Data were acquired with ChromPerfect 4.4.0 software (Justice Laboratory Software, Fife, UK).

The XTerra ™ RP C_{18} column (5 μm, 250 mm × 4.6 mm i.d.) (Waters, Milford, MA, USA) was immersed in a water bath at 70 °C, controlled by means of a Julabo thermostat (Seelbach, Germany). The mobile phase consisted of acetonitrile–isopropanol–0.2 M ammonium acetate pH 7.0–water (165:105:50:680, v/v/v/v). The mixture was degassed by sparging with helium. A flow rate of 1.0 mL/min was used.

The LC/MS apparatus consisted of a SpectraSYSTEM P1000XR quaternary pump, a SpectraSERIES AS100 autosampler equipped with a 20 μL loop, a variable wavelength Spectra 100 UV–vis detector set at 215 nm, all from Thermo Separation Products (Fremont, CA, USA), connected to a computer with ChromPerfect 4.4.0 software. The LC system was coupled to a LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionisation (ESI) source operated in the positive ion mode. Data were recorded with Xcalibur software (Thermo Finnigan). The chromatographic conditions were the same as above, except for the column dimensions (5 μm, 250 mm × 2.1 mm i.d.) and the flow rate (0.2 mL/min).

The EA reference substance was used to tune the instrument. This tune file was used during the subsequent investigation of erythromycin fractions. Nitrogen supplied by a Nitroprime TM Membrane unit, type SNIFF (AGA, Lidingö, Sweden) was used as auxiliary and sheath gas. Helium was used as collision gas in the ion trap. Xcalibur software was used for data acquisition and processing. For MS/MS investigation, the protonated EA was isolated in the ion trap and collisionally activated with different collision energies (CE) to find the optimal CE for a distinct fragmentation. The CE, which generated the highest intensity of the product ions needed for further collision-induced dissociation (CID) experiments, was chosen as the final value. A CE value of 28% was optimal. CID spectral data or LC/MS^3 spectra were obtained for the product ions relevant to the identification of the unknowns. CE was optimized as described above for MS/MS. All the reference substances were injected on

