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Thin-layer chromatography–matrix-assisted laser desorption ionisation–time-of-flight mass spectrometry using particle suspension matrices

Anna Crecelius[a], Malcolm R. Clench[a,*], Don S. Richards[b], Vic Parr[c]

[a]*Biomedical and Molecular Sciences, School of Science and Mathematics, Sheffield Hallam University, Pond Street, Sheffield S1 1WB, UK*

[b]*Pfizer Global R&D, Ramsgate Road, Sandwich, Kent, UK*

[c]*SAI Ltd, New Elm Lane, Manchester, UK*

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Abstract

Particle suspension matrices have been successfully utilized for the analysis of tetracycline antibiotics by thin-layer chromatography–matrix-assisted laser desorption ionisation–time-of-flight mass spectrometry (TLC–MALDI–TOF–MS). Particles of different materials and sizes have been investigated (Co-UFP, TiN, TiO_2, Graphite and Silicon) by applying particle suspensions to eluted TLC plates. Mass spectra and mass chromatograms have been recorded directly from the TLC plates. Strong cationization by sodium and potassium was obtained in the positive ion mode, with $[M+Na-NH_3]^+$ ions being the predominant signals. The TLC–MALDI mass spectra recorded from graphite suspensions showed the lowest background noise and the highest peak intensities from the range of suspension matrices studied. The mass accuracy from graphite films was improved by adding the peptide Phe–Phe to the graphite suspensions. This allowed internal recalibration of the TLC–MALDI mass spectra acquired during a run. One major potential advantage of TLC–MALDI–TOF–MS has been demonstrated in the analysis of chlortetracycline and tetracycline in a mixture of oxytetracycline, chlortetracycline, tetracycline and minocycline. Examination of the TLC plate prior to MALDI analysis showed only an unresolved spot for chlortetracycline and tetracycline. However by investigation of the MALDI mass spectra and plotting of single ion chromatograms separate peaks for chlortetracycline and tetracycline could be obtained.

Keywords: Particle suspension matrix; Mass spectrometry; Inorganic matrix; Matrix-assisted laser desorption ionisation; Chlortetracycline; Tetracycline

1. Introduction

Matrix-assisted laser desorption/ionisation (MALDI) is a powerful and widely used mass spectrometry method for the analysis of biopolymers. The process of using a UV absorbing organic compound as matrix material to aid laser desorption of intact protein molecular ions was introduced by Karas and Hillenkamp [1]. Mass spectra of various proteins, such as albumin (67 kDa), were recorded

*Corresponding author. Tel.: +44-11-4225-3072; fax: +44-11-4225-3066.

E-mail address: m.r.clench@shu.ac.uk (M.R. Clench).

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by mixing the analyte with nicotinic acid. Since then, different compounds, including cinnamic acid derivates and aromatic carbonyl derivates, have been considered as potential MALDI matrices [2].

Besides organic matrices, metal particles of different materials and sizes have been investigated as possible MALDI matrices. The major advantage of metal particles is the absence of matrix interference in the lower mass range (below 500 Da) hence allowing the analysis of low mass analytes. In 1988, Tanaka et al. [3] first demonstrated laser desorption/ionisation (LDI) spectra of proteins and polymers with molecular masses up to 25 kDa, using 30 nm diameter fine cobalt powder, suspended in a glycerol dispersant. Schürenberg et al. [4] were inspired by Tanaka's work and investigated recently several nano-particles as matrices for the analysis of proteins and peptides. Mass spectra of cytochrome *c* and myoglobin were obtained by this research group using a suspension of titanium nitride (35 nm diameter) in glycerol. Sunner et al. [5] and Dale et al. [6,7] have investigated the use of micro-particles instead of nano-particles. In their experiments particles of 2–150 μm diameter of graphite and silicon with a range of dispersants including glycerol were employed to ionise compounds such as peptides, proteins, oligosaccharides, synthetic polymers and anionic analytes. The term SALDI for surface-assisted laser desorption/ionisation was introduced by Sunner et al. to distinguish this technique from MALDI employing organic matrices. Kinumi et al. [8] investigated commercially available metal and metal oxide micro-particles (Al, Zn, TiO_2, ZnO, etc.) as matrices for the analysis of PEG 200 and methyl stearate. Michalak et al. [9] reported that the fullerene C_{60} with a diameter of a few micrometers was a good matrix for protein analysis and Huang et al. [10] pursued this technique for the screening of diuretics in urine. More recently, laser desorption/ionisation has been achieved without a matrix by depositing the analyte on a UV absorbing silicon substrate (DIOS) [11–14].

The coupling of thin-layer chromatography (TLC) to mass spectrometry (MS) combines the simplicity of TLC with the specific detection capabilities of MS [15,16]. Unlike other hypernated techniques, e.g. liquid chromatography (LC–MS), solvent consumption is low and additionally the TLC plate can act as a storage device for samples and chromatograms. In TLC, unknowns, which might be missed in LC due non-elution from the column, are readily detected as spots that have not moved from the origin. For the analysis of tetracycline antibiotics, a further advantage, is that the non-volatile compound, disodium ethylenediaminetetraacetate, which is required to improve the separation, remains on the TLC plate and hence does not cause any of the problems such as clogging of the interface and deposits in the ion source, that have been reported in their analysis by LC–MS [17]. Fast atom bombardment–mass spectrometry (FAB-MS) was successfully employed by Oka et al. [18] for the TLC–MS analysis of tetracyclines. However, the lateral analyte spreading caused by the use of a liquid matrix in FAB, required the sample spots to be concentrated by ''condensing'' them using a solvent focusing technique [19]. The chromatographic information contained in the TLC plate is therefore lost in this technique.

