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Preliminary assays to elucidate the structure of oxytetracycline's degradation products in sediments
Determination of natural tetracyclines by high-performance liquid chromatography–fast atom bombardment mass spectrometry

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Abstract

A very specific high-performance liquid chromatography–mass spectrometric method for the determination of natural tetracyclines was developed in order to characterise the degradation products of oxytetracycline in sediments. First, extraction used a clean up step with a Bond Elut Certify® LRC cartridge. A 3 μm Spherisorb® ODS1 column was then used with a methanol, acetonitrile and oxalic acid mobile phase gradient. Chromatographic resolution in these conditions was 3.31 between oxytetracycline and tetracycline. Two liquid chromatography–mass spectrometry methodologies based on a particle beam and a frit fast atom bombardment interface were developed. In the first approach, ionisation was performed in the negative chemical mode using methane as reacting gas. In the other case, glycerol–thioglycerol mixture was used as matrix to ensure good sensitivity. MS–MS experiment was performed to determinate oxytetracycline fragmentation pattern in the perspective of degradation product study.

Keywords: Oxytetracycline; Tetracycline; Chlortetracycline; Mass spectrometry; LC-MS; Frit FAB; Particle beam

1. Introduction

Bacterial diseases are a major problem in intensive fish farming. Consequently, antibiotics, such as oxytetracycline (OTC) (Fig. 1a), are widely used to increase productivity. For example, in 1994, 12 tons of antibiotics were used in French aquaculture for 50 000 tons of fish produced [1]. OTC, administred to fish as medicated pelleted feed, is characterised by a low oral bioavailability [2,3]. Thus, about 80% of OTC reaches the environment and may contaminate sediments. Little knowledge about the fate of OTC in marine sediments is available. The aim of our work was to elucidate OTC degradation product structures. Consequently, the first part of the work was the development of a selective method to study OTC and its degradation products in marine sediments.

Microbiological assays have been commonly used

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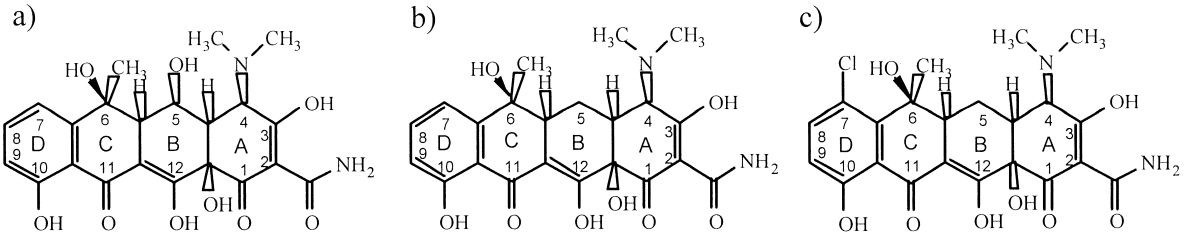

Fig. 1. Structures of the three natural tetracyclines: (a) oxytetracycline (MW=460); (b) tetracycline (MW=444); (c) chlortetracycline (MW=478).

for the determination of tetracyclines (TCs), but their sensitivity and specificity are limited. Chromatographic methods generally allow better sensitivity and specificity than microbiological assays. High-performance liquid chromatography (HPLC) was preferred to gas chromatography because of TCs thermolability. Sediment preparation must include an extraction step and a clean up step by using a solid-phase extraction (SPE) cartridge. Because of the need of structural information on OTC degradation products in sediments, mass spectrometry (MS) was the appropriate methodology. Combination of MS with suitable chromatographic separation was necessary to reach necessary selectivity and LOD. Two parameters were optimised. Sensitivity was studied through the LOD. Specificity was studied through chromatographic resolution between OTC and TC, the two analytes being very close one from the other (Fig. 1b). Chlortetracycline (CTC) was used as external standard in the extraction procedure. In consequence a method for TCs identification is described in detail here.

2. Experimental

2.1. Sediments

Sediments used in this study came from Bourgneuf Bay (Loire-Atlantique, France) and were taken in the Pornic (Loire-Atlantique, France) harbour. Some of the physico-chemical and microbiological properties of this sediment were determined (Table 1).

2.2. Reagents and chemicals

Acetonitrile (Merck, Darmstadt, Germany), ethyl acetate (BDH, Poole, UK) and methanol (Carlo Erba, Milan, Italy) were HPLC-grade reagents. Disodium ethylenediaminetetraacetate (Na_2EDTA) (Merck), disodium hydrogen phosphate (Merck), 25% ammonia (Panreac, Barcelona, Spain), oxalic acid (Merck), glycerol and thioglycerol (Aldrich, Gillingham Dorset, UK) were analytical-grade reagents. Ultrapure water was obtained from a Elgastat Spectrum RO2 apparatus (Elga Ltd., Buckinghamshire, UK).

McIlvaine buffer pH 3.0 was prepared mixing 0.1 *M* citric acid and 0.2 *M* disodium hydrogen phosphate aqueous solutions. The pH was adjusted to pH 3.0 using a Acumet® model 15 pH-meter (Denver Instrument Company, CO, USA)

Oxytetracycline was supplied by Pfizer (Amboise, France), tetracycline and chlortetracycline were purchased from Sigma (Saint Louis, MO, USA). Metha-

Table 1
Some physico-chemical and microbiological characteristics of mud

Dry matter (g/100g)	39.04
Inorganic matter (g/100g)	34.58
Organic matter (g/100g)	4.46
Calcium (g/kg)	13.15
Magnesium (g/kg)	6.22
Zinc (g/kg)	0.234
Iron (g/kg)	0.039
Aluminium (g/kg)	0.013
Bacteria number (×1000/g)	55

nolic solutions of TCs were prepared at a concentration of 1 μg l^{-1} and stored at −20°C in darkness for 1 month. Aqueous dilutions and mixtures of these solutions were performed daily and stored in darkness at +4°C.