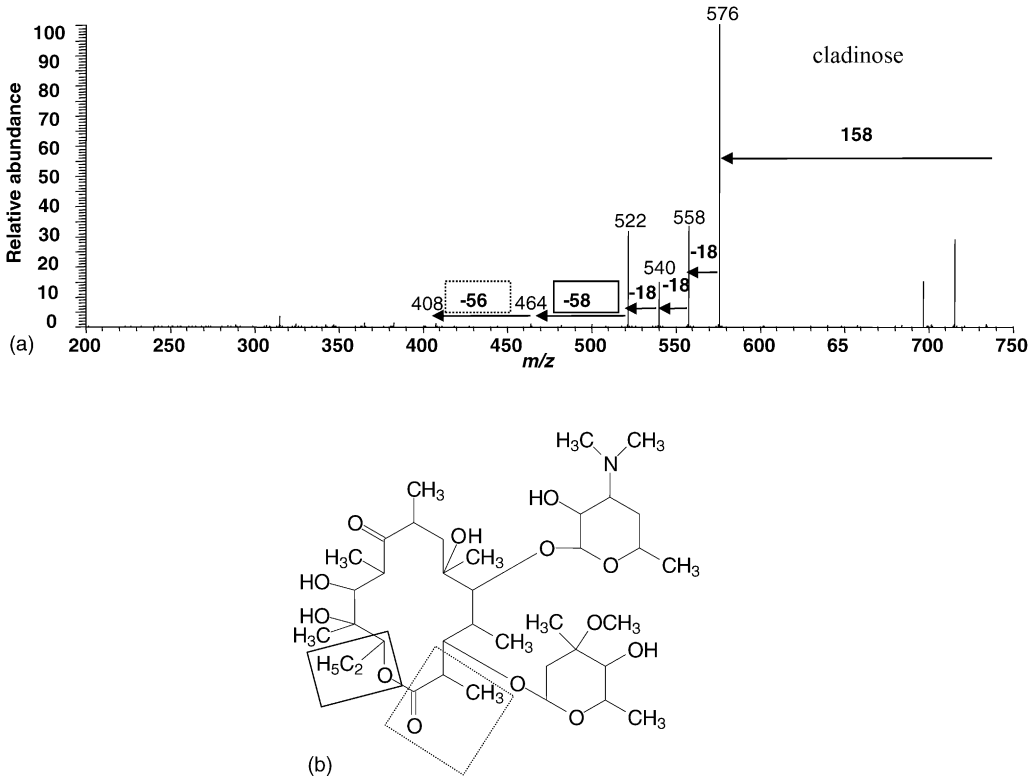


Fig. 2. (a) Product ion spectrum (MS/MS) acquired for EA, the result of isolation and collisional activation in the ion trap with 28% CE of the precursor ion with *m/z* 734. The spectrum shows the masses for the obtained fragments. The mass losses are indicated above the arrows. (b) According to the described biosynthetic routes, the aglycone of erythromycin is formed by incorporation of seven propionate units, one of which is used as starter acid. The starter acid of erythromycin, observed as a loss in the MS/MS spectrum is boxed with a full line. The neighbouring oxygen-containing unit is boxed with a dotted line.

the column to avoid misinterpretation of spectra due to the impurity of some samples. On those reference substances, analogous experiments were performed as for EA.

3. Results and discussion

3.1. Development of the LC method

Preliminary method development was performed using LC combined with an ELSD detector because this technique shows lower investment and operating costs, easier operation and less maintenance compared to LC/MS. Since the chromatographic requirements are similar, methods developed with ELSD are easily transferable to MS. Compared to UV detection, ELSD has the advantage to detect compounds with a weak chromophore and it allows the use of strong UV absorbing organic modifiers like acetone.

Based on the non-volatile method developed by Chepkwony et al. [20], a mobile phase with a volatile ammonium acetate buffer was developed instead of the original potassium phosphate buffer. Simply replacing the non-volatile buffer with the volatile one did not separate all peaks separated in the non-volatile method. EANO, which is eluted as first component in the non-volatile method is co-eluted with EA and also EE and ED are co-eluted with EA. Adaptation of the original amount of acetonitrile and incorporation of isopropanol in the mobile phase resulted in the separation of EA and EE. Ammonium hydrogen carbonate was also evaluated in combination with different organic modifiers, but resulted in longer retention times and a decreased sensitivity.

3.2. MS of the reference substances

The fragmentation behavior of the erythromycins was studied using reference substances. The product ion spectrum of the main compound EA ($[M+H]^+$ precursor ion at *m/z* 734) is shown in Fig. 2 as an example. The spectrum shows the loss of the cladinose sugar (formation of *m/z* 576 by loss of 158 u) and further water losses from the macrolide ring.

Important information was obtained from the CID analysis of the product ion at *m/z* 522. The CID spectra were analogous to the spectra obtained in previous studies of the fragmentation behavior by Gates et al. [29] and Roddis et al. [30]. The subsequent losses of 58 u (indicated in Fig. 2 with the full box) and 56 u (indicated in Fig. 2 with the dotted box) to, respectively, 464 and 408, have been clarified by Gates et al. [29]. Their deuterated experiments with high resolution FTICR apparatus showed that the first loss of 58 u is due to the loss of the starter acid unit of the polyketide ring and the