The use of micro-particles in TLC–SALDI–TOF–MS has recently been described [20–22]. The appropriate zone of the developed TLC plate was coated with a suspension of activated carbon particles in glycerol and analysed. Using this approach spectra were obtained for a variety of peptides (bradykinin, angiotensin II) and low molecular mass organic compounds (hydrochorothiazide and prometryn). Limitations in analytical sensitivity and spectra quality led Han and co-workers [22] to create a carbon activated surface on the aluminium support of the TLC plate, so that the separated analytes could migrate towards the particle surface after elution. The sensitivity as well as the mass resolution could be readily improved by this new methodology, (a similar approach was used by Mehl et al. [23] who used organic matrices to create the activated surface). However, the chromatographic integrity of a separation is destroyed in this technique and hence there is no longer the possibility of ''scanning'' the TLC plate to produce chromatograms or ''imaging'' spots of the analyte.

In this paper, results for the TLC–MALDI–TOF–MS analysis of tetracyclines using different particle suspensions are reported. Micro-particles as well as nano-particles were examined for their suitability for TLC–MALDI–MS. The majority of the results were obtained for a suspension of graphite (1–2 μm

diameter) in ethylene glycol which was found to yield better sensitivity in comparison to the other tested materials and dispersants. Using this system the major ion species observed in both positive and negative ionisation modes were fragment ions. Fragmentation did not occur to the same extent when organic matrices, such as DHB or α-CHCA, were used.

Extracted ion chromatograms have been constructed from the scanned TLC plates. Using the extracted ion chromatograms, obtained from the TLC–MALDI analysis of different tetracyclines, it was possible to calculate the R_f-value of the detected analyte spots. These showed good agreement with the R_f-values obtained by UV detection.

2. Experimental

2.1. Materials

Oxytetracycline (OTC, MW 460), Tetracycline (TC, MW 444), Chlortetracycline (CTC, MW 478) and Minocycline (MC, MW 457) were purchased from Sigma–Aldrich (Dorset, UK). OTC was used as dihydrate, CTC and MC as hydrochloride. The five tested nano- and micro-particle powders of different materials and particle diameters are listed in Table 1. All chemicals were used as purchased from commercial suppliers.

2.2. TLC separation

The tetracycline antibiotics were separated using the procedure described by Naidong et al. [24]. Pre-treatment of the aluminium-backed TLC plates coated with 0.2 mm layers of silica gel 60 F_{254} (Merck, Germany) was necessary in order to avoid the formation of metal–tetracycline complexes and hence to improve the separation. The TLC plates (10×10 cm) were sprayed with ca. 5 ml of an aqueous disodium EDTA solution (0.27 mol/l, pH 8), air dried for 30 min in a horizontal position and then activated in an oven (120 °C) for another 30 min.

The mobile phase dichloromethane–methanol–water (59:35:8, v/v) was saturated for 2 h prior to use. The plates were eluted over a distance of 7.0 cm, air dried and visualised under UV light (254 nm).

2.3. Matrix application

In all experiments a 60×2 mm strip of the developed TLC plate was attached to a modified MALDI target with double sided tape before the matrix was deposited on to the silica gel surface.

Particle matrix suspensions were prepared by dispersing powders of nano- or micro-particles (10–100 mg/ml) in ca. 1 ml of ethanol–ethylene glycol (1000:1, v/v) or methanol–ethylene glycol (1000:1, v/v). The suspensions were homogenised by sonication for 15 min and then applied (30 μl) to the developed TLC strip using a 10 μl syringe. Experiments with the crystalline chemical matrices 2,5-dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxy cinnamic acid (α-CHCA) were performed for comparison. In these cases the matrix solutions (300 mg/ml DHB in ethanol–water containing 0.1% TFA (80:20, v/v) or 20 mg/ml α-CHCA in methanol containing 0.1%TFA) were electrosprayed on to the TLC plates using an in-house modified commercial robotic *x*–*y*–*z*-axis motion system (PROBOT, BAI, Germany). The instrument was modified to act as an electrospray deposition device by connection of a 0–6 kV power supply to the sample needle (110 μm

Table 1
Matrix properties of different nano- and micro-particles

Material	Particle colour	Particle diameter	Supplier
Cobalt ultra fine powder (Co-UFP)	Black	20 nm	Kratos, UK
TiN	Black	36 nm	HC Starck GmbH, Germany, (kind gift by the supplier)
TiO_2	White	1 μm	Fluka, USA
Graphite	Black	1–2 μm	Sigma–Aldrich, UK
Silicon	Grey	45 μm	Sigma–Aldrich, UK

I.D.) and adding an earthed metal plate (7×15 cm) to the sample table. The matrix solutions were electrosprayed on to the silica gel surface with a flow-rate of 10 μl/min, as the sample table was moved with a speed of 0.25 mm/s. An area of 2×60 mm on the TLC strip was typically covered with matrix crystals and a good matrix coverage was obtained using this modified device. Note: Particle suspension matrices cannot be successfully electrosprayed owing to capillary blockages.