2.3. Extraction and clean-up procedure

A 1 g fortified sediment sample, into which was added 0.2 g of Na_2EDTA was homogenised three times for 5 min with 5 ml of McIlvaine buffer. After centrifugation at 3000 *g* for 5 min at +4°C, the supernatants were combined and centrifuged again at 3000 *g* for 10 min at +4°C. The supernatant was purified by passing through Bond Elut Certify® LRC (300 mg, 10 ml, part number 1211-3052, lot number 292905, Varian, Harbor City, CA, USA) SPE cartridge. Before use, the cartridge was activated with methanol (4 ml) and McIlvaine buffer (4 ml). After passing the sample, the cartridge was rinsed with methanol (10 ml). TCs were then eluted with methanol (10 ml) after raising the pH with 10 ml of a mixture of ethyl acetate and 25% ammonia (96:4, v/v). After addition of the external standard (CTC), the eluate was evaporated to dryness in SpeedVac® system SC 210A (Savant, Framingdale, NY, USA) for 1 h at 70°C and reconstituted to 0.5 ml in methanol. The sample was then centrifugated at 16 000 *g* for 5 min at +4°C.

2.4. HPLC

The separation of TCs was performed on a Spherisorb® S3 ODS1 column (80 Å, 150×2.1 mm, Waters, Milford, MA, USA) using a binary HP 1100 series pump (Hewlett-Packard, Palo Alto, CA, USA) with a variable wavelength UV detector (Hewlett-Packard) operating at 355 nm. A solvent gradient with two eluents was performed in 20 min. A mixture of a 0.01 *M* oxalic acid aqueous solution and methanol (80:20, v/v) was used for eluent A. A mixture of acetonitrile and methanol (80:20, v/v) was used for eluent B. The reversed-phase separation used a linear gradient from 12.5% eluent B to 62.5% eluent B over 20 min. The column was set to initial in 10 min and stabilised over 5 min before next injection. The flow-rate was 0.2 ml min^{-1}. For both standard solutions and extracts, 10 μl were injected. Detectors were UV and MS.

2.5. Frit FAB HPLC–MS

All FAB–MS measurements were performed on a JEOL-SX 102 (Jeol, Tokyo, Japan) electromagnetic mass spectrometer. The instrument was equipped with a frit-FAB ion source at a temperature of +60°C. The FAB gun was operated with xenon gas at 10 kV using an acceleration voltage of 3 kV for measurement of all spectra. LC/MS data were obtained by scanning from *m/z* 50 to 500. The LC and the mass spectrometer were interfaced by a Jeol flow splitter, connection tubing and a frit FAB probe. The flow-rate was 10 μl min^{-1} and the effluent was split in a ratio of 1:20. The FAB matrix (glycerol/thioglycerol, 50:50, v/v) was added to the mobile phase at a concentration of 0.5‰. The instrument was manually tuned using matrix ions with the source voltages adjusted to maximise matrix cluster ions.

2.6. Particle beam HPLC–MS

MS detection was realised on a HP 5989A quadrupolar mass spectrometer in the chemical ionisation mode. The LC was interfaced to the mass spectrometer with a HP 59980B particle beam interface (Hewlett-Packard) (desolvatation temperature 70°C). The negative chemical ionisation (NCI) was selected with methane as reagent gas at 2 10^{-4} Torr source pressure; the electron energy beam was set at 230 eV. Ion source and quadrupole temperatures were 300°C and 150°C respectively. Helium pressure in the nebuliser was set at 100 p.s.i.. The instrument was calibrated and manually tuned with perfluorotributylamine (PFTBA) on *m/z* 452, 595 and 633. The skimmers were cleaned daily.

3. Results and discussion

3.1. Extraction and clean-up procedure

The extraction procedure was derived from the procedure developed by Pouliquen et al. [10]. Pre-

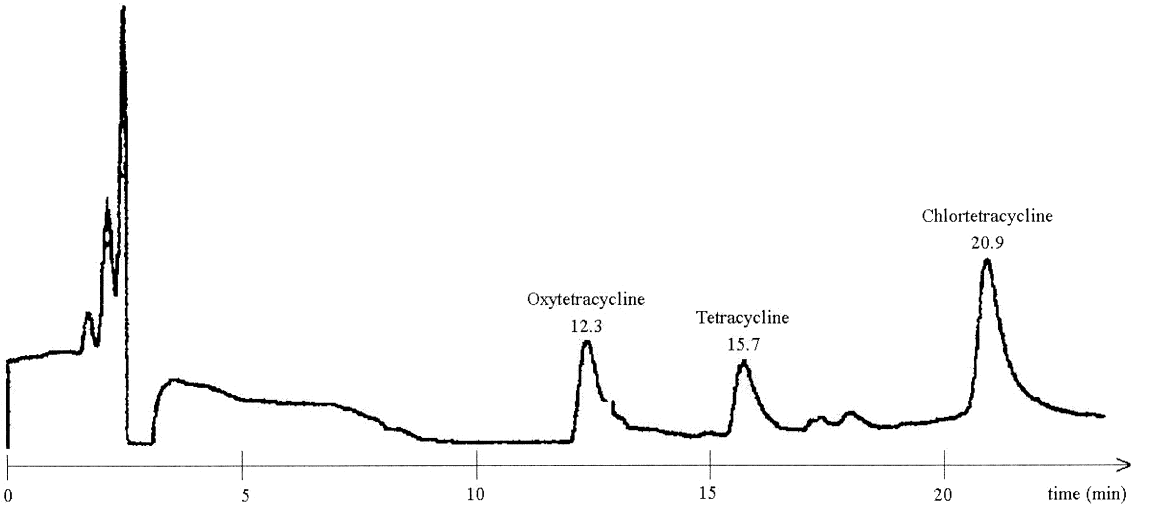

Fig. 2. HPLC chromatogram obtained for sediment (1 g) fortified with TCs (100 ng) after solid-phase extraction on a Bond Elut Certify® LRC cartridge. Chromatographic resolution between OTC and TC is 3.31. LC conditions: linear solvent gradient, 0.01 *M* aqueous oxalic acid solution, methanol, acetronitrile, 70:20:10 (v/v/v) to 30:20:50 (v/v/v) over 20 min, pH 2.30–2.55; column 150×2.1 mm, Spherisorb® ODS1 (3 μm); flow-rate, 0.2 ml/min; injection volume 10 μl; wavelength 355 nm. The clean-up and concentration process developed allowed an absolute recovery near from 30%.