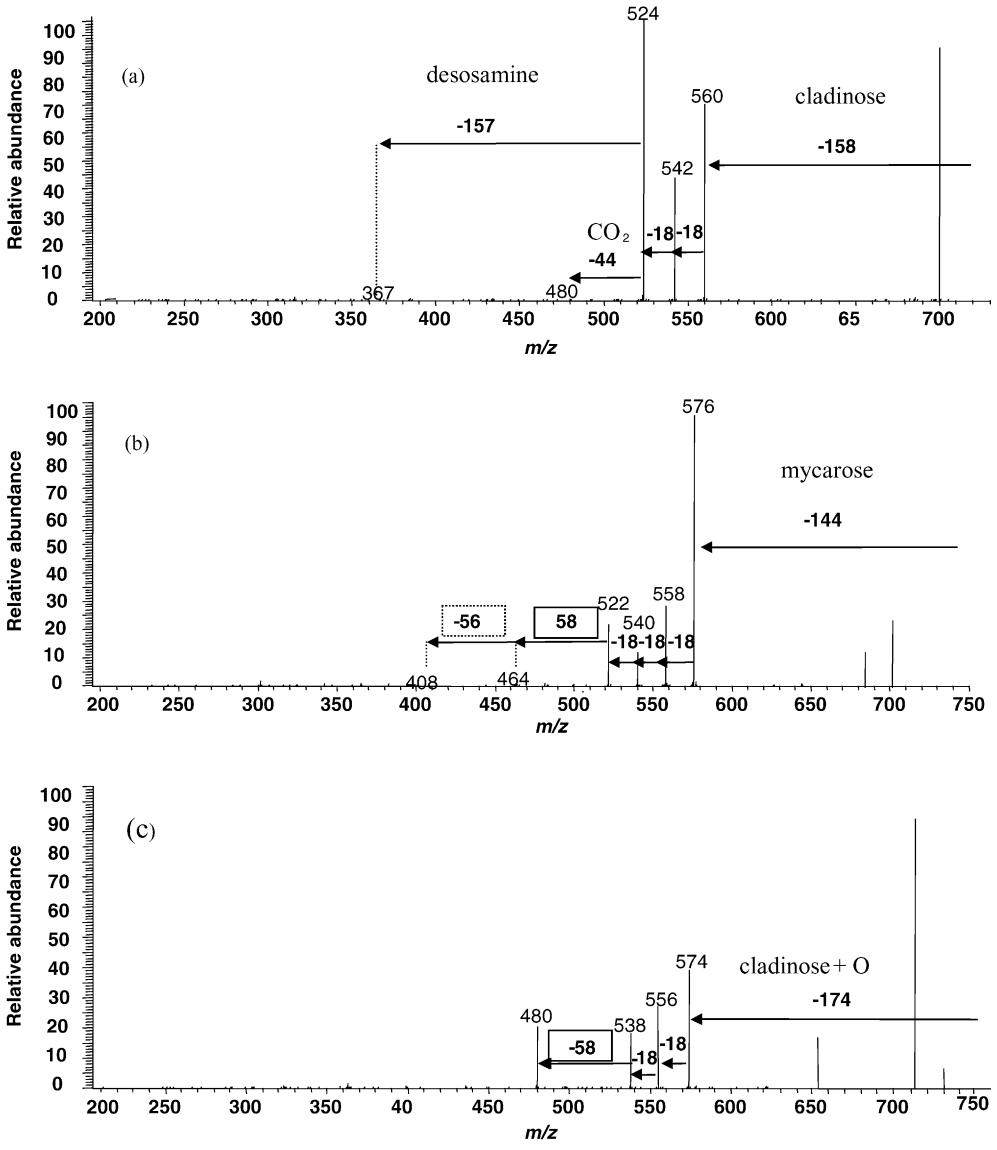


Fig. 3. Product ion spectra for (a) EB, (b) EC and (c) EE, obtained with 28% CE. The observed losses are clarified above the arrows.

second loss of 56 u is assumed to arise from the neighbouring oxygen-containing unit of the ring under the form of a propionaldehyde.

Mass spectra of EB, EC, ED, EE, EF, NdMeEA, EANO, PsEAEN, PsEAHK, AEA and EAEN were similarly recorded. These mass spectrometric data were incorporated in a library with as main objective the confirmation of their presence in erythromycin fractions and the structure elucidation of unknowns by comparison. In this paper, only those related substances that were used for structure elucidation of unidentified peaks will be discussed.

The mass spectrometric data of erythromycin B ($[M+H]^+$ *m/z* 718) are shown in Fig. 3(a). Initial losses are identical to EA. Since this compound does not have a hydroxyl group at position C-12, the fragmentation route is different. Instead of the loss of the starter acid, a loss of CO_2 (44 u) disrupts the starter acid unit of the ring system most probably so that it cannot be lost intact. Further loss is assumed to be from the desosamine unit, i.e. loss of 157 u to *m/z* 367.