2.4. Mass spectrometry

Mass spectra and ion mass chromatograms were recorded directly from the TLC plate with a modified linear Laser TOF 1500 mass spectrometer (SAI, UK), equipped with a nitrogen laser (λ=337 nm). The modifications to the instrument and its software for use in TLC–MALDI–MS have been previously described by this group [25]. The positive and negative ion mode was used in these investigations and the mass spectra acquired from the TLC surface were the results of the cumulative acquisition of 16 shots. The TLC strips were scanned over a distance of 60 mm and mass spectra were recorded each 0.5 mm. A data set of 120 mass spectra was obtained for each sample, from which single ion mass chromatograms were constructed.

3. Results and discussion

3.1. Comparison of particle suspension matrices with organic matrices

For the MALDI–MS analysis of TC in a range of particle suspension matrices, mixtures containing equal volumes of particle suspensions (10–100 mg/ml in ethanol–ethylene glycol (1000:1, v/v)) and analyte solutions (1 mg/ml in methanol) were added to the stainless steel targets (typically 0.25 μl) and analysed.

The MALDI mass spectra obtained for TC using nano- and micro-particles are presented in Fig. 1. The inorganic matrices showed the following characteristics, compared to the crystalline organic matrices DHB and α-CHCA. No protonated molecule of TC could be observed, except when silicon powder was used, as shown in Fig. 1e. However strong cationisation by sodium and potassium was typically obtained when particle suspension matrices were used. Hence the molecular related ions of TC appeared as $[M+Na]^+$ at m/z 467 and as $[M+K]^+$ at m/z 483.

Fragment ions, which have been identified as $[M+Na–NH_3]^+$ at m/z 450, $[M+K–NH_3]^+$ at m/z 466 and $[M+Na–NH_3–H_2O]^+$ at m/z 432, could also be detected for TC. Furthermore, the ion intensity of the most abundant ions of TC was typically lower than that achieved when organic matrices were used. However the mass spectra were not dominated by complex signals at the lower mass range, as shown in Fig. 1.

In order to get some idea of the relative sensitivity obtained from TLC–MALDI–MS employing suspension matrices in comparison to the sensitivity obtained from conventional targets the same quantity of TC (10 μg) was analysed on both silica gel TLC plates and stainless steel targets, (three samples were analysed on each substrate and the results averaged). The suspension matrix used in this case was 40 mg/ml graphite, dissolved in methanol–ethylene glycol (1000:1, v/v). Comparison of the peak areas of the $[M+Na–NH_3]^+$ of TC at m/z 450 showed that the relative sensitivity obtained by MALDI–MS from conventional targets was two times better than that obtained by TLC–MALDI–MS.

3.2. Liquid dispersants of suspension matrices

Three different viscous liquids are generally described in the literature as particularly suitable for use as dispersants with particle suspension matrices, i.e. glycerol [3], ethylene glycol [26] and liquid paraffin [8]. The dispersant glycerol shows a characteristic background level in the low-mass region, and hence is in general not considered a good choice for the analysis of low mass analytes [8]. In our studies, ethylene glycol showed an advantage to paraffin, since the latter showed a degree of analyte suppression. Therefore, all data were collected by using ethylene glycol as dispersant.

The role of glycerol in supporting the phase transition is well documented in fast atom bombardment (FAB) and liquid secondary ion mass spectrometry (LSIMS). It is not clear if the function of