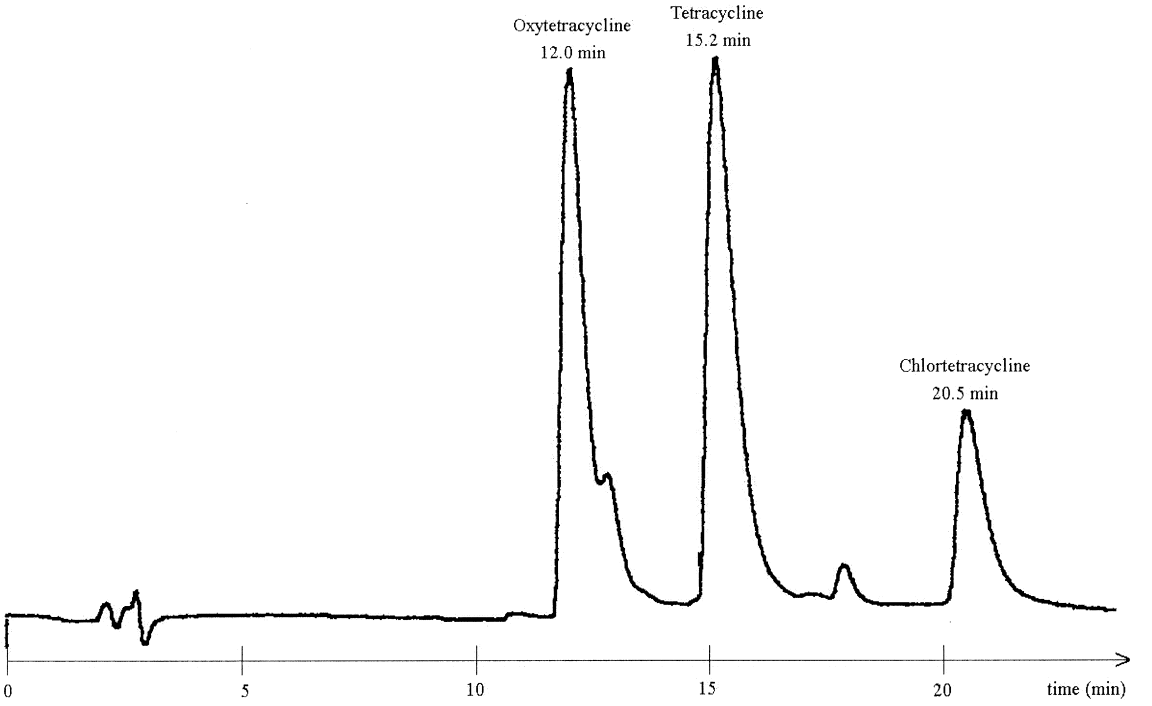

Fig. 3. HPLC chromatogram of the natural TCs (100 ng injected) obtained with an UV detection (peak height in mV in term of time in min). LC conditions were the same than in the Fig. 2.

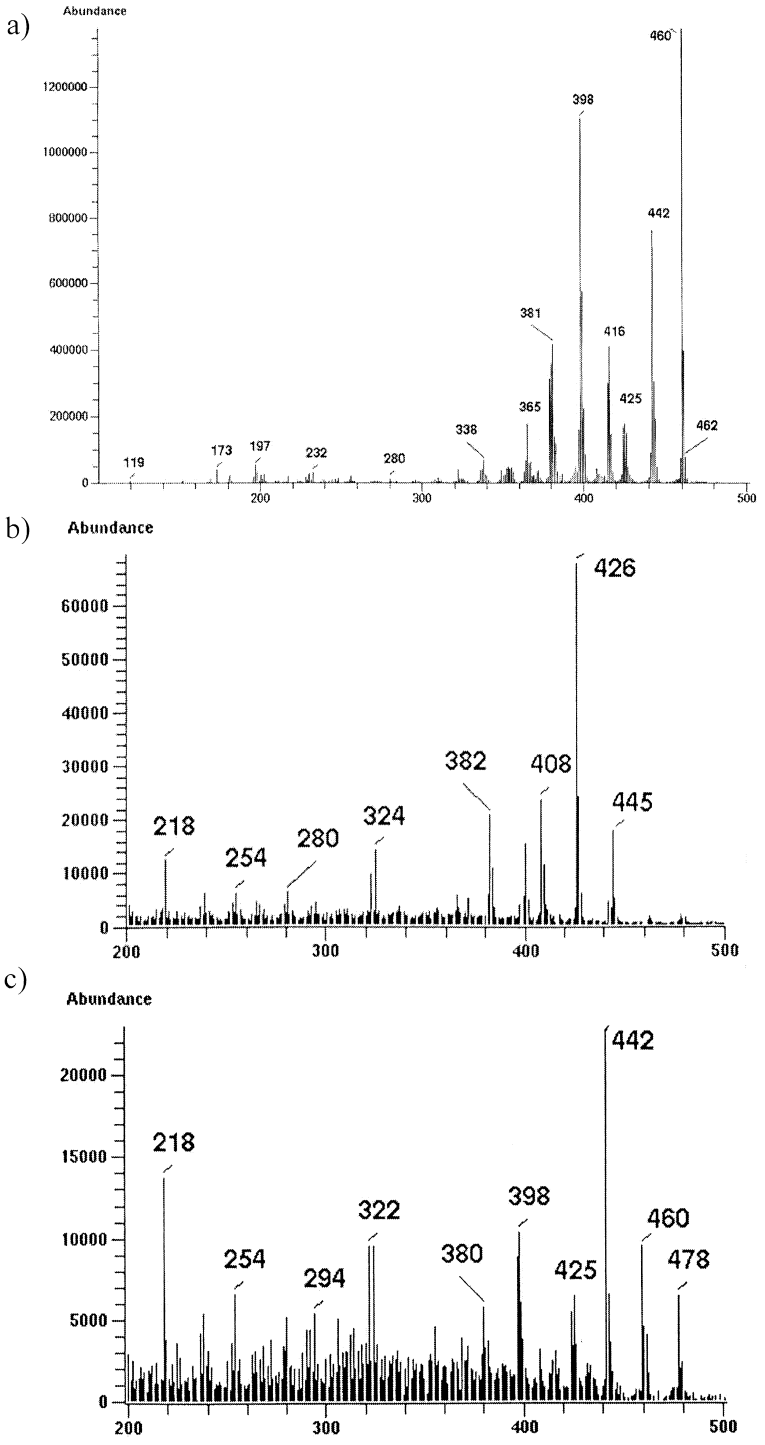

Fig. 4. Background-subtracted NCI (CH4) mass spectra of the natural TCs (10 μg injected in infusion mode): (a) OTC; (b) TC; (c) CTC. High mass fragments should permit to deduce structural informations using HR–MS and MS–MS studies.

liminary work (results not shown) showed that the best SPE cartridge was the Bond Elut Certify® LCR cartridge. This cartridge contained a mixture of octadecylsilyl and benzensulphonic acid (strong cation exchange) and gave the best recovery and clean up extracts. The third pKa of TCs is 3.3; so, after activation of the SPE sorbent, TCs were loaded under their cationic form in the McIlvaine buffer (pH=3.0). Polar compounds of sediments were removed by using methanol and pH then was raised with ethyl acetate containing ammonia (96:4). TCs were then under their zwitterionic form (isoelectric pH=5.0) and were eluted by methanol. The clean-up and concentration process developed for sediments allowed a 30% absolute recovery (Fig. 2).

