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Thin Layer Chromatography – Matrix Assisted Laser Desorption Ionisation – Mass Spectrometry of Pharmaceutical Compounds

Anna Christina Crecelius

Dipl. - Ing. (FH)

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The great things in this world is,
not so much where we are,
but in what direction
we are going.

Oliver Wendell Holmes

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Abstract

Thin-layer chromatography (TLC) is of great importance for the pharmaceutical industry as a simple, quick, and low cost analytical method. Considerable effort has been made over the past decades to combine the simplicity of TLC with the selectivity and sensitivity of mass spectrometry (MS) detection. In the pharmaceutical industry sensitivity is an especially important factor, since the allowed impurity level of most drugs is under 0.1%.

The aim of the present thesis was to develop methods for the direct examination of pharmaceutical compounds from TLC plates by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI TOF MS). The study was started by comparing several approaches for the application of the matrix for direct TLC-MALDI including a newly developed electrospray matrix deposition method. This new method was found to be superior to the other techniques studied. It produced a stable signal, minimised analyte spreading, and hence allowed the scanning of a TLC plate to obtain chromatographic as well as mass spectral data. The plotted mass chromatograms assisted in spot location, and allowed the calculation of R_f-values. These showed good agreement with the R_f-values determined by UV detection. The decrease in mass resolution and mass accuracy commonly observed in TLC-MALDI TOF MS due to the uneven nature of the silica gel layer was corrected by internal recalibration on selected matrix ions during the scanning of the TLC plate. To enhance the signals recorded directly from a TLC plate the use of an extraction solvent prior the matrix application was explored. Further improvements in sensitivity were obtained by modifying a robotic x-y-z axis motion system to act as an electrospray deposition device and by use of special Si 60 F_{254} HPTLC-MALDI targets. Using both approaches sensitivities in the high fmol range were obtained. To minimise matrix interference, which can suppress analyte signals, the application of suspensions of particles of different materials and sizes (Co-UFP, TiN, TiO_2, graphite and silicon) onto eluted TLC plates were investigated. The structural analysis of pharmaceutical compounds was achieved by post-source decay – matrix-assisted laser desorption/ionisation (PSD-MALDI) mass spectrometry performed directly on the separated spots. TLC-MALDI MS is not only applicable to the qualitative analysis of pharmaceutical compounds. The generation of quantitative data by using a structural analogue as an internal standard is also described. Different approaches to the incorporation of the internal standard into the TLC plate were tested. The most successful approach was to develop the TLC plate in the mobile phase to which the internal standard was added. Good accuracy, precision, linearity and sensitivity was obtained using this approach.

Contents

The pharmaceutical industry has produced hundreds of new compounds for the market over the last decade. This development would certainly not have been possible without the corresponding improvements in analytical techniques, e.g. of vibrational spectroscopy (Raman and IR), column chromatography (GC and HPLC), planar chromatography (TLC and HPTLC), and hyphenated techniques of column or planar chromatography with mass spectrometry (MS).

Thin-layer chromatography (TLC) is of great importance for the pharmaceutical industry as a simple, quick, and low cost analytical method [reviewed in 1-5], particularly in three areas: (i) research and development of new drugs, (ii) manufacturing process of drugs, and (iii) detection of residues and monitoring of poisoning cases [6]. As an off-line method TLC offers the possibility of analysing many samples simultaneously, and hence is often used in routine investigations. Even though high performance liquid chromatography (HPLC) is usually conducted as a complementary technique, with TLC the chances of observing the presence of unknowns is greater. In column chromatography unknowns might be missed due to non elution from the stationary phase or migration within the solvent front. TLC is also commonly performed as a cleanup step, which is simultaneously carried out with the separation, offering a further advantage to this relatively low technology technique.

To improve the sensitivity and to allow chemical identification of the compounds, considerable effort has been made over the past decades to combine TLC with MS [reviewed in 7-10]. Especially in the pharmaceutical industry sensitivity is crucial, since the allowed impurity level of most drugs is under 0.1% [11].

The whole range of modern ionisation techniques has, at one time or another, been used for coupling TLC to MS. From this whole range, there are essentially two modes of TLC-MS, which can be broadly categorised as either indirect or direct methods. In indirect methods, the analyte is removed from the TLC plate by scraping it off with the stationary phase, followed by extraction into a suitable solvent for MS analysis. In direct methods the TLC plate, or a large portion of it, is left undisturbed and introduced into the mass spectrometer for analysis. This increases the information available from the TLC plate. Chromatograms or images of the analyte spots on the TLC plate can be obtained. Furthermore, chromatographically overlapping analyte spots with different molecular weights can be fully resolved by MS detection.

The following ionisation techniques have been applied to direct TLC-MS methods: fast atom bombardment (FAB) [12], liquid secondary ion mass spectrometry (LSI MS) [13], laser desorption (LD) [14] and matrix-assisted laser desorption/ionisation (MALDI) [15-25]. TLC-FAB MS and TLC-LSI MS normally require the use of a vacuum stable liquid matrix, e.g. glycerol. The liquid matrix provides extraction of the analyte from inside the TLC plate towards the top of the TLC plate where it can be desorbed during the analysis. In addition, the liquid matrix aids in soft ionisation of the analyte. However, liquid matrices cause analyte spreading in the plane of the TLC plate. TLC-LD MS is performed without the addition of a liquid matrix due to the greater penetration depth of a laser beam compared to an atom or ion beam. Even though TLC-LD MS provides high sensitivity and spatial resolution, it suffers from poor signal reproducibility and significant molecular fragmentation [15]. The latter disadvantage can be overcome by using an IR laser for desorption and an UV laser for ionisation, but this technique requires complex instrumentation [26]. The coupling of TLC with MALDI can potentially overcome these drawbacks. TLC-MALDI MS has been shown to be useful for the analysis of a variety of biopolymers, including peptides and proteins [15-18], nucleotides [19], glycosphingolipids [20], lipopolysaccharides [21], and oligomers [22]. Low molecular weight compounds, such as dyes [16-17], drugs [23], metal acetylacetomates [24], and pesticides [25] have been also investigated. In recent years the interest in the use of TLC-MALDI MS for the direct analysis of TLC plates has increased [27].

The aim of the present thesis was to develop methods for the analysis of pharmaceutical compounds by direct TLC-MALDI TOF MS. The thesis is divided in ten chapters. *Chapter 2* gives an overview of the evolution of TLC-MS, involving the different ionisation techniques employed, with special emphasis on MALDI mass spectrometry. Six main chapters follow, in which several issues for successful TLC-MALDI coupling are addressed:

Chapter 3 is concerned with the method used for the deposition of the MALDI matrix onto the TLC plate. The matrix facilitates the ionisation of the analyte molecules, separated on the TLC plate. The application of the matrix directly onto the TLC plate has to be done in such a manner that analyte spreading along the silica gel layer is avoided. Thus, the chromatographic integrity of the analyte spots is maintained. Several approaches for the application of the matrix for TLC-MALDI coupling have been

compared, including a newly developed electrospray deposition method. This electrospray method was found to be superior to the other techniques studied. It produced a stable signal, minimised analyte spreading and hence allowed the scanning of a TLC plate to obtain chromatographic as well as mass spectral data.

Chapter 4 discusses the requirement for efficient extraction of the analyte from the interior of the silica gel layer to increase the sensitivity. To enhance the signal recorded directly from a TLC plate the use of an extraction solvent prior matrix application was investigated. The porosity of the silica gel layer can also lead to problems. Ions starting from different points on the surface can have a slight variation in their flight times, and hence a decrease in the mass resolution of spectra recorded is observed, when a TOF analyser is employed. The mass measurement inaccuracies, typically obtained in TLC-MALDI TOF MS analysis, were corrected by internal recalibration on selected matrix ions during the MS scan.