The MS/MS spectrum of EC ($[M+H]^+$ at *m/z* 720) is presented in Fig. 3(b) with the typical losses mentioned above the arrows. The MS/MS spectrum of EE is presented in Fig. 3(c) with the clarified losses above the arrows. Typical for the protonated EE molecule with *m/z* 748 is the product ion at *m/z* 574 by loss of 174 u, corresponding to the cladinose sugar and the oxygen atom of the glycosidic linkage.

3.3. MS of new erythromycin related substances (IMPs)

Screening of different fractions of erythromycin at a concentration of 4 mg/mL revealed several unknown related substances of which the fragmentation pattern did not match any

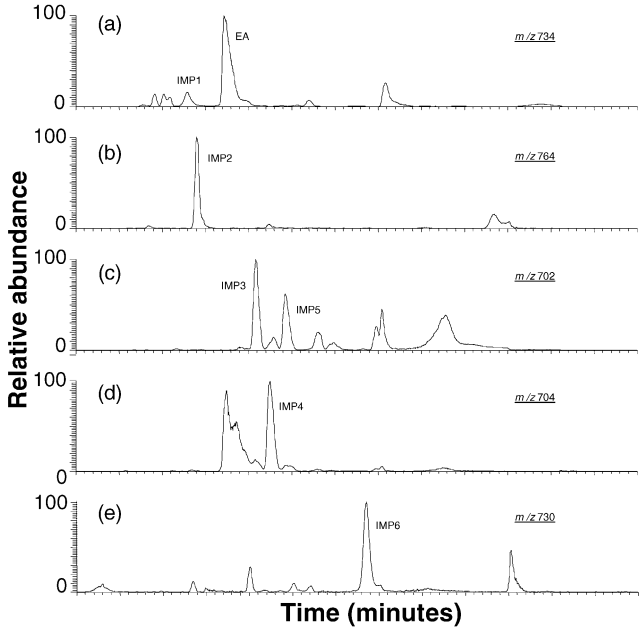


Fig. 4. Typical extracted mass range chromatograms of novel erythromycin impurities IMP1 to IMP6. Stationary phase: XTerra™ RP C_{18} (5 μm, 250 mm × 2.1 mm i.d.); column temperature: 70 °C; mobile phase: acetonitrile–isopropanol–0.2 M ammonium acetate pH 7.0–water (165:105:50:680, v/v/v/v), flow rate: 0.2 ml/min; sample concentration: 4.0 mg/mL; injection volume: 20 μL; extracted mass range chromatograms are shown in (a) for IMP1 (*m/z* 734), (b) for IMP2 (*m/z* 764), (c) for IMP3 and IMP5 (*m/z* 702), (d) for IMP4 (*m/z* 704) and (e) for IMP6 (*m/z* 730). Detection: Ion trap equipped with ESI source operated in the positive ion mode. IMP: impurity.

of the fragmentation spectra of the reference standards in the library. The compounds with *m/z* values 734, 764, 702, 704, 702, 730, 592 and 576 are referred to as IMP1, IMP2, IMP3, IMP4, IMP5, IMP6, IMP7 and IMP8, respectively. Extracted mass range chromatograms of IMP1 to IMP6 are shown in Fig. 4.

The MS/MS spectrum of IMP1 with *m/z* 734 is presented in Fig. 5(a). Compared to EE, the product ions in the product ion scan are shifted down by 14 u. The subsequent losses noted in the figure, are comparable to EE. Additonal information about the structure could be obtained from the fragmentation of the product ion at *m/z* 466 (MS^3 data not shown). A loss of 143 u is in agreement with the loss of *N*-demethyldesosamine. Hence the identity and structure of IMP1 is thus established as *N*-demethylerythromycin E (NdMeEE) (Fig. 1).

Compared to EE, the *m/z* values of the product ions in the product ion spectrum of IMP2 ($[M+H]^+$ at *m/z* 764) in Fig. 5(b) are increased by 16 u. Losses are identical to the losses observed for EE. Fragmentation of the product ion at *m/z* 554 (MS^3 data not shown) results in a loss of 58 u, which corresponds with the starter acid unit, to yield the product ion at *m/z* 496. One of the product ions of the MS^3 spectrum is at *m/z* 173, which indicates the presence of an extra oxygen attached to the desosamine sugar. This sugar is thus the same as that present in EANO. Hence, IMP2 is identified as erythromycin E *N*-oxide (EENO) (Fig. 1).