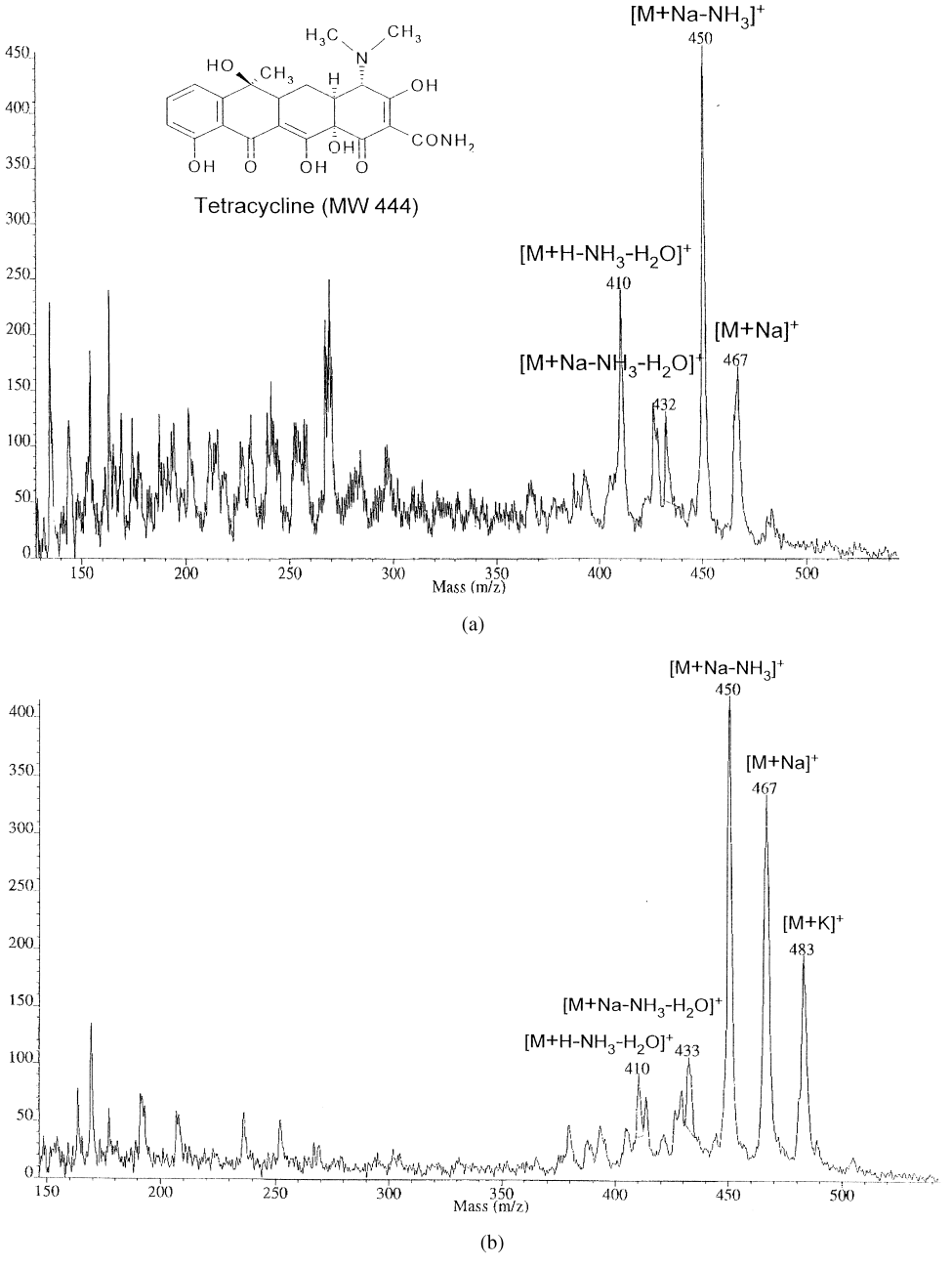
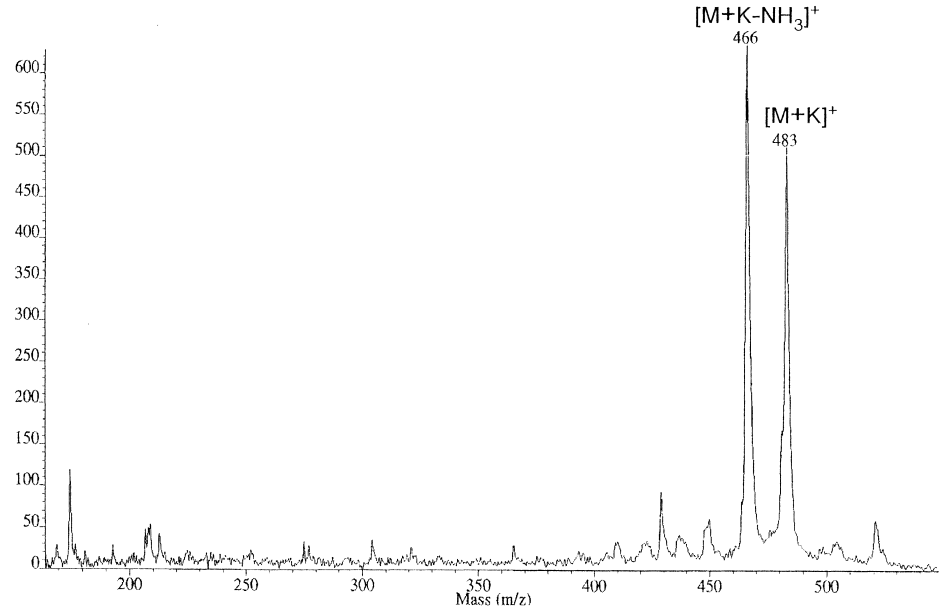
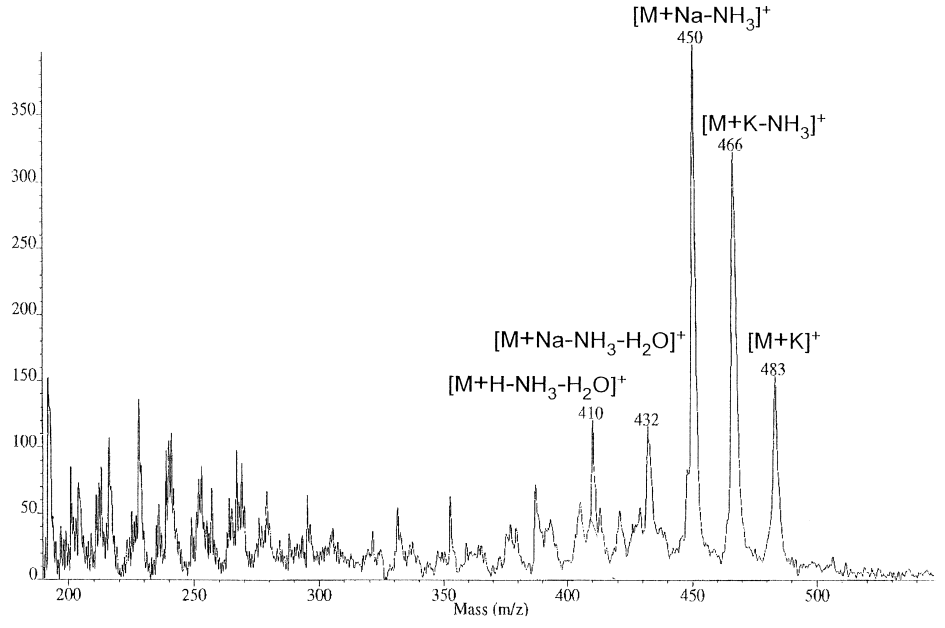