In *chapter 5* the generation and characterisation of an impurity profile of a pharmaceutical compound by TLC-MALDI TOF MS at the 0.1% level is reported. Mass chromatograms could be constructed down to 2.4 pmol spots of the impurity. Further improvements in sensitivity were achieved by using a special Si 60 F_{254} HPTLC-MALDI target. Using this special target mass spectra in the high fmol range were obtained.

For the analysis of low molecular weight compounds, such as pharmaceuticals, strong matrix peaks can interfere with the analyte signal. *Chapter 6* presents a study of the minimisation of such matrix interference by applying suspensions of different materials and sizes (Co-UFP, TiN, TiO_2, graphite, and silicon) onto eluted TLC plates.

The combination of TLC with MALDI is not only applicable to the qualitative analysis of pharmaceuticals. *Chapter 7* describes the generation of quantitative data by using a structural analogue as an internal standard. Different approaches to the incorporation of the internal standard into the TLC plate were investigated and are presented. The most successful approach was to develop the TLC plate in the mobile phase to which the internal standard was added. Good accuracy, precision, linearity and sensitivity could be obtained using this approach.

In *chapter 8* the use of post-source decay (PSD) – MALDI, directly performed on TLC plates, is presented as a method to aid the identification of compound spots. Two matrices were tested for the TLC-PSD MALDI analysis of several small drug molecules, the organic matrix α-cyano-4-hydroxy cinnamic acid (α-CHCA) and the particle suspension matrix graphite.

The results presented in chapters 3 to 8 are finally concluded in *chapter 9* and some possible suggestions for future studies were made.

1.1 References

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2.1 Thin-layer Chromatography - Mass Spectrometry

2.1.1 Introduction

Thin-layer chromatography (TLC) combined with mass spectrometry (MS) has a long and distinguished history dating back to the late 1960s, where much of the work involved scraping off the separated sample spot from the support (plastic, glass or aluminium backing) and subsequent analysis by MS (with or without extraction of the adsorbent). A detailed summary of the earlier TLC-MS work, including this indirect or off-line approach, can be found in the review by Busch [1].

A wide range of compounds, including small molecular weight compounds (drugs and metabolites, porphyrines, surfactants, toxins and dyes) and biomolecules (peptides, lipids, bile acids, oligosaccharides, polysaccharides and glycosides), have been analysed by TLC-MS. The whole range of ionisation techniques, has at one time or another, been used for the MS detection of TLC spots in a direct or indirect approach. An overview of the ionisation techniques applied to TLC-MS is given in **Figure 2-1**.

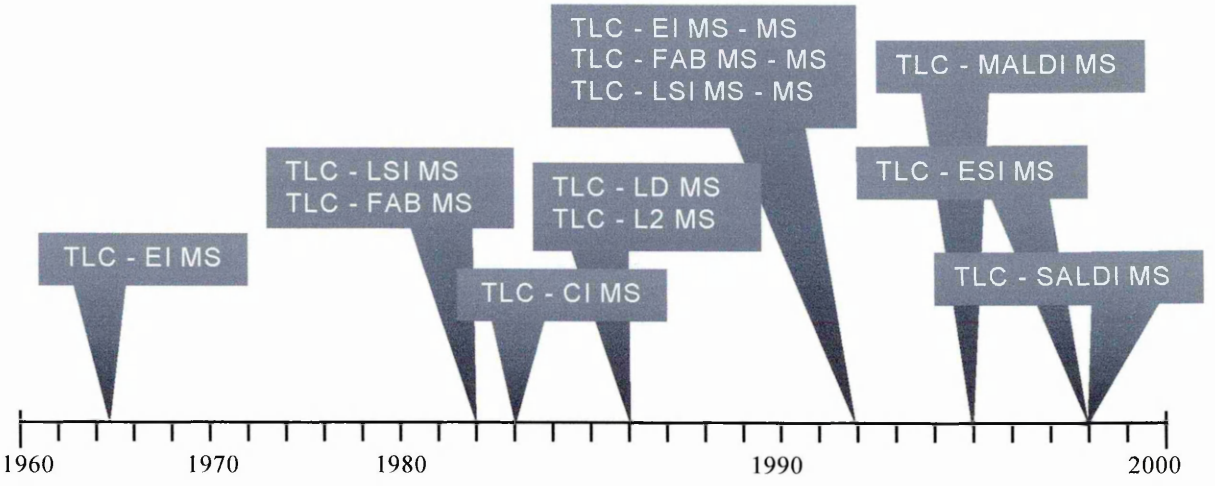

Figure 2-1. Evolution of ionisation techniques in TLC-MS.

Here only the recent developments and applications will be described on the basis of the ionisation methods employed. For a more detailed coverage of this topic the reader is directed to several reviews [2-4].

2.1.2 Ionisation Techniques

TLC-EI MS. The use of the classical ionisation technique electron ionisation (EI) [5-7], in conjunction with the "traditional" scrape and elute method in TLC-MS, is generally limited to the analysis of relatively non-polar and thermally stable compounds. The reasons are that polar involatile compounds have a strong adsorption to the silica gel layer, and hence high temperatures are required to evaporate them, and at these temperatures they decompose. One possibility to overcome this problem is the use of less adsorbent phases, such as polyamide TLC plates [8].

Recently Brzezinka *et al.* [9] have described the screening of biological samples for drugs and metabolites by indirect TLC-EI MS. The appropriate zone of the silica gel TLC plate was scraped off and extracted in methanol. The extracts were then transferred to the direct insertion probe of the mass spectrometer. Evaporation of the drug molecules were accomplished by a source temperature of 200°C. The gas-phase molecules were ionised by collision with unusually energetic electrons (100 eV), emitted from a heated filament. Typically, the radical cation $M^{+\bullet}$ as well as several fragment ions of the drug molecules were present in the recorded mass spectra. The formation of fragment ions was caused by the release of the excess energy of the radical cations.

TLC-CI MS. Chemical ionisation (CI) [10-11] combined with TLC is considered as an alternative to TLC-EI MS for the analysis of volatile samples. However, TLC-CI MS has not been widely used. The most comprehensive work in this area has been reported by Ramaley *et al.* [12-13], introducing a TLC-MS scanner. The instrument was based on thermal or laser desorption of analytes, separated or spotted on a TLC plate, into a gas stream, which carried the sample molecules into the ion source of a quadrupole mass spectrometer. Methane was used as CI reagent gas to form protonated molecules $[M+H]^+$ of the sample by proton transfer reactions between the highly acidic reagent gas CH_5^+ ions and the neutral sample molecules. The TLC plate was moved at a constant speed using stepper motors to enable the evaporation of all analyte spots present on the plate. Only low resolution chromatograms were obtained by this approach, due to redistribution of desorbed material within the TLC plate.

TLC-LSI MS and TLC-FAB MS. The sputtering techniques, liquid secondary ionisation (LSI) [14-15] and fast-atom bombardment (FAB) [16-17] have been coupled to TLC to avoid the separate and discrete evaporation step, necessary in TLC-EI MS and TLC-CI MS. The sample molecules are transferred directly from the condensed-phase into the gas-phase, enabling the analysis of polar, involatile, thermally unstable compounds, e.g. biomolecules. A variety of reports, including indirect and direct coupling methods by means of one- or two-dimensional images, are described in the literature [3].

Chang *et al.* [18] have described a TLC-FAB MS method that involved the transfer of an analyte spot within the silica gel layer to a FAB probe tip. After the TLC separation, the TLC spot was localised with UV fluorescence and removed from the backing by pressing the probe tip, covered with double-sided tape, onto the silica gel layer, as shown in **Figure 2-2.**

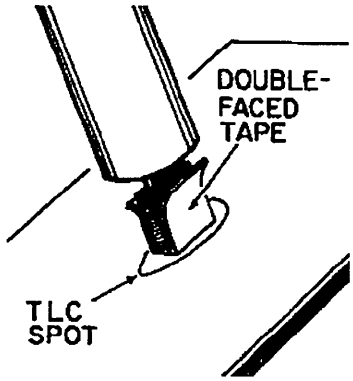

Figure 2-2. Transfer of the TLC spot to the FAB probe tip [18].