3.2. HPLC

The presence of a chlorine atom and the absence of one hydroxyl (Fig. 1c) confers to CTC the longer retention time of natural TCs; consequently this molecule was also used to evaluate analysis runtime. It was supposed that a chromatographic method allowing the separation of TC and OTC would allow the separation of OTC from its degradation products. So the chromatographic method should permit a good separation between OTC and TC. The resolution and the numbers of theoretical plates were calculated thanks to the European Pharmacopoeia [4] recommendation.

All the stationary phases recently used to analyse TCs were reversed bonded silica because of the high polarity of these molecules. A large number of hydrophobic HPLC stationary phases have already been used for TCs determination, i.e. propyl [5], octyl [6,7] and octadecylsilyl sorbents [8–12]. On the one hand, a too polar reversed-phase (silica, alumina) induced peak tailing and sometimes definitive adsorption of TCs. On the other hand, a less polar reversed-phase (end-capped C_{18}) did not efficiently retain TCs. Waters SymmetryShield® and Waters Spherisorb® ODS1 columns seemed to be a

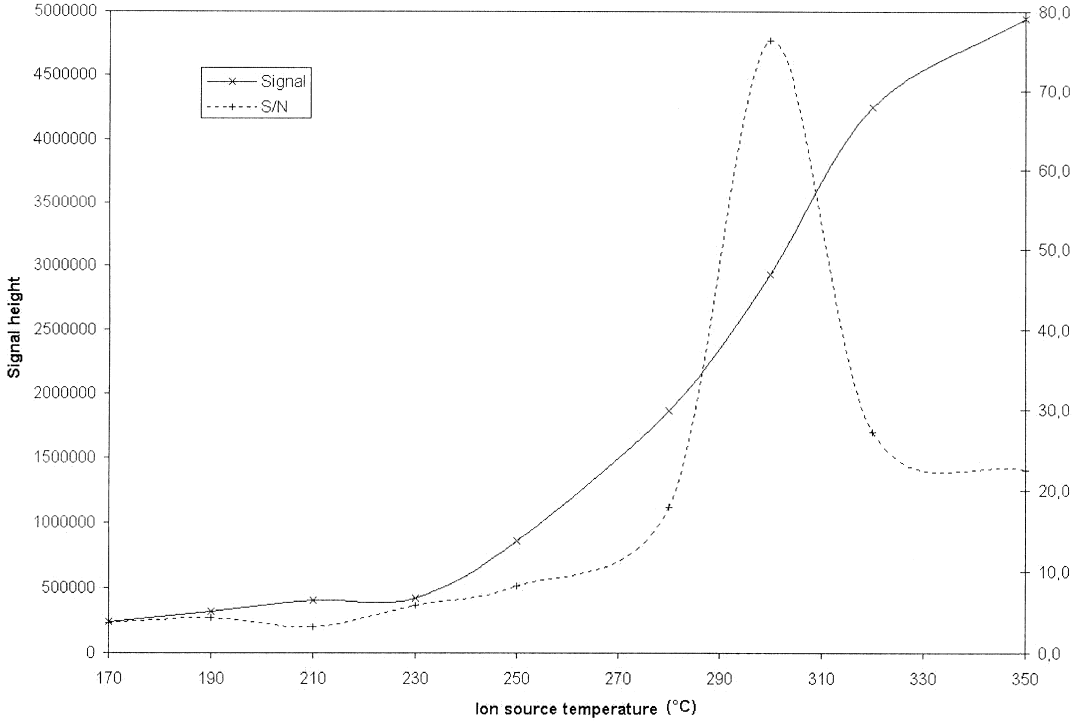

Fig. 5. Evolution of OTC signal height and S/N ratio with ion source temperature (10 μg injected in infusion mode, acquisition in scan mode, $n=3$). Ion source temperature was optimal at 300°C.

good compromise in terms of polarity. The particularity of the Waters SymmetryShield® stationary phase is the complementary action of the apolar octadecyl groups and the polar carbamates. The Waters Spherisorb® ODS1 column contains octadecyl non end-capped bonded silica and so high polar silanols competing with apolar C_{18}.

Preliminary assays (results not shown) determined that Spherisorb® ODS1 (150×2.1 mm, 3 μm particle size) column allowed better specificity for natural TCs. Because of the low silica purity and TCs chelating properties, oxalic acid was added to the mobile phase in order to prevent interactions between divalent cation impurities and TCs. Mobile phase was then optimised with a 0.01 *M* oxalic acid aqueous solution, methanol and acetonitrile. Moreover, small internal diameter allowed a lower flow-rate more suitable for LC–MS coupling, and a smaller particle size gave a better column efficiency. The characteristics of the column were 150×2.1 mm with 3 μm particle size. Resolution was improved by using a linear solvent gradient. The chromatogram obtained is shown in Fig. 3. The retention times of OTC, TC and CTC were 12.6, 15.7 and 21.1 min respectively. The numbers of theoretical plates for OTC, TC and CTC were 2397, 2502 and 4883 respectively. Resolution between OTC and TC in these conditions was 3.31. For comparison, the best resolution found in the literature was 1.9 [13]. The method presented here constitutes a significant improvement in the separation capabilities between natural TCs and would probably allow the separation between OTC and its hypothetical degradation products.

3.3. Mass spectrometry

Two mass spectrometric techniques based on different LC–MS interfaces were studied.

● Determination of TCs by LC–MS using particle beam interface (PB) has been studied by Kijaj et al.