The losses in the MS/MS spectrum of IMP3 ($[M+H]^+$ at *m/z* 702) in Fig. 5(c) and the product ions in the MS^3 spectrum are identical to those observed for EC, except for one water loss less for IMP3. The presence of the desosamine residue and the loss of the mycarose residue indicate that there is no change in the sugar residues. Hence the identity and structure of IMP3 was established as anhydroerythromycin C (AEC) (Fig. 1). IMP3 has previously been described by Volmer et al. in their study on erythromycin A decomposition products in aqueous solution by solid-phase microextraction/liquid chromatography/tandem mass spectrometry [31].

The protonated IMP4 molecule at *m/z* 704 fragments (Fig. 5(d)) to the product ion at *m/z* 546 by means of loss of 158 u, identified as cladinose sugar. Further fragmentation of the product ion at *m/z* 510 (MS^3 data not shown) is comparable to that of erythromycin B. Instead of the loss of the starter acid from *m/z* 510, loss of CO_2 (44 u) disrupts the starter acid unit of the ring system and generates the product ion at *m/z* 466 similar to that observed with erythromycin B. Further loss from *m/z* 466 was confirmed to be from a demethylated desosamine residue (loss of 143 u) and leads to *m/z* 323. Hence the identity and structure of IMP4 is established as *N*-demethylerythromycin B (NdMeEB) (Fig. 1).

The protonated IMP5 molecule at *m/z* 702 fragments (Fig. 5(e)) to the product ion at *m/z* 544 by means of a loss of 158 u, identified as a cladinose residue. Fragmentation of the product ion at *m/z* 508 (MS^3 data not shown) resulted in a loss of 58 u (*m/z* 450) and a subsequent loss of 56 u (*m/z* 394), which confirmed the presence of starter acid unit and the neighbouring unit. Loss of 161 u from *m/z* 394 yields the product ion at *m/z* 233 corresponding to the loss of demethyldesosamine and the oxygen of the glycosidic bond. One of the product ions in the MS^3 spectrum is at *m/z* 144 which confirms the presence of a demethylated desosamine sugar. Based on the MS/MS and MS^3 spectra of IMP5 it is postulated that this compound has one water molecule less compared to *N*-demethylerythromycin A and can be anhydro-*N*-demethylerythromycin A or *N*-demethylerythromycin A enol ether. However, since its relative retention time (1.86) compared to *N*-demethylerythromycin A is similar to that of AEA versus EA (1.96) (see Table 1), the identity of IMP5 is assumed to be anhydro-*N*-demethylerythromycin A (ANdMeEA) (Fig. 1). Similar to IMP3, this compound has been previously discussed by Volmer et al. [31].

The protonated IMP6 molecule at *m/z* 730 fragments into the product ions shown in Fig. 5(f). The presence of the desosamine moiety (in the CID spectrum of *m/z* 538) and the loss of the cladinose moiety immediately followed by the oxygen of the glycosidic bond, indicate that in comparison to EE, there is no difference except for one water loss less. Based on the lack of one water molecule compared to EE, it is postulated that this impurity can be anhydroerythromycin E, pseudoerythromycin E enol ether or erythromycin E enol ether. Based on its relative retention time (2.61) compared to erythromycin E, which is similar to that of the relative re-