To extract the analyte from the absorbent and to aid in soft ionisation, a small amount of the FAB matrix, glycerol, was added to the removed spot. Desorption and ionisation of the sample was accomplished by irradiation with a fast neutral beam (6 kV xenon atoms). The exact mechanisms of the ion formation is not clear, however proton-transfer reactions are believed to play an important role [19]. The mass spectra of the antibiotic samples showed protonated molecules $[M+H]^+$, as well as $[M+Na]^+$ and $[M+K]^+$ adducts. The matrix glycerol employed gave high background signals in the low mass range. However, no interference with the analyte signals were reported.

To enable the trace analysis of tetracyclines in bovine tissues and milk by TLC-FAB MS, Oka *et al.* [20-22] developed a technique to concentrate the analyte in the spot into a smaller area. The so called "trapezoid condensation technique" involves scraping a

trapezoidal shape around the TLC spot followed by the application of the polar solvent methanol, causing the migration of the analyte towards the tip of the trapezoid. The procedure is presented in **Figure 2-3**. A FAB matrix is then applied to the condensed zone to enable direct MS analysis.

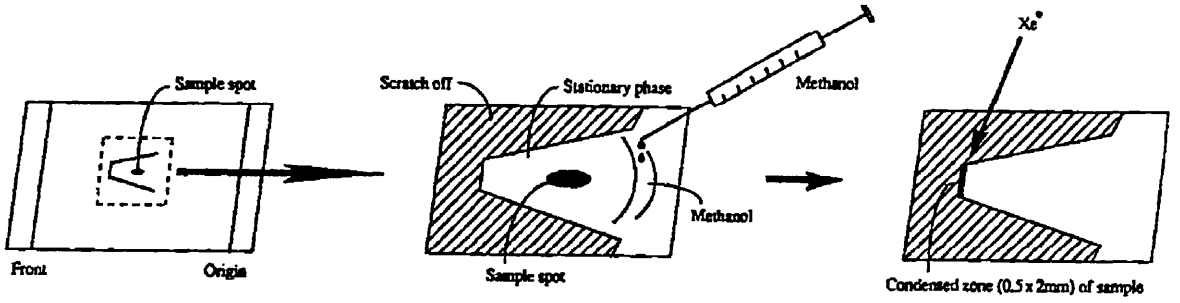

Figure 2-3. Procedure of the "trapezoid condensation technique" [20].

The acquisition of one-dimensional images of developed TLC plates by TLC-FAB MS was reported by Tamura *et al.* [23]. The insertion probe inlet of the mass spectrometer was modified to hold a 10 mm × 65 mm TLC plate, as shown in Figure **2-4**.

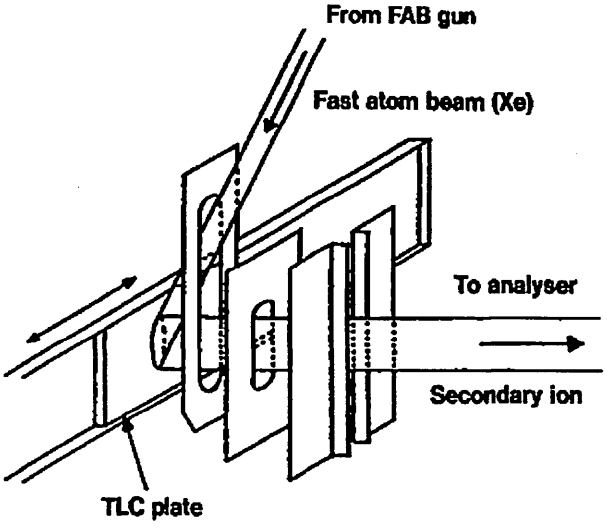

Figure 2-4. Design of the modified insertion probe inlet of a FAB mass spectrometer, allowing the scanning of a TLC plate in one dimension [23].

Since the incident particle beam was fixed, the various spots on the TLC plate were subsequently brought into focus by stepper motors, which moved the TLC plate along the y-axis. The obtained chromatograms exhibited reduced resolution due to diffusion

processes of the analyte caused by the liquid matrix; particularly if analysed a long time (10 minutes) after application.

A similar approach was reported by Nakagawa and Iwatani [24] for the direct TLC-LSI MS determination of several drug metabolites. The design of the movable direct insertion probe was similar to the type described by Tamura *et al.* [23]. The only obvious difference was the particle beam used for the deposition and ionisation process; an ion beam (cesium ions) was used, instead of a neutral beam.

Besides one-dimensional imaging, TLC-LSI MS has been successfully applied to record two dimensional images of a variety of compounds, including drugs, peptides, bile acids, diuretics and alkaloids [reviewed in 1, 25-27]. Busch *et al.* [27] first introduced such a mass spectrometer enabling the scanning of a TLC plate in the x- and y-axis. For the collection of the images in most experiments a gallium ion gun was employed due to its small beam diameter (1 μm). Thus, the spatial resolution could be readily improved compared to a cesium ion gun (20 μm) or an argon FAB gun. To minimise the limitation in the spatial resolution, arising from the liquid matrix, the phase-transition matrix threitol (melting point: ~ 71°C) was tested [26]. The matrix was applied to the TLC spot by means of spray deposition. The energy of the primary ion beam in the mass spectrometer was sufficient to melt threitol precisely at the point of analysis, therefore providing a spatially discrete extraction.

TLC-LD MS. Another desorption-ionisation technique, which also allows the analysis of polar thermally unstable substances, is laser desorption (LD) [5-6]. Coupling to TLC has the advantage that no liquid matrix is required, and hence the spatial resolution for imaging of TLC plates is only limited by the incident laser beam. The imaging capabilities of such a technique has been shown for triphenylmethane dyes, distributed on a polymer membrane, using a laser-desorption based microprobe (LAMMA) [28]. The LAMMA instrument was equipped with a ultraviolet (UV) laser, focused to a spot size of 5 μm. The mass analysis was accomplished in a TOF analyser. The ions are believed to be desorbed and ionised with the UV laser due to excitation processes and individual electronic and photochemical reactions [29]. A thermal process was also suggested by transfer of the energy through substrate absorption followed by heating up of the sample layer [30]. The recorded mass spectra were dominated by molecular cations $M^{\bullet+}$ of the dye molecules and several fragment ions.

The ionisation of a range of low molecular mass organic compounds directly from polyamide TLC plates was reported with this microprobe [31]. However, the polyamide stationary phase caused background ions in the low mass range of the recorded mass spectra. Furthermore, a high degree of fragmentation was obtained, due to the high laser power required for desorption of the analytes from such a material. The described fragmentation combined with poor reproducibility [32] encouraged the development of two-step laser mass spectrometry (L2 MS) for the surface analysis of TLC plates, since it also offers the advantage of no sample preparation.

TLC-L2 MS. The detection of various thermally labile biological molecules from silica gel TLC plates has been presented by Li and Lubmann [33] using L2 MS. In L2 MS the desorption and ionisation process is separated from each other. In the first step (desorption step), an infrared (IR) laser pulse is employed to ablate the analyte with the silica gel from the TLC support. The desorbed material is entrained in a supersonic jet of CO_2 and transported towards a time-of-flight (TOF) mass analyser. The supersonic jet has three main functions [33]: (i) transport of the desorbed species away from the TLC plate to prevent sample condensation; (ii) cooling of the desorbed neutral molecules to avoid thermal decomposition, and (iii) increase in mass resolution of the recorded spectra by minimisation of the energy spread. The last point will be explained in more detail in section 2.2.4 of this thesis, where TOF mass spectrometry is discussed. In the second step (ionisation step), a pulse from a tuneable UV laser is used for resonance-enhanced multi-photon ionisation (REMPI) [5] of the neural gaseous molecules. In the resonance-two-photon ionisation (R2PI) [34], which takes place, the gaseous molecules in the ground-state, absorb one photon to reach their first excited-state. When they absorb a second photon, their internal energy is above the ionisation potential (IP) and dissociation of an electron occurs, resulting in the formation of radical cations. This soft ionisation scheme prevents extensive fragmentation, and therefore the recorded spectra are dominated by molecular ions, of those compounds that strongly absorb the selected ionisation wavelength. Since the ionisation wavelength is tuneable, it is possible to minimise the background level caused by the silica gel and to detect selectively analytes in unresolved TLC spots. The application of this technique has also been demonstrated for the determination of the drug naproxen [35] and the construction of one-dimensional images of small peptides, developed on a TLC plate [36].