Fig. 1. MALDI mass spectra of tetracycline using nano- and micro-particles as suspension matrices, (a) Co-UFP, (b) TiN, (c) TiO_2, (d) Graphite and (e) Silicon.

glycerol is the same in particle assisted desorption/ ionisation. Dale et al. [6] considered that the addition of glycerol to graphite particles fulfils several roles. Besides increasing the signal lifetime at a particular sample position, it acts as a proton source in the case of peptides and proteins. In our initial studies we found that the absence of dispersant in graphite (or any other particle material) caused the ion intensity



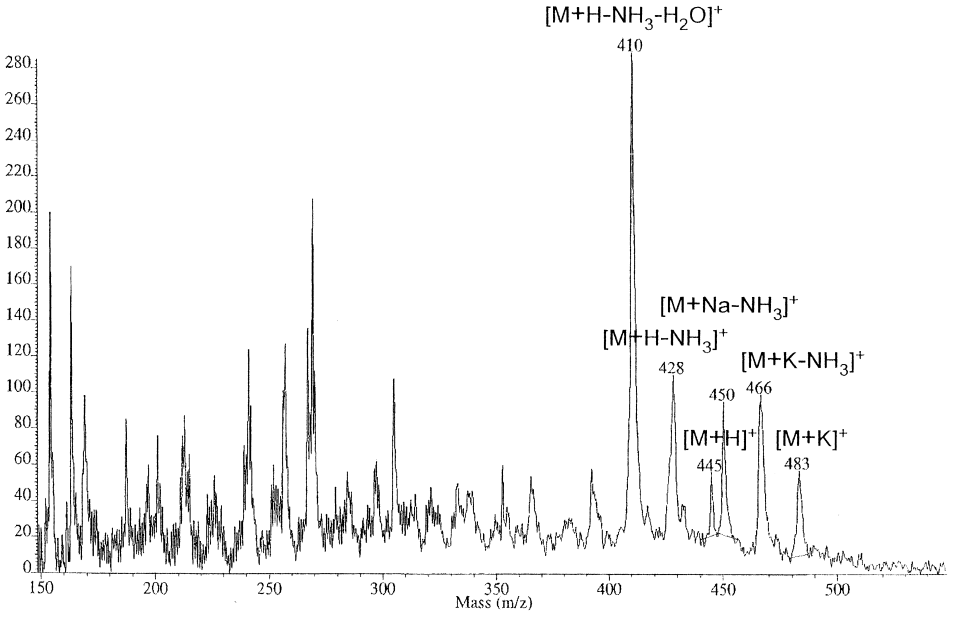
(c)



(d)

Fig. 1. (*continued*)

to rapidly decrease after firing the laser repeatedly at the same position. An increase of the concentration of ethylene glycol (from 0.1 to 1% in ethanol) caused an increase in the lifetime of the analyte signals obtained for TC. However, this also led to a faster contamination of the ion source extraction



(e)

Fig. 1. (*continued*)

grid. As described earlier, typically no protonated signals of TC could be detected when particle–ethylene glycol systems were used (Fig. 1). This was also the case when small peptides, such as Phe–Phe, Tyr–Tyr and Tyr–Tyr–Tyr were analysed (data not shown).

This would appear to indicate that ethylene glycol does not function as a proton source, but simply serves to mobilize the analyte by remaining liquid under vacuum.

3.3. TLC–MALDI–TOF–MS of tetracycline

From the whole range of particles studied, the TLC–MALDI mass spectra recorded from graphite suspensions showed the lowest background noise and the highest peak intensities. The peak intensities of TC obtained from graphite suspensions were even higher than the ones obtained when DHB was used (Fig. 3 and the corresponding ion mass chromatogram is shown in Fig. 4).

In TLC–MALDI–TOF–MS the extraction of the analyte from the TLC plate and its adsorption on the particle surface plays an important role. Micro-particles were found to be superior to nano-particles, in this case the particle size was of the same order as the particle size of the TLC silica gel layer (10 μm). However, if the particle diameter was higher than 10 μm, as in the case of silicon with a diameter of around 40 μm, the particles did not adhere to the silica gel layer after solvent evaporation. The addition of an additive, e.g. sucrose [20,21], can improve the adhesion between particles and silica gel layer. But this causes extra signals in the recorded mass spectra, which can interfere with the analyte peaks in the low mass range.

Fig. 2 shows the TLC–MALDI mass spectra of TC (200 μg) developed on a TLC plate using graphite particles as matrix material. In the positive ion mode several fragment ions and sodium adducts of TC were observed. In the negative ion mode, high intensity carbon clusters in the low mass range were present and fragment ions were the dominant species. A level of 200 μg of analyte was chosen in order to detect all possible fragment ions in reasonable peak intensities, so that mass chromatograms of the corresponding analyte ions could be constructed with a low background noise level. Mass chromatograms of the identified analyte peaks were recorded in the positive and negative ion mode.