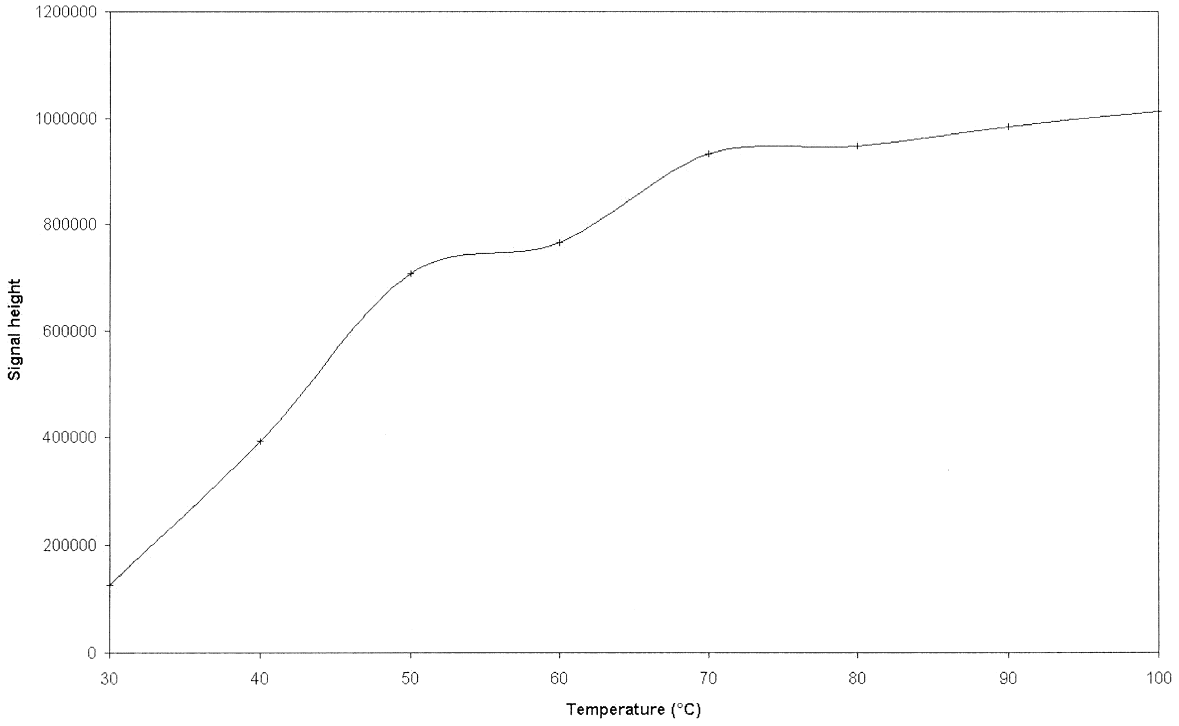

Fig. 6. Evolution of OTC signal height with PB interface's helium pressure in the nebuliser (10 μg injected in infusion mode, acquisition in scan mode, $n=3$, 1 p.s.i.=6.8948 kPa). Signal increase with helium pressure so nebuliser helium pressure was set at 100 p.s.i..

[14] and Carson et al. [15]. PB allowed detection limits as low as 20 ng injected (SIM mode, methane NCI, quadrupole mass spectrometer). Preliminary assays were realised on different ionisation modes: electronic impact, positive and negative chemical ionisation using methane, isobutane and ammonia as reacting gases. Signal-to-noise ratio (S/N) and signal height were better with chemical ionisation than with electronic impact conditions. The negative chemical ionisation increased the signal and allowed a more important fragmentation than the positive chemical ionisation. It was important to induce high fragmentation (Fig. 4) in order to be able to obtain structural information on the hypothetical degradation products of OTC. The fragmentation was more specific when methane was used as reagent gas with ion source pressure adjusted to $2 \cdot 10^{-4}$ Torr.

Ion source temperature consequences on the mass spectrum have been discussed by some authors. Kijaj et al. [14] and Carson et al. [15] described an optimum at 250°C for signal sensitivity while Mylcreest [16] was in favour of a higher temperature which did not exceed 280°C. Our data confirmed that higher temperatures increased the S/N ratio [16]. An optimal value was reached at 300°C (Fig. 5).

The interface parameters were also optimised. Signal increased with helium pressure in nebuliser as shown in Fig. 6, so the helium pressure was set to the maximum (100 p.s.i.) and the position of the capillary in the nebuliser was optimised after each changing. The study of the desolvatation chamber temperature showed that the signal increased with temperature (Fig. 7) because of more efficient desolvatation [17,18]; temperature was set to 70°C. The flow-rate was 200 μl min^{-1} in order to reduce split ratio and, consequently, to obtain better LOD. Scan mode acquisition was chosen because it gave structural information. In these conditions, the detection limits of TCs were 100 ng in the infusion mode and 1 μg after HPLC separation on the Spherisorb®