TLC-MS-MS. Tandem mass spectrometry (MS-MS) [5-6, 37] applied in combination with TLC offers the advantage that structural information of the compounds of interest can be obtained and that the background noise is eliminated. In TLC-FAB MS and TLC-LSI MS the background noise is typically obtained due to the use of a liquid matrix, and in TLC-LD MS arising from the material of the TLC plate. A wide range of compound classes, including drugs and their metabolites, polymer additives, and glycolipids have been investigated in a direct or indirect approach [reviewed in 38]. For those compounds, which were not amenable by EI, FAB or the related ionisation technique LSI was used. In the TLC-MS-MS experiments a molecular ion species of the analyte was selected in the first mass analyser, and thus separated from the interfering background ions. Excitation of the selected molecular ion species occurred in a collision cell with neutral gas molecules (usually He, Ar or Xe). The dissociated ions were then analysed in a second mass analyser. The only disadvantage of the tandem MS analysis was the higher sample quantity required compared to single stage TLC-MS.

TLC-MALDI MS. Matrix-assisted laser desorption/ionisation (MALDI), as a technique to ionise analytes separated on a TLC plate, has been examined by several groups in order to compensate for the limitations of the previously discussed ionisation methods. In comparison to LD, the sample preparation by MALDI involves the use of a matrix. However a greater versatility in the MS analysis is provided, by enabling the formation of larger ionic species from thermally labile non-volatile compounds with nearly no fragmentation. The fundamentals of MALDI mass spectrometry are separately discussed in section 2.2 of this chapter. Here, only the important features of direct TLC-MALDI coupling are covered. For the indirect TLC-MALDI approach (scrape and extract technique) the reader is directed to two recent publications [39-40].

As mentioned in the introduction (chapter 1) of this thesis, the challenge of direct TLC-MALDI coupling is the application of the matrix solution onto the TLC plate without causing planar diffusion. Several possible application procedures are described in the literature, and as the reader will see in chapter 3 of this thesis, have been investigated for their suitability in order to obtain chromatographic data.

In the original work, by Hercules and co-workers [32], the matrix solution was directly deposited on the separated TLC spot, using a pipette or syringe. Before matrix application, an extraction solvent was applied to the TLC spot to move the analyte

molecules to the surface, thus enhancing the sensitivity. Once matrix and analyte were co-crystallised, portions of the TLC plate were introduced in the mass spectrometer for analysis. Although the technique enabled the construction of two-dimensional images, the shape of the TLC spots were altered due to spot diffusion, caused by the matrix deposition. Hence, a new technique [41] was introduced, which is presented in **Figure 2-5**.

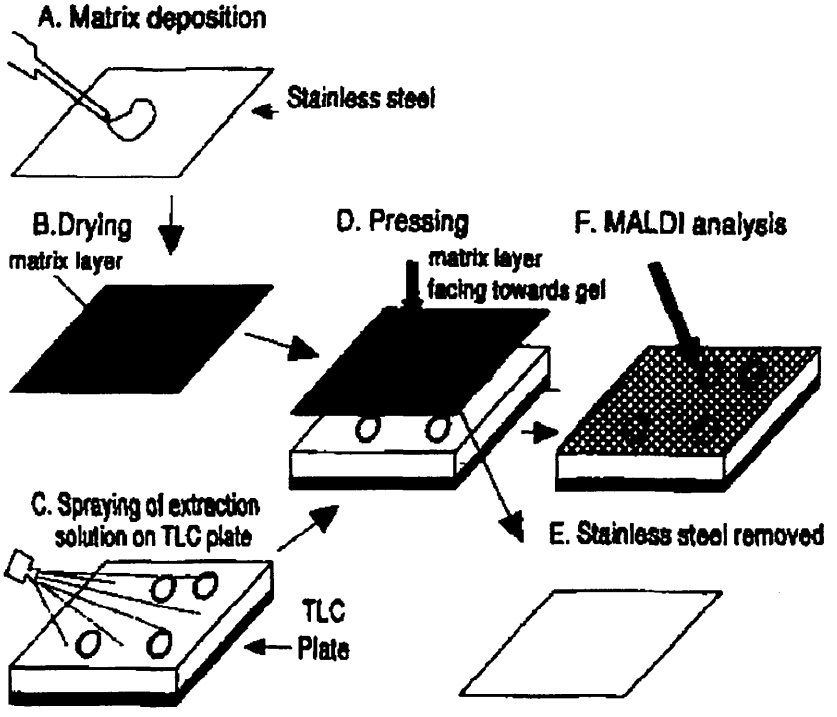

Figure 2-5. Matrix deposition for direct TLC-MALDI analysis [41].

The new, very complex, technique involved pressing a previously prepared layer of matrix crystals into a pre-wetted TLC plate. The selection of the extraction solvent was based on its retardation factor (R_f) [42]. A high R_f value appeared to correlate to a high extraction efficiency. The signal intensity of a selected analyte peak, recorded from a TLC plate, showed typically a maximum at mid R_f values, since too high extraction efficiencies caused analyte spreading. Several follow up studies were carried out, **including** the qualitative determination of pesticides [43], carcinogen DNA adducts **[44], and** the quantitative analysis of drugs [45].

Since sensitivity is an important factor for the usefulness of an analytical method, the same group has recently presented a TLC-MALDI hybrid plate [46]. The use of this

special plate enabled, according to the authors, the recovery of 100 % of the deposited or developed analyte spot. This was achieved by eluting the separated analyte spot to an area, where the silica gel layer was removed and a matrix layer was created. With this hybrid plate the detection of the analyte in the rough and polar silica gel absorbent was avoided, and hence detection limits in the low femtomol range were obtained. Another approach, for improving the sensitivity in TLC-MALDI, is shown in **Figure 2-6.**

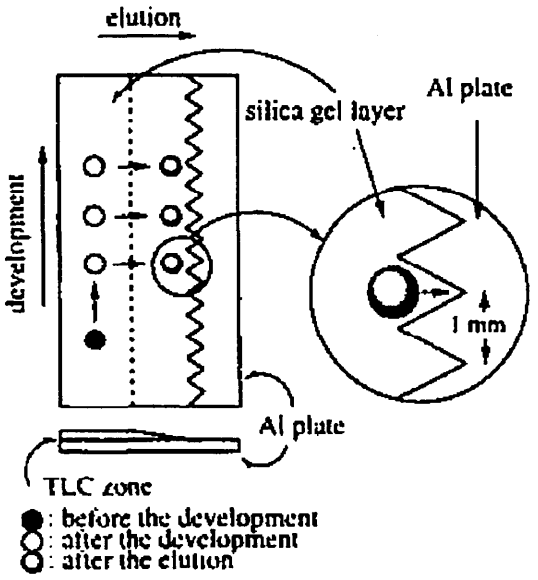

Figure 2-6. Pre-concentration of analyte spots for direct TLC-MALDI coupling to enhance sensitivity [47].