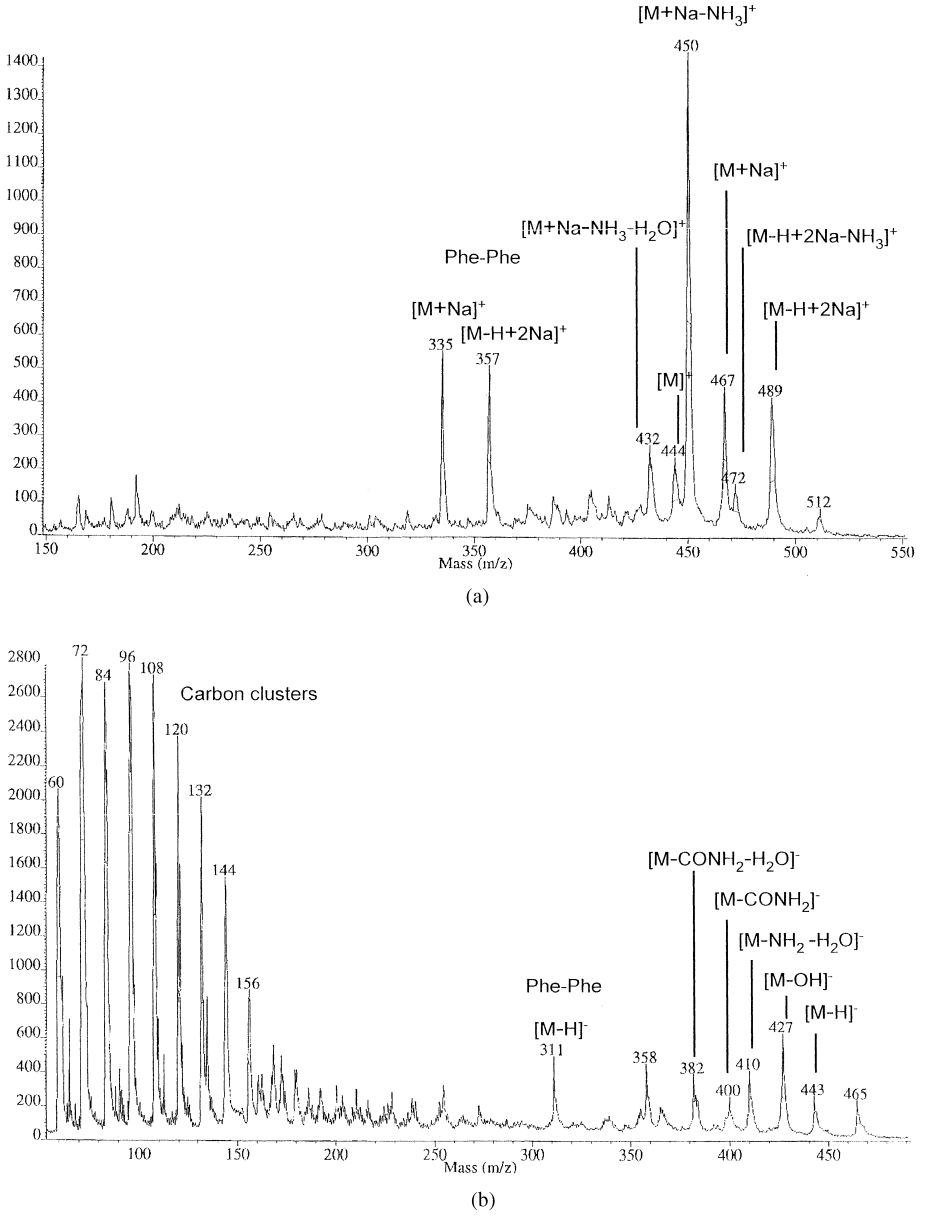


Fig. 2. TLC–MALDI mass spectra of tetracycline (200 μg) using a graphite matrix in: (a) positive; and (b) negative ion mode.

Since the absolute mass accuracy obtained was limited by thickness variations in the graphite film (this effect has been observed by Zumbühl et al. [27], resulting in a mass inaccuracy of ±2 mass units), it was decided to employ a ''lock mass'' and to recalibrate each mass spectrum acquired. Initially three small peptides (Phe–Phe, Tyr–Tyr and Tyr–Tyr–Tyr) and mixtures containing two of the peptides were tested as internal calibration standards. Phe–Phe was found to give the best results: optimum