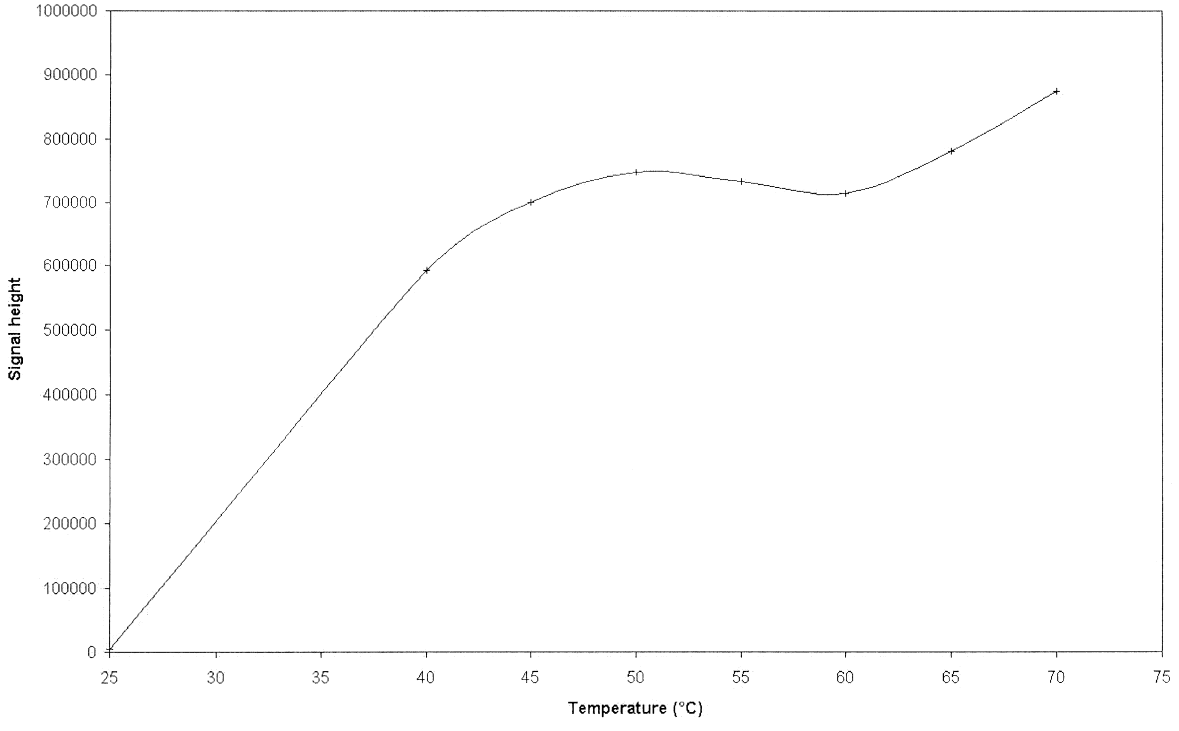

Fig. 7. Evolution of OTC signal height with PB interface's desolvatation temperature (10 μg injected in infusion mode, acquisition in scan mode, $n=3$). Signal increase with desolvatation temperature was set at 70°C.

ODS1 column. This high LOD was due to the low transmission of PB interface, but also to the low sensitivity of an old generation of quadrupole mass filter, especially in the scan mode.

● Determination of TCs by LC–MS using frit FAB interface in accordance with the literature gave a LOD of 10 ng in scan mode [19,20]. The choice of the FAB matrix was correlated to the ionisation yield. Diethanolamine (DEA), triethanolamine (TEA), *o*-nitrophenyl octyl ether (NPOE) were not able to ionise TCs. Dithioethanolamine (DTE), *m*-nitrobenzyl alcohol (NBA), glycerol and magic bullet (1,4-dithiothreitol/1,4-dithioerythritol 3:1 v/v) generated adduct ions near the OTC mass [21]. Thioglycerol was judged to be a good matrix according to several authors [20,14] as was glycerol [22].

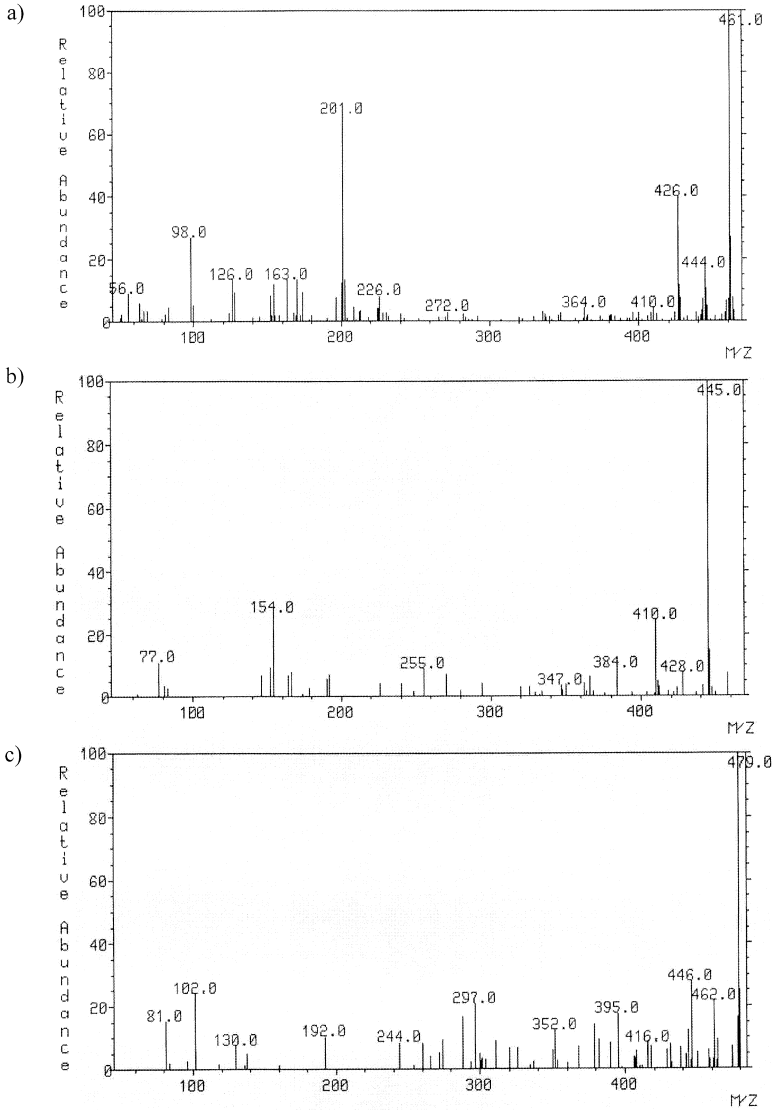

Fig. 8. Positive frit FAB (glycerol:thioglycerol matrix) mass spectra (100 ng injected in infusion mode) of (a) OTC, (b) TC, (c) CTC. Molecular ions are $(M+H)^+$. MS–MS fragmentation was studied to purpose fragmentation pattern (Fig. 10).