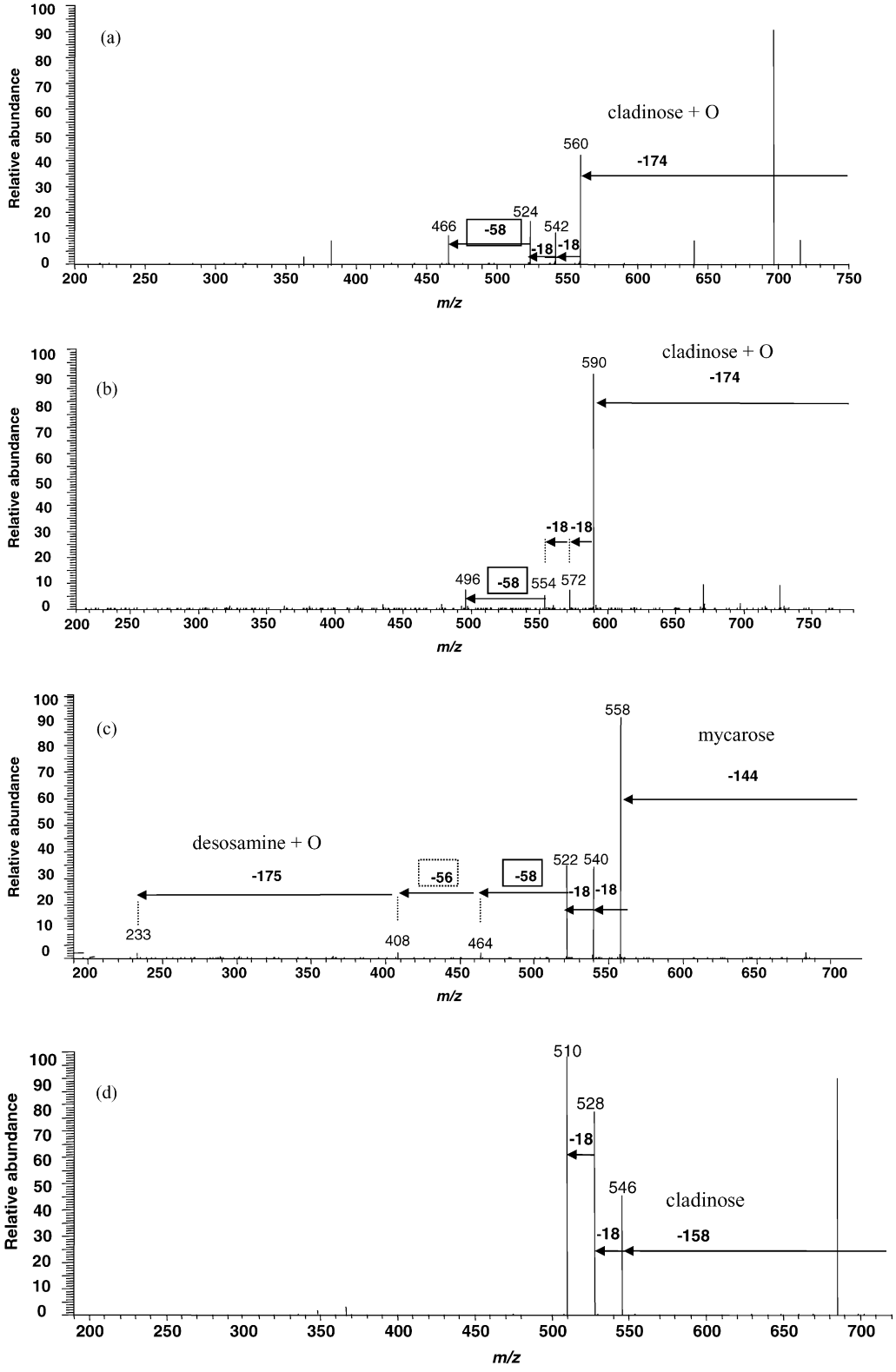


Fig. 5. Product ion spectra (MS/MS) of novel erythromycin impurities, the result of isolation and collisional activation in the ion trap of the precursor ions with *m/z* 734 for IMP1 (a), *m/z* 764 for IMP2 (b), *m/z* 702 for IMP3 (c), *m/z* 704 for IMP4 (d), *m/z* 702 for IMP5 (e) and *m/z* 730 for IMP6 (f). The observed losses are clarified above the arrows.