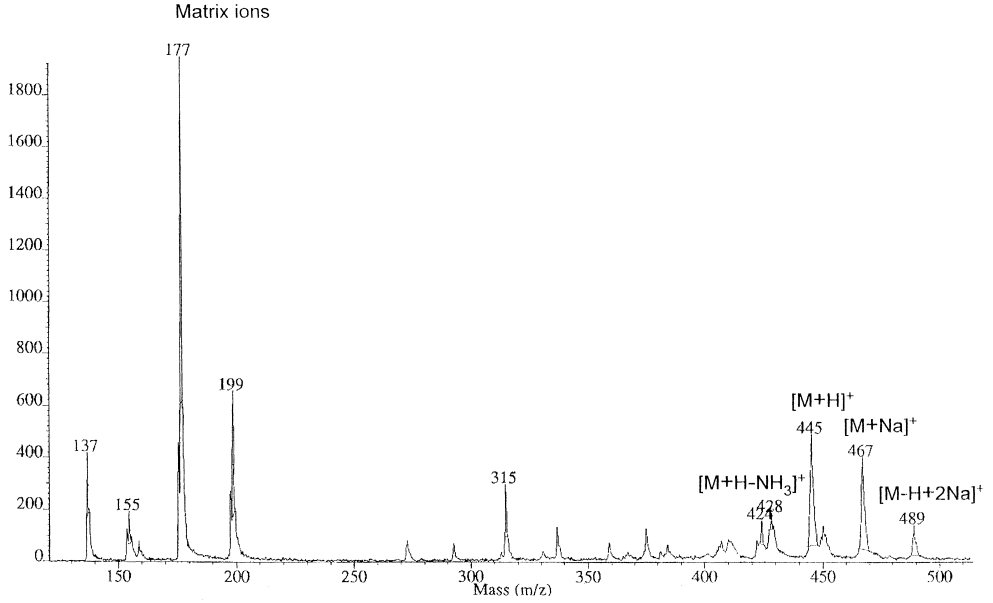


Fig. 3. TLC–MALDI mass spectrum of tetracycline (200 μg) using DHB.

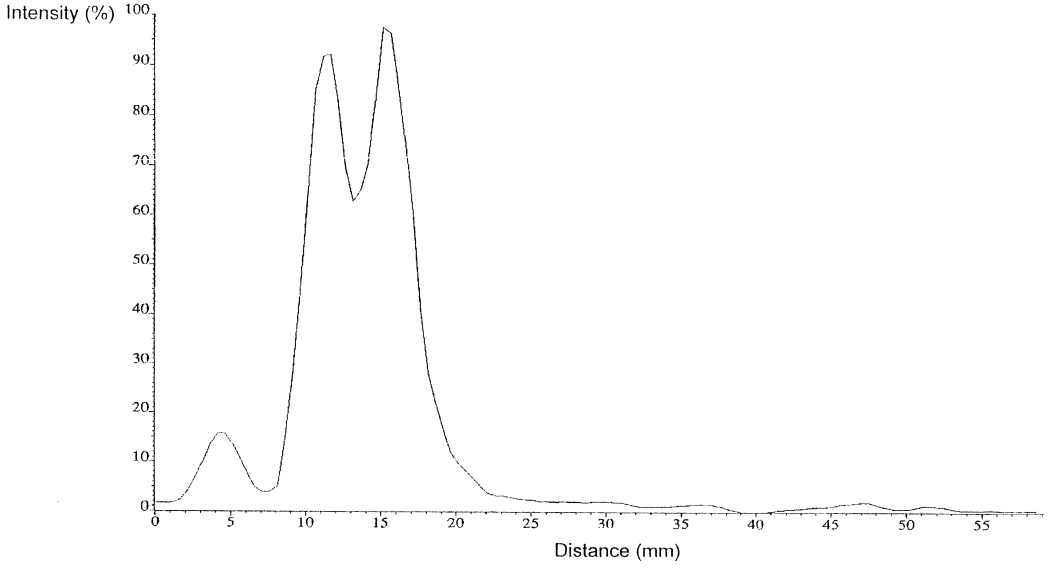


Fig. 4. The smoothed ion mass chromatogram of tetracycline (200 μg) from the experiment shown in Fig. 3. The $[M+H]^+$ ion (m/z 444–446) was used to construct the ion mass chromatogram.