Matusumoto *et al.* [47] pre-concentrated the analyte spot by scraping off the silica gel layer on one side of the TLC plate and development of the TLC plate in a second polar solvent, after a 90° rotation of the plate (an approach similar to two-dimensional TLC). Thus, the analyte migrated towards the edge of the silica gel layer and was simultaneously concentrated. This technique shows similarity to the "trapezoid condensation technique", developed by Oka *et al.* [20]. Finally for MALDI analysis the matrix was directly applied on the edge of the silica gel layer at the position of the analyte.

Direct TLC-MALDI mass spectrometry has not only been applied to model compounds, "real" samples, such as the identification of unknowns in a synthesised organic mixture [48], or biological material such as glycosphingolipids [49] or lipopolysaccharides [50], have also been successfully analysed. In the two latter applications, the biological samples were not only directly detected on the TLC plate, but also after heat transfer to

a membrane. The sample preparation steps for the so called TLC blotting-MALDI MS experiments is explained in **Figure 2-7**. After the TLC plate was wetted in an extraction solution, the TLC spots were transferred to the membrane by employing heat.

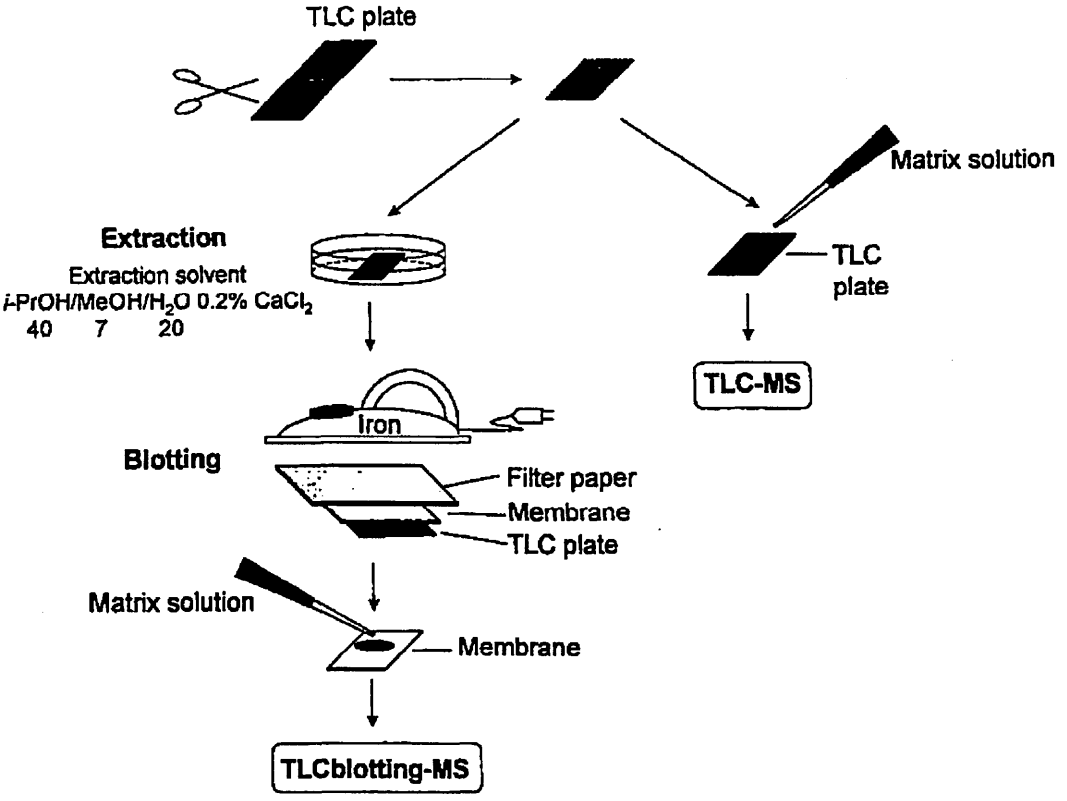

Figure 2-7. Sample preparation for TLC-MALDI MS and TLC blotting-MALDI MS experiments [49].

The direct TLC-MALDI MS experiments presented so far have all been obtained by employing a TOF analyser. Recent developments in MALDI have introduced the possibility of mass analysing ions, formed in an external ion source, in an ion trap or fourier transform ion cyclotron mass spectrometer. Further information about the different types of mass analysers used for MALDI, can be found in section 2.2.4 of this chapter. TLC-MALDI analysis of dyes in an ion trap mass spectrometer [51], as well as the determination of gangliosides in a fourier transform instrument [52] have been demonstrated.

TLC-SALDI MS. One disadvantage of using a matrix in TLC-MALDI is the matrix related background, occurring in the low mass range (below 500 m/z). Attempts to reduce these inference signals were reported by Cheng *et al.* using surface-assisted laser desorption/ionisation (SALDI) coupled to TLC [53-57]. In this technique the

appropriate zone of the TLC plate was in general covered with carbon particles (graphite, or carbon activated particles), suspended in glycerol, and introduced in the mass spectrometer, where the sample was irradiated with a UV laser. Using this methodology a variety of low molecular weight compounds could be detected, for example peptides [53-54], organic reactions products [55], polymers [56], and porphyrine derivatives [57]. A more detailed description of this technique can be found in chapter 6 of this thesis, where a similar approach was chosen for the analysis of tetracyclines.

TLC-ESI MS. Electrospray ionisation (ESI) [5-7, 58], another fairly new soft ionisation technique, was recently used for the detection of analytes, separated by reversed-phase TLC [59]. The design of the TLC-ESI interface is shown in **Figure 2-8.**

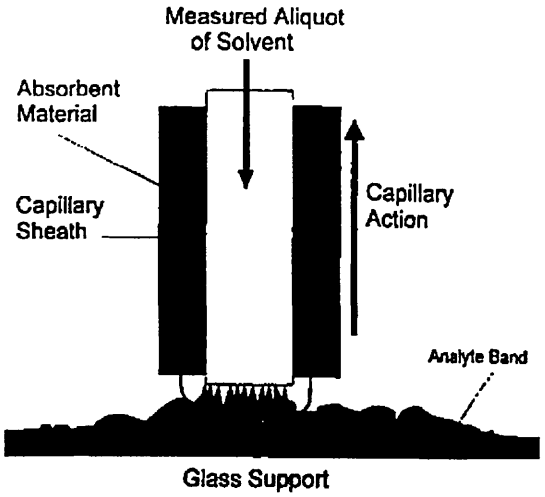

Figure 2-8. TLC-ESI MS interface [59].

The extraction solvent (methanol-water) was delivered through the central capillary tube to the analyte spot on the TLC plate. After a short time, the solvent extract was absorbed by capillary action in the absorbent material, placed in a further tube, surrounding the solvent delivery capillary. For mass spectrometric analysis, the analyte was extracted from the absorbent with an aliquot of solvent and directly injected in a triple-quadrupole mass spectrometer. The fine needle, which was held at a high potential (3-5 kV) produced a spray consisting of charged solvated aerosol particles, which evaporated and reduced their size till they were unable to "hold" the electrical charge on the rapidly decreasing surface area. The solvent clusters of the resulting single ions were removed by heating to 80°C. After ionisation of the sample molecules at atmospheric pressure,

they passed several skimmers entering the high vacuum system of the analyser. Since the recorded mass spectra showed several abundant ions in the low mass range, produced from the material of the TLC plate, which was also extracted, tandem MS was performed on the protonated analyte species. To minimise the spreading of the analyte during the extraction step, a hydrophobic barrier by means of melted wax was tested.

A newer TLC-ESI interface directly pumps the analyte extract in the ESI mass spectrometer [60-61]. The described extractor works similar to a HPLC pump valve and forms a strong sealing to the TLC support by cutting the absorbent layer. Qualitative and quantitative data were obtained with this set-up. Both TLC-ESI interfaces are at an very early stage and seem rather complex.