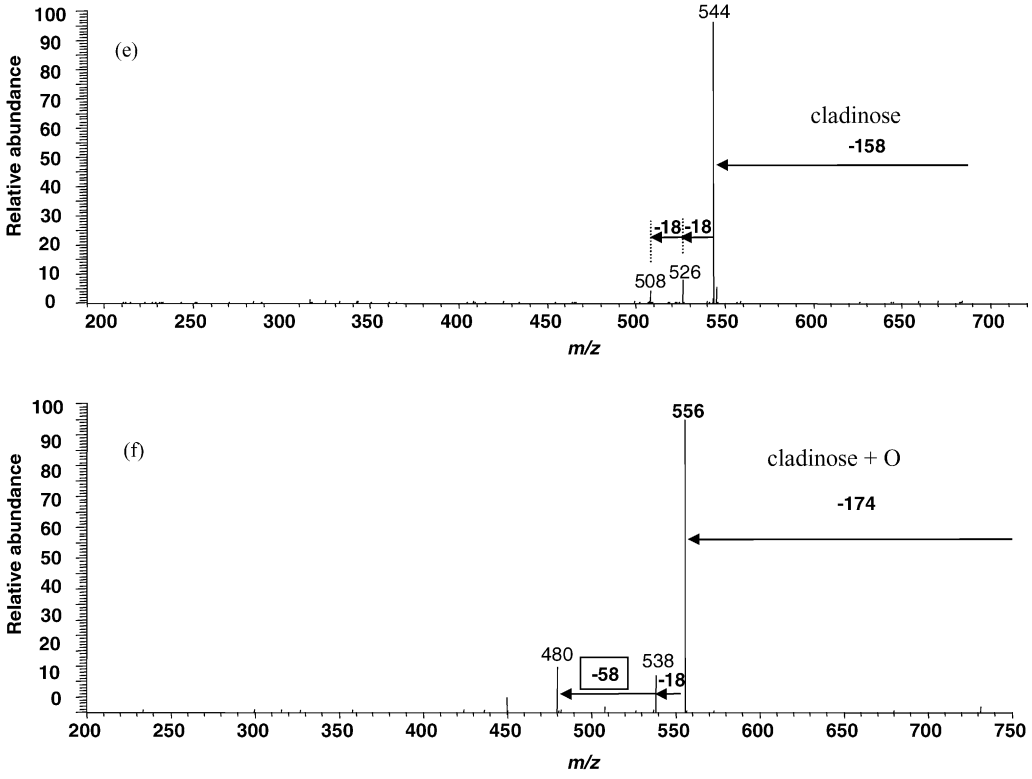


Fig. 5. (*Continued*).

Table 1
Overview of all compounds detected in erythromycin samples with their retention times, their *m*/*z* values and the proposed names

Peak number	Retention times	Retention time relative to EA	*m/z*	Proposed name (abbreviations: see text)
1	5.04	0.15	592	EF without neutral sugar (IMP7)
2	5.74	0.20	720	Isomer[a] of EC
3	7.32	0.30	750	EF
4	7.84	0.34	576	EA without neutral sugar (IMP8)
5	9.26	0.44	716	Isomer[a] of AEA/EAEN/PsEAEN
6	11.64	0.60	720	EC
7	13.05	0.69	734	NdMeEE (IMP1)
8	15.31	0.84	764 + 720 + 748	EENO (IMP2) + NdMeEA + EE
9	17.67	1.00	734	EA
10	21.82	1.28	702	AEC (IMP3)
11	23.55	1.39	704	NdMeEB (IMP4)
12	25.51	1.53 (1.86 compared to NdMeEA)	702	ANdMeEA[a] (IMP5)
13	26.77	1.67	732	Not defined
14	27.69	1.67	564	Not defined
15	29.88	1.87	718	EB
16	31.98	1.96	716	AEA
17	35.49	2.20 (2.61 compared to EE)	730	PsEEEN[a] (IMP6)
18	36.97	2.30	702	Isomer[a] of IMP5
19	37.97	2.36	734	PsEAHK
20	39.03	2.43	716	PsEAEN
21	46.18	2.91	702	Isomer[a] of IMP5
22	54.35	3.46	748	Not defined
23	59.29	3.80	732	Not defined
24	60.86	3.90	716	EAEN

The peak numbers correspond with the peak numbers in the chromatogram shown in Fig. 6.
[a] Probable structure.