Assays gave better results for a mixture of glycerol and thioglycerol (50:50, v/v) than for glycerol or thioglycerol alone. A 0.5‰ matrix concentration in the mobile phase gave better results than the 1–10% usually applied [19,23]. Optimisation of the spectrometer parameters showed that the increase of ion

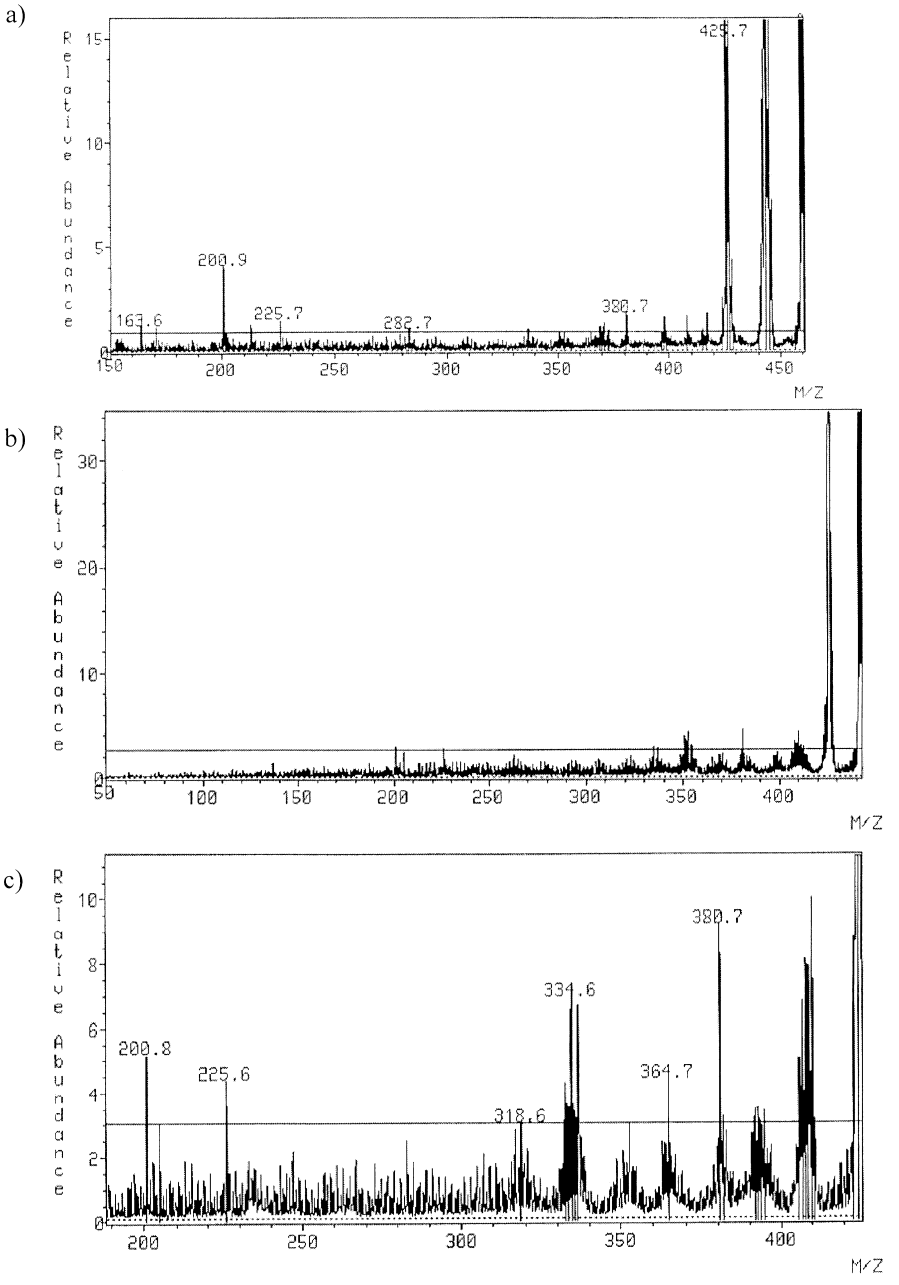

Fig. 9. LC–MS–MS spectra of OTC. Daughter scan study on ion (a) m/z=461, (b) m/z=443, (c) m/z=426. Parent scan study on ion (d) m/z=398, (e) m/z=381. The two principal ions displayed on mass spectra (Fig. 8) (m/z 461 $[M+H]^+$ and m/z 426 $[M+H-H_2O-NH_3]^+$) induce nearly all fragments.

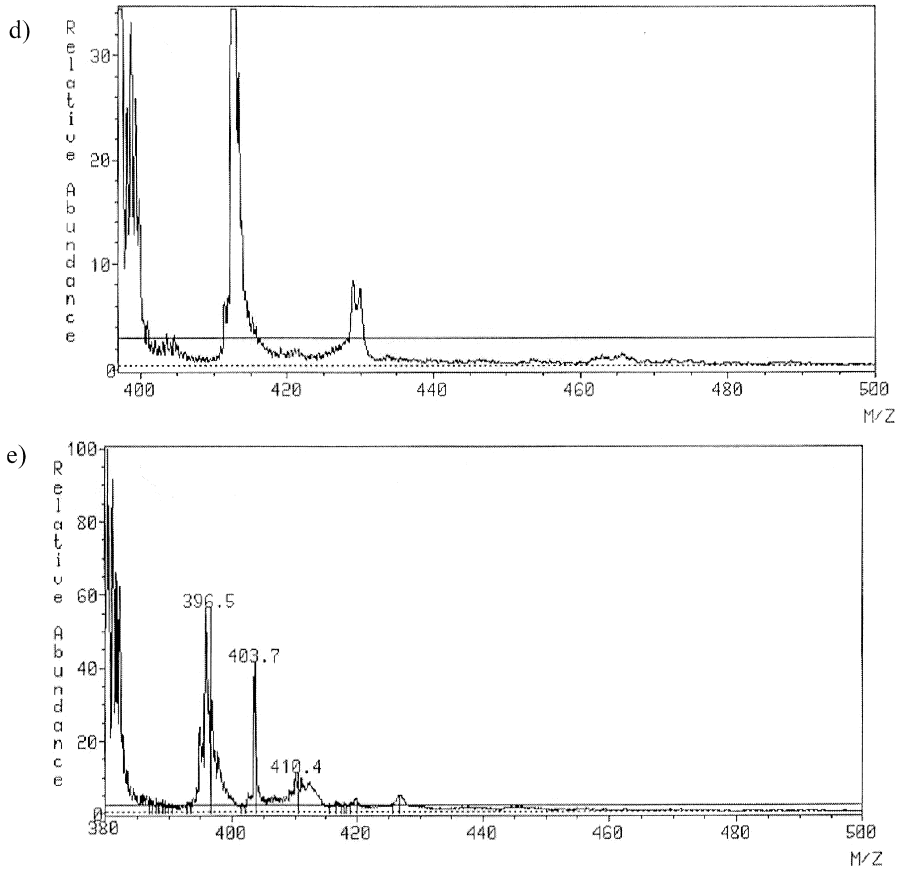

Fig. 9. (*continued*)

source temperature reduced peak tailing. Above 60°C, signal height continued to decreased so ion source temperature was set to 60°C. The frit probe position, optimised after each frit cleaning, was set back to near 1.5 mm from ion source. Split ratio was 1:20 in order that the flow-rate reaching mass spectrometer was 10 μl min^{-1}. In these conditions, the detection limits of TCs were 1 ng in the infusion mode and 10 ng with Spherisorb® ODS1 column. Frit FAB LC–MS sensitivity on an electromagnetic instrument was a hundredfold better than PB, this being mainly explained by the mass filter sensitivity in spite of the split applied on the mobile phase. Furthermore, many high mass ions were generated in FAB ionisation mode (Fig. 8); another reason for the selection of frit FAB for determination of TCs and its degradation products.