2.2 MALDI Mass Spectrometry

2.2.1 Introduction

The introduction of matrix-assisted laser desorption/ionisation mass spectrometry (MALDI MS) as a soft ionisation technique in the late 1980s by Karas and Hillenkamp [62-64] and by Tanaka *et al.* [65] has greatly helped to bring mass spectrometry into biomolecular science. MALDI MS enables the mass analysis of large, non-volatile and thermally labile compounds, such as proteins, oligonucleotides and synthetic polymers [66]. In the thirteen years since its introduction MALDI has become a standard method for the mass spectrometric analysis of biological macromolecules [67-68], due to its high sensitivity (attomole-range) [69], the accessible mass range (above 300 kDa) [66] and improvements in the resolution of time-of-flight (TOF) analysers [70-71]. Although MALDI is a powerful technique for the production of gas-phase ions from large molecules, the ion formation pathway remains still not fully understood. In a typical MALDI analysis, the analyte of interest is co-crystallised with an excess of a solid matrix, which has a strong extinction coefficient at the wavelength of the laser and hence directly absorbs the laser energy. The laser pulse induces desorption and ionisation processes of matrix and analyte molecules in a very dense plume, as shown in **Figure 2-9**.

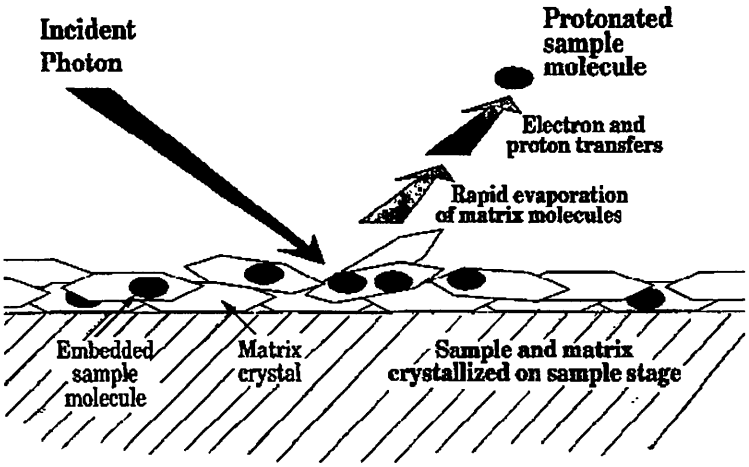

Figure 2-9. Schematic representation of the desorption/ionisation process. Matrix molecules absorb the laser energy, volatilise explosively and liberate the analyte ions to form the MALDI plume [72].

The ions formed in the MALDI plume are extracted by a strong electric field into the field-free region of the TOF analyser, where they are separated according to their mass-to-charge (m/z) ratio. Depending on the polarity of the electric field, positive or negative ions are extracted. Each ion induces a current at the detector, which is then monitored as a function of time yielding a mass spectrum. Several tens of mass spectra are summed up to improve the signal-to-noise ratio.

In the following sections, the experimental factors effecting the outcome of a MALDI spectrum are presented and the current knowledge on desorption-ionisation processes in the MALDI plume are discussed. A brief overview of the basic instrumentation currently available for TOF mass spectrometry is also given. This should give the reader an insight into the great diversity of MALDI TOF mass spectrometry.

2.2.2 Experimental Parameters in MALDI Mass Spectrometry

The number of parameters influencing the outcome of a MALDI experiment is enormous. Here, only the laser wavelength (A), the choice of matrix (B), and the sample preparation technique (C) are discussed, since they are considered to be the most significant [73].

A. Laser wavelength. The laser wavelength is a particularly important parameter in MALDI. Traditionally MALDI uses ultraviolet (UV) fixed frequency lasers

with nanosecond pulses. In the original work, Karas and Hillenkamp [62] employed a frequency-quadrupled Nd:YAG laser at 266 nm to detect proteins with molecular masses above 10 kDa embedded in nicotinic acid. Later, lasers emitting at 337 nm (nitrogen laser) and 355 nm (frequency-tripled Nd:YAG laser) [74] were used. Although all UV laser wavelengths have been successfully applied to MALDI MS, nitrogen lasers are found in most commercial instruments, since they are reliable and inexpensive. A nitrogen laser was also used in this work. In a parallel development, lasers in the infrared (IR) wavelength range have been applied: Er:YAG lasers emitting at 2.94 μm [75] and CO_2 lasers, emitting at 10 μm [76]. The introduction of tuneable free-electron lasers (FEL) [77] and optical parametric oscillator (OPO) based systems [78] have made the use of IR-MALDI very attractive, since they may be used in conjunction with ice as the matrix. The visible (VIS) wavelength range is not often used in MALDI due to a limited choice of good matrices. Dye compounds, e.g. rhodamine 6G [79] or graphite plates [80] have been shown to be effective, when a frequency-doubled Nd:YAG laser (532 nm) is employed.

B. Matrix. In addition to the selection of the laser wavelength, the matrix plays a key role in MALDI mass spectrometry. The functions of the matrix are characterised as follows [64, 81]:

- The high molar excess of the matrix (typically in the range of 1000 fold) provides separation of analyte molecules from each other, and therefore reduces the intermolecular interactions between individual analyte molecules.
- The energy deposition into the analyte molecules occurs through the matrix, thus the matrix must show a strong resonance absorbance at the applied laser wavelength. (In the case of particle suspension matrices, this is discussed extensively in chapter 6 of this thesis.)
- The matrix promotes analyte ionisation. (This will be described in detail in section 2.2.3 of this chapter.)

Besides this three properties, further requirements of the matrix, such as vacuum stability and compatible solubility with the analyte, are necessary. It is impossible to predict *a priori* if a compound is a good matrix or not, due to the limited understanding of the desorption-ionisation process. Thus, the discovery of new matrix materials with the desired properties has been a matter of empirical search [73]. There are several

compounds, which have been used as MALDI matrices, however only a few compounds have established themselves as "common matrices". Frequently used UV matrices are derivatives of benzoic acid, e.g. 2,5 dihydroxy benzoic acid (DHB) [82-84], and derivatives of cinnamic acids, e.g. α-cyano-4-hydroxy cinnamic acid (α-CHCA) [85]. For the analysis of oligonucleotides, 3-hydroxy picolonic acid (3-HPA) [86] is a potential matrix. Glycerol [87], succinic acid [88] and water [89] have been proven as useful IR matrices. The structures of some common UV and IR matrices, including those used in this work, are shown in **Figure 2-10**.

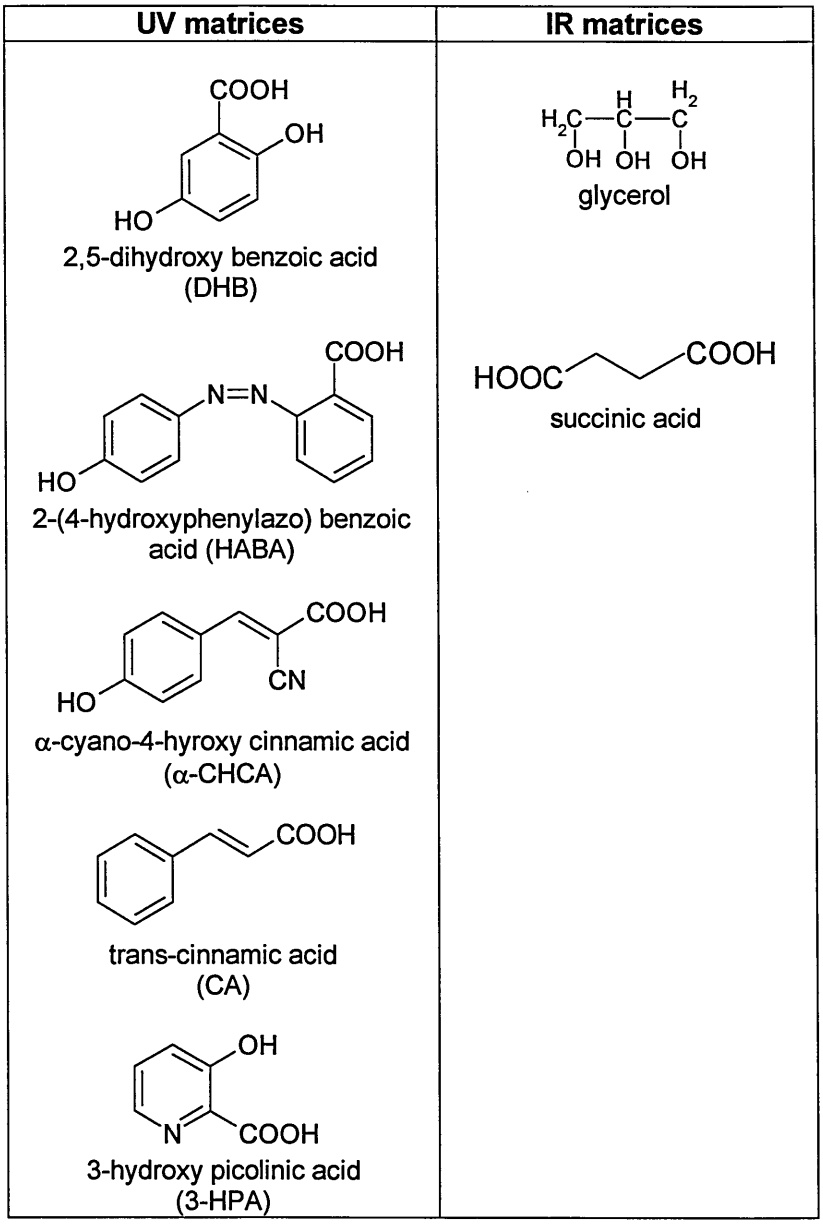

Figure 2-10. Structures, chemical names and abbreviations of common MALDI matrices. The abbreviations are usually used throughout this thesis.

The mass spectral quality can often be improved by addition of a low molecular weight compound to the analyte-matrix mixture. On examination of the physio-chemical properties of these compounds, one can roughly distinguish between two different groups. The first group is embodied by substances, like fructose [90] or diamonium hydrogen citrate [91], which show no properties required for a matrix compound and hence are called as "matrix additives" or "co-matrices". Fructose for example improves the mass spectral quality by decreasing the fragmentation rate of peptides and proteins. It is believed that the laser energy causes the pyrolysis of fructose molecules, which then absorb excess energy causing cooling during the desorption-ionisation process [92]. A very well known representative of the second group is "super DHB" (DHBs), which is a mixture of 2,5-dihydroxy benzoic acid (DHB) and 2-hydroxy-5-methoxy benzoic acid (HMB) in the volume ratio 9:1 [93]. HMB is a compound, which has the properties of a matrix, and hence the resulting mixture of these two matrices is named a "binary matrix system". In principal more then two matrices can be mixed together, however each compound added increases the possibility of interference with analyte signals in the low mass range.

C. Sample Preparation Technique. A variety of different sample preparation techniques have been developed, since the introduction of MALDI mass spectrometry [62-65]. A summary of the most common sample preparation methods is given in **Table 2-1**.

Table 2-1. Different sample preparation techniques applied in MALDI mass spectrometry.

Solid matrix	Dried-droplet
	Fast-evaporation
	Pellet
	Spin-coating
	Electrospray
	Air-spray
	Matrix-precoated targets
Liquid matrix	Chemical liquid
	Particle suspension
Special preparation	Solid supports

The *dried-droplet method* [82], originally developed by Karas and Hillenkamp, is nowadays used routinely in combination with the matrices DHB, DHBs and 3-

HPA. A drop (1µl) of an aqueous matrix solution is mixed with a drop (1µl) of an analyte solution and dried. The resulting analyte-doped matrix crystals show typically a heterogeneous morphology, and therefore induce a lower mass accuracy, especially in linear TOF mass analysers.

Therefore the *fast-evaporation method* [94] was introduced to improve the mass accuracy and mass resolution of the MALDI measurements. Matrix and sample are handled separately: a drop (1µl) of the matrix solution (e.g. a saturated solution of α-CHCA in acetone) is applied to the target and is dried, and on top of it a drop (1µl) of an aqueous analyte solution is deposited.

If the analyte is insoluble and it seems impossible to embed it in a matrix environment by the two previously described methods, the *pellet method* [95] is a possible solution. A mixture of finely ground matrix and analyte is pressed together in a hydraulic press.

There is a natural interest in the improvement of spectral quality by means of increased reproducibility and signal intensities. This enables the quantitative analysis of different compound classes by MALDI mass spectrometry. The sample preparation techniques, *spin-coating* [96], *electrospray* [97-99] and *air-spray* [100], have been developed to create a more homogeneous layer of equally sized matrix-analyte crystals, since even with the *fast-evaporation method* [94] a certain degree of heterogeneity within the MALDI sample is obtained, as revealed recently by mass spectrometric imaging [101]. Equally sized matrix-analyte crystals are believed to enhance the overall MALDI performance [102]. In *spin-coating*, a large volume (3-10 µl) of a premixed analyte-matrix solution is deposited on the target, yielding an even distribution of the analyte.

In the *electrospray method* [97-98] a small amount of the analyte-matrix mixture is electrosprayed from a HV-biased (3-5 kV) stainless steel capillary onto a grounded metal sample plate, mounted 0.5-3 cm away from the tip of the capillary. The diameter of the capillary, the flow rate, as well as the distance between the capillary tip and the table effects the size of the micro-crystals. Not only a mixture of analyte-matrix solution can be electrosprayed, Caprioli *et al.* [99] used this method to electrospray a matrix only solution onto tissue sections for molecular imaging of individual compounds distributed in the biological sample. An alternative, simple, low-cost sample preparation technique is the *air-spray method* [100], in which the pre-mixed matrix-analyte mixture is deposited onto a heated stainless steel target with a compressed air-brush device. The

two latter presented sample preparation techniques have been further studied for matrix deposition onto TLC plates, as described in chapter 3 of this thesis.

Often only a small amount of the analyte is available and the addition of the matrix solution to the analyte decreases the analyte concentration. In this situation the use of *matrix-precoated targets* [103-104] is advantageous; an aqueous solution of the analyte is spotted directly onto a matrix-precoated target. Most efforts have been focused on the development of thin-layer matrix-precoated membranes, e.g. nitrocellulose, anion- and cation-modified cellulose, nylon, PVDF or regenerated cellulose dialysis membranes [103]. This method offers the possibility of off-line coupling of MALDI to liquid separation techniques, such as capillary electrophoresis (CE) [105] or liquid chromatography (LC) [reviewed in 106-107].

The preparation of analytes with *chemical liquids* [63, 87] is rather straightforward. An appropriate molar ratio of the analyte is dissolved in an UV-absorbing liquid, e.g. o-nitrophenyl octyl ether [63] or an IR-absorbing liquid, e.g. glycerol [87]. The requirements for co-crystallisation and solubility, when employing solid matrices, are thus circumvented.

The addition of fine metal or carbon particles to a non-absorbing liquid (e.g. glycerol, liquid paraffin or ethylene glycol) is also a way of preparing samples for MALDI. The solid particles absorb the laser energy and assist in analyte desorption and the liquid dissolves and ionises the analyte. This concept offers more flexibility compared to a laser energy absorbing liquid or solid matrix. The use of such *particle suspensions* [108-114] was first introduced by Tanaka *et al.* [65] and since then several combinations of particles and liquids have been used to analyse proteins, oligosacccharides, synthetic polymers, and dyes. Further information about this approach and its application in TLC-MALDI MS can be found in chapter 6 of this thesis.

An extension of the particle suspension technique is the *solid support method* [80, 106, 115-116]. The laser energy absorbing particles are compressed or sintered to form an "active" MALDI support. A variety of materials, including Si, TiN, CuO, graphite, and carbon particles have been tested [80, 106]. However, best known is the use of porous silicon as sample support (desorption/ionisation on porous silicon or DIOS) [115-116]. This concept is very promising for practical applications, although current limitations

are a limited mass range, poor reproducibility of the fabrication process, and surface contamination.