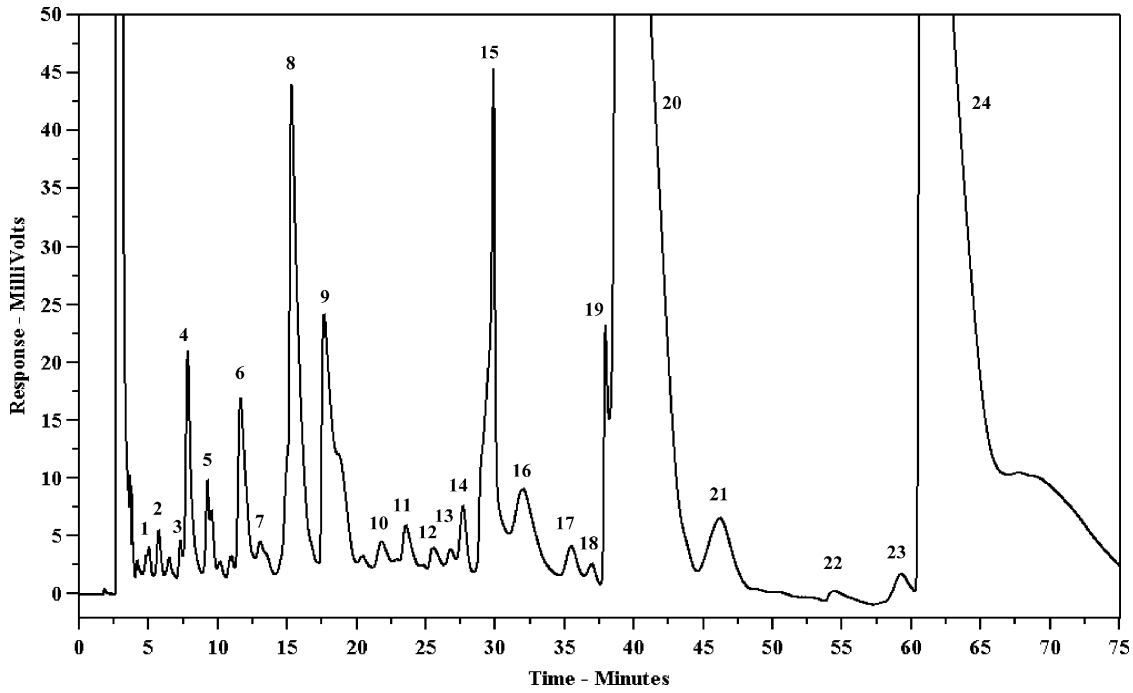


Fig. 6. Typical UV chromatogram of an erythromycin fraction containing all novel erythromycin related impurities (IMPs). Stationary phase: XTerra™ RP C_{18} (5 μm, 250 mm × 2.1 mm i.d.); column temperature: 70 °C; mobile phase: acetonitrile–isopropanol–0.2 M ammonium acetate pH 7.0–water (165:105:50:680, v/v/v/v), flow rate: 0.2 mL/min; sample concentration: 4.0 mg/mL; injection volume: 20 μL; detection: UV at 215 nm (see Table 1 for information about the peaks).

tention time of PsEAEN versus EA (2.43) (see Table 1), the identity of IMP6 is likely to be pseudoerythromycin E enol ether (PsEEEN) (Fig. 1).

IMP7 is a component at *m/z* 592. The MS/MS spectrum (data not shown) does not show the loss of the cladinose sugar. The CID spectrum of the ion at *m/z* 538 formed after loss of three water molecules (data not shown) is exactly the same as that of EF and confirms the presence of desosamine. Hence, this compound was identified as EF without the neutral sugar.

The MS/MS spectrum of IMP8 with $[M+H]^+$ *m/z* 576 (data not shown) shows the absence of the neutral sugar. The MS^3 spectrum of the fragment ion at *m/z* 522 (data not shown) is exactly the same as that of EA and confirmed the presence of the desosamine sugar. So, this component corresponds to EA without the neutral sugar.

All these compounds were present in varying amounts in many of the fractions examined. The UV chromatogram of one such fraction containing all the identified impurities is shown in Fig. 6. The peak numbers correspond to the peaks in Table 1. Peak number 8 is a co-elution of three components at the same retention time: IMP2, NdMeEA and EE. Peaks 2, 5, 18 and 21 are probably isomers as indicated in Table 1.

4. Conclusion

The simple isocratic volatile LC method combined with ELSD was used for the screening of many erythromycin fractions. Selected samples containing unknown peaks were further investigated with LC/MS. In total eight impurities, of which the fragmentation spectra did not or only partly match the fragmentation spectra of the reference substances, were identified.

Acknowledgement

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