3.4. Oxytetracycline fragmentation in FAB ionisation

Knowledge of OTC fragmentation could supply information for further studies on its degradation. For example, some degradation products could give the same fragment ions as the precursor molecule, so filiation of the main ions were studied on a JEOL SX 102 magnetic mass filter (inverse geometry) by MS–MS acquisition (Fig. 8a). Daughter scan and parent scan of OTC fragment ions were acquired in the accumulation mode. Molecular ion $(M+H)^+$ (m/z 461) was fragmented into 6 main daughter ions (m/z 201, 226, 398, 416, 426 and 443) as shown in Fig. 9. Principal secondary fragmentation came from m/z 426 ion which gave four principal daughter ions (m/z 201, 226, 381 and 398) (Fig. 9). Fig. 10 shows the

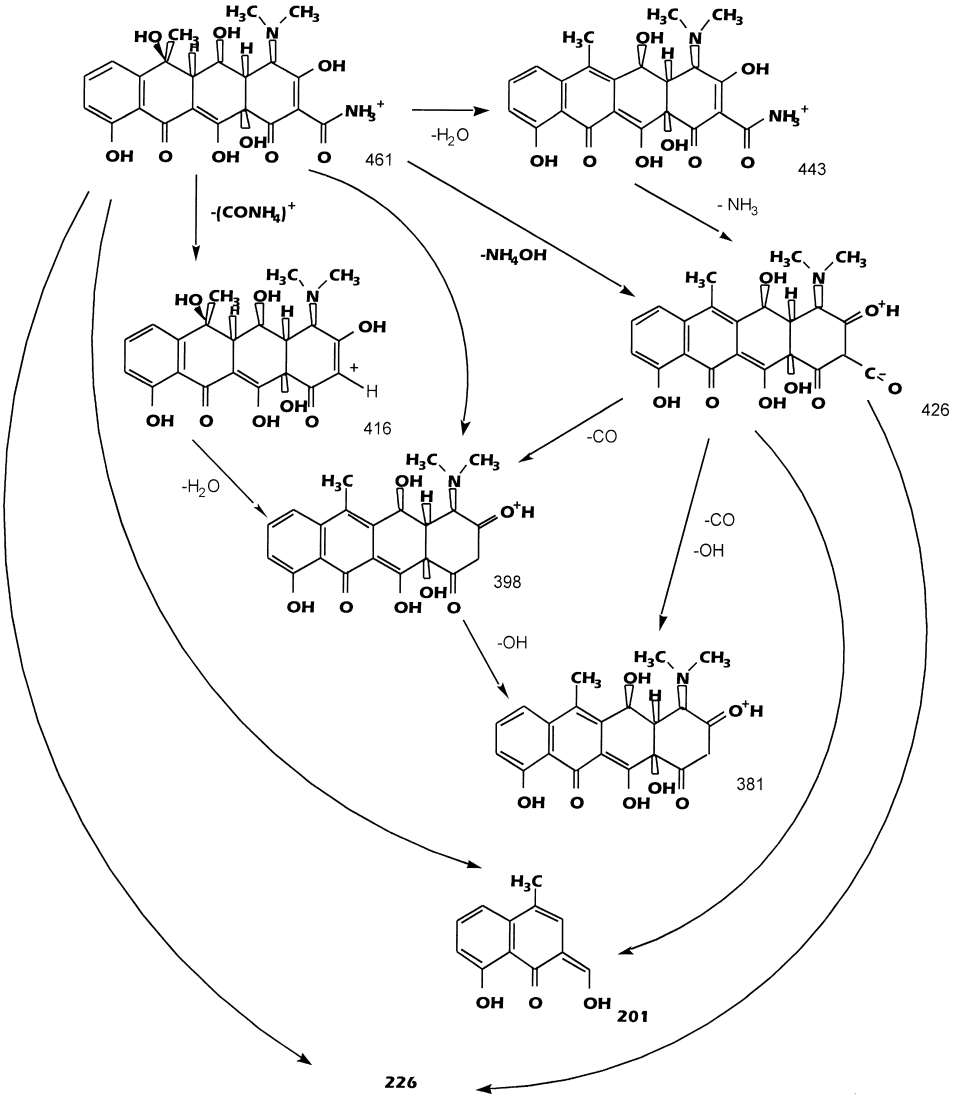

Fig. 10. Fragmentation pattern suggested for OTC by positive FAB ionisation. Fragmentation mainly came from losses of ammonia, water and carbonyl. NCI fragmentation of OTC should be very near from this pattern.

fragmentation pattern suggested for OTC. Principal fragmentations probably resulted from loss of water, ammonia and carbonyl. All these ions were also observed in the NCI mode (Fig. 4).

4. Conclusion

A very specific HPLC–MS method was developed for the determination of tetracycline, oxytetracycline and chlortetracycline. This method significantly improved the resolution between the natural TCs (3.31 between OTC and TC) and gave a very low LOD (10 ng injected in scan mode with frit FAB). The superiority of frit FAB LC–MS interface over particle beam interface have been demonstrated for TCs in terms of detection limit, 100-fold lower. Furthermore, the FAB fragmentation of oxytetracycline has been studied. Structure of OTC fragment ions would probably be helpful to identify OTC degradation

products. This method open numerous possibilities for the further study of OTC degradation products. Electrospray and atmospheric pressure ionisations were also used for LC/MS determination of TCs [12,24–29] and will shortly be investigated.

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