2.2.3 Desorption and Ionisation Mechanisms in UV MALDI

Investigation of the MALDI mechanism has been the focus of interest of a number of research groups. Several models explaining the desorption and ionisation processes occurring in the MALDI plume have been proposed, and these are discussed in the following section. Each is considered separately for simplicity reasons, although it certainly does not reflect reality, where they probably work in combination.

Desorption Mechanisms. A quasi-thermal desorption model was recently suggested by Hillenkamp and co-workers [117-118]. The first step involves a pressure-driven decomposition of the matrix-analyte material into smaller particles. The smaller particles consist of clusters and single molecules [119-120]. In a second step the clusters and individual molecules are thermally desorbed from the sample surface forming the MALDI plume. At the first few seconds, the MALDI plume is characterised by high density and high collision rates. After a few nanoseconds an explosive phase transition occurs, in which molecular gas-phase species of matrix and analyte are formed through expansional cooling. The initial velocities of ions formed in the plume are about 300-500 m/s [121], and such high initial velocities are assumed to indicate a soft desorption process.

Ionisation Mechanisms. The ionisation processes in MALDI mass spectrometry are less well understood then the desorption processes. Models for ion formation were summarised in a recent review by Zenobi and Knochenmuss [73] and discussed again more recently by the same authors [122] and by Karas *et al.* [123].

The current view is that several different pathways are contributing to the formation of ions, as summarised in **Table 2-2**.

Table 2-2. Possible pathways for MALDI ion formation.

Primary ion formation	Desorption of pre-formed ions
	Multi-photon ionisation
	Energy pooling
	Excited-state proton transfer
	Disproportionation reactions
	Thermal ionisation
Secondary ion formation	Proton transfer
	Electron transfer
	Cationisation

The term *primary ion formation* stands for pathways in which gas-phase ions are created from neutral molecules or aggregates (often derived matrix species) in the MALDI plume. Many of these ions will undergo collisions, which is believed to be particularly important for the formation of analyte ions. These reactions will be described later in the section on *secondary ion formation.*

Primary ion formation. An important primary ion formation pathway is the *desorption of pre-formed ions* [124]. In this mechanism the ions observed in the MALDI spectrum, e.g. peptide-metal adducts, are believed to be already present in the solid sample and are ejected as those in the gas-phase by the laser pulse.

The formation of matrix radical cations ($M^{+\bullet}$) by *multi-photon ionisation* is considered by many authors as a strong candidate for the UV-MALDI primary ionisation step, following the equation:

$$M \xrightarrow{n\,(h\nu)} M^{+\bullet} + e^-$$

Ionisation of the matrix molecule can only occur if the absorbed energy given by the laser is higher then the ionisation potential (IP) of the matrix molecule. The IP of the common matrix DHB was recently determined as 8.05 eV [125]. This means, that three photons (each 3.7 eV) of a nitrogen laser (commonly used for MALDI experiments) would be required to ionise one DHB molecule, which is not possible with the laser irradiance typically used. Nevertheless, ionisation could occur via the described two-photon absorption and an assisting thermal step, as described by Allwood *et al.* in a

photothermal model [126] or by *energy pooling* [125]. In the latter model two nitrogen photons are absorbed by two neighbouring matrix molecules, which go into the excited state (M*M*) and pool their energy to yield one matrix radical cation ($M^{+\bullet}$) as follows:

$$2M \xrightarrow{2\,(h\nu)} M^*M^* \longrightarrow M + M^{**} \longrightarrow M + M^{+\bullet} + e^-$$

This model is more likely, since clusters are known to be liberated from the MALDI sample [119-120].

The *excited-state proton transfer* pathway is also a frequently proposed MALDI ionisation model [127]. A single excited matrix molecule (M*) is presumed to be much more acidic compared to its ground state. An analyte or matrix molecule in the ground-state in the proximity of an excited matrix molecule can then accept the labile proton:

$$M \xrightarrow{1\,(h\nu)} M^* \begin{cases} \xrightarrow{+A} [M-H]^- + [A+H]^+ \\ \xrightarrow{+M} [M-H]^- + [M+H]^+ \end{cases}$$

Since this model only requires one nitrogen photon it is favoured. However, the knowledge of the local environment in the MALDI plume is still too small to fully evaluate this approach [73].

Disproportionation reactions [128] have been suggested to explain the simultaneous formation of positive and negative ions in the MALDI experiment. Proton transfer of two coupled matrix molecules in the excited–state is believed to occur, following the equation:

$$2M \xrightarrow{2\,(h\nu)} MM^{**} \longrightarrow [M-H]^- + [M+H]^+$$

This reaction was found to be energetically accessible (5 eV) with two 337 nm photons [128].

For particle suspension matrices *thermal ionisation* [65, 73, 111] is a very probable mechanism. The liquid is generally transparent to the laser radiation, which is absorbed by the particles. Smaller particles have been found to work better, since the reflection of the laser light is reduced and the enhanced surface area results in a higher absorbance efficiency of the laser light. Both factors are presumably responsible for the higher

peak temperatures reached with smaller particles [65, 111].

Secondary ion formation. In the gas-phase *proton transfer reactions* [122] are believed to occur between matrix ions and neutral molecules (analyte or matrix molecules).

If non polar compounds with IPs lower then that of the matrix are analysed in MALDI, *electron transfer reactions* [129] between a matrix radical cation ($M^{+\bullet}$) and an analyte molecule (A) can take place to form an analyte radical cation ($A^{+\bullet}$):

$$M^{+\bullet} + A \xrightarrow{\Delta G} M + A^{+\bullet}$$

The secondary ionisation steps discussed so far involve the charge transfer in the gas-phase. For compounds that have an ionic character the release of pre-formed ions (cations) upon laser irradiation possibly contributes to the total ion yield [130]. The mechanism describing this pathway of ion formation in MALDI is called *cationisation* [130].

A uniform model for ion formation in MALDI was introduced in 2000 by Karas and co-workers [123], and needs mentioning. The dominant ionisation process in this hypothetical *"lucky survivor model"* is considered to be the desorption of multiply charged clusters. After evaporation of neutral matrix molecules, the desolvated and highly charged analyte ions are neutralised or charge reduced by electron capture or proton-transfer reactions with reactive matrix species. Thus, only singly charged analyte ions are believed to have a chance to survive in the dense MALDI plume. The model was later extended in accordance to different compound classes and preparation techniques [131].

After ions have been formed in the MALDI source, they are accelerated towards the mass analyser. A common type of mass analyser coupled to MALDI sources is the TOF analyser, which will be discussed now.

2.2.4 Time-of-Flight Mass Spectrometry

The TOF mass analyser [132] is possibly the simplest analyser, and is ideally suited for MALDI ion sources, due to its virtually unlimited mass range and the high duty

cycle. Many reviews have been published, outlining its principles [133-135].

Linear TOF mass spectrometer. Essentially in a linear TOF instrument (**Figure 2-11**) ions, which are generated by the laser pulse, are accelerated by an accelerating potential (V) as they enter the drift region (D). The kinetic energy the ions gain in the source region is given by:

$$z \cdot e \cdot V = \frac{1}{2} m \cdot v^2$$

Where

z = number of charges

e = charge of an electron

m = mass of an ion

v = velocity of an ion

The flight times (t) for the ions to reach the detector is proportional to the square root of their mass-to-charge (m/z) values, as shown below:

$$t = \frac{D}{v} = D\sqrt{\frac{m/z}{2 \cdot e \cdot V}}$$

D = length of the drift region

A mass spectrum is created because ions of different masses arrive at the detector at different times. Heavier ions arrive at the detector later than lighter ones, assuming the ions are singly charged.