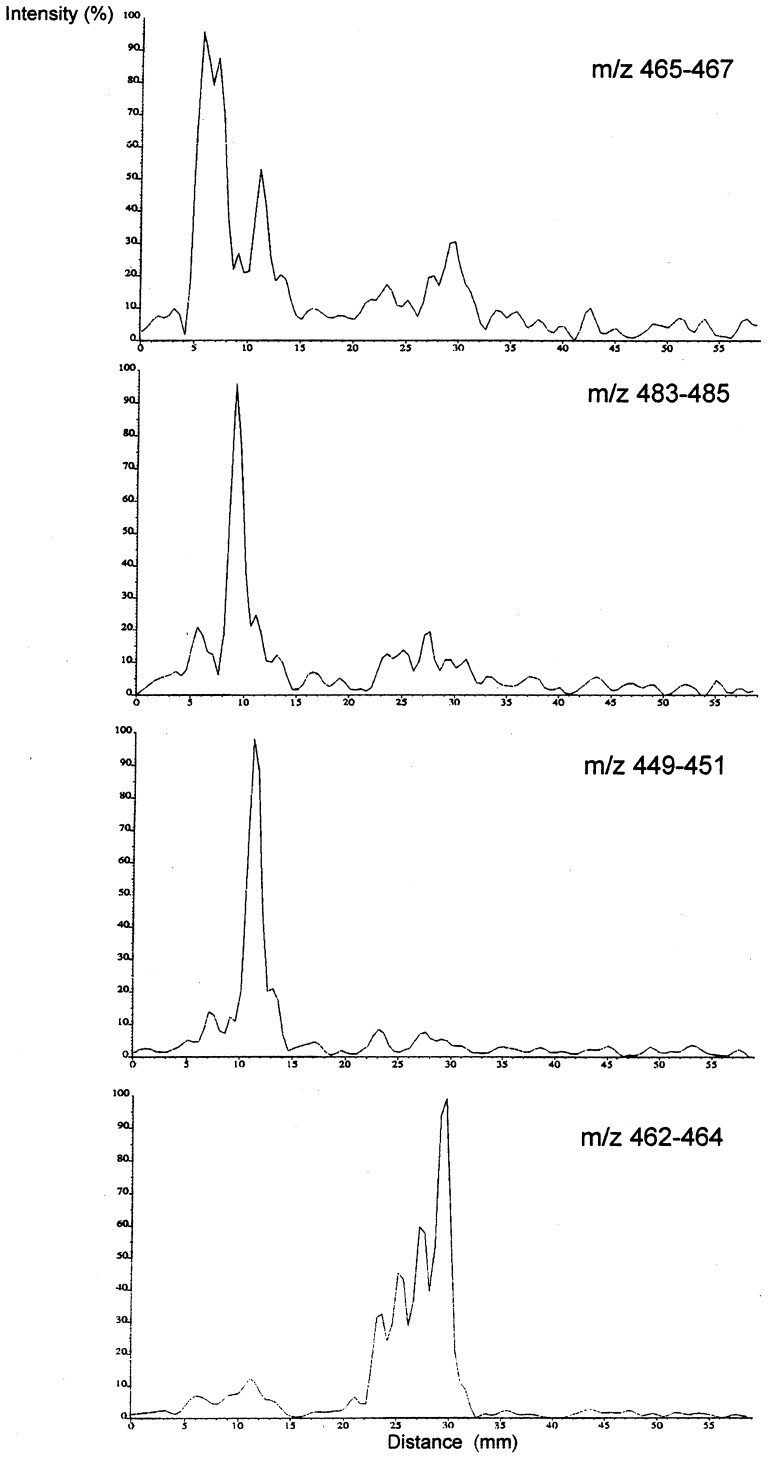


Fig. 5. Smoothed single ion mass chromatograms from the TLC–MALDI–TOF–MS analysis of OTC, CTC, TC and MC (10 μg per component).

signal response and no suppression of analyte signals and hence was used in further experiments.

Phe–Phe (3 m*M* in ethanol) was added to the graphite suspensions in order to recalibrate the TLC–MALDI mass spectra acquired during a run. This was achieved by use of the $[M+Na]^+$ and $[M-H+2Na]^+$ signals of the peptide as ''lock masses'' in the positive ion mode and by use of the $[M-H]^-$ signal in the negative ion mode. The mass accuracy and mass resolution achievable by internal recalibration with Phe–Phe was comparable with that obtained in similar TLC–MALDI–TOF–MS experiments employing DHB (Fig. 3 and also Fig. 4).

3.4. TLC–MALDI–TOF–MS of a mixture of OTC, CTC, TC and MC

A typical chromatogram obtained for a mixture containing 10 μg of OTC, CTC, TC and MC, using the mobile phase described earlier, showed one unresolved spot for both CTC and TC ($R_f = 0.17$). From reference runs it was established that CTC has an R_f value of 0.16 and TC one of 0.18. The TLC–MALDI–MS analysis of such a mixture confirmed this, as shown by the single ion mass chromatograms (Fig. 5). This demonstrates the potential of TLC–MALDI using a graphite suspension matrix for detecting unresolved analyte spots on a TLC plate. All four tetracycline antibiotics gave one characteristic fragment ion, $[M+Na-NH_3]^+$ in the TLC–MALDI mass spectra recorded. Hence the $[M+Na-NH_3]^+$ was used for each tetracycline antibiotic to construct the corresponding ion mass chromatogram (Fig. 5). The detection limit of tetracyclines in TLC–MALDI–MS employing graphite suspensions was found to be under 10 μg, however at lower levels the reproducibility was poor.

It should be noted that the present work was undertaken to demonstrate the applicability of particle suspension matrices to TLC–MALDI–TOF–MS. It is anticipated that improvements in sensitivity and reproducibility could be achieved by finding procedures with higher and more consistent extraction efficiency.

Increased in-source fragmentation of tetracyclines antibiotics was observed when particle suspension matrices were used, compared to crystalline organic matrices. The reasons for this are probably that higher peak temperatures are reached when particles are employed. Zenobi et al. [28] found that peak temperatures of 700–900 K could be reached in a few nanoseconds with 2 μm graphite–glycerol samples and Schürenberg et al. [4] has estimated that peak temperatures above 10 000 K are possible with 35 nm TiN particles.

4. Conclusion

The acquisition of chromatographic as well as mass spectral data from eluted TLC plates via TLC–MALDI–TOF–MS using different particle suspension matrices for the analysis of tetracyclines has been successfully demonstrated. A suspension of graphite (1–2 μm) in ethylene glycol was found to yield superior data to the other tested matrices and dispersants. The mass accuracy on graphite films was improved by adding Phe–Phe as ''lock mass'' to the graphite–ethylene glycol suspensions in order to allow internal recalibration of the acquired TLC–MALDI mass spectra.

Furthermore, it could be shown that the specific detection capabilities of TLC–MALDI–MS from graphite suspensions assisted in the analysis of the antibiotics CTC and TC in a mixture of OTC, CTC, TC and MC.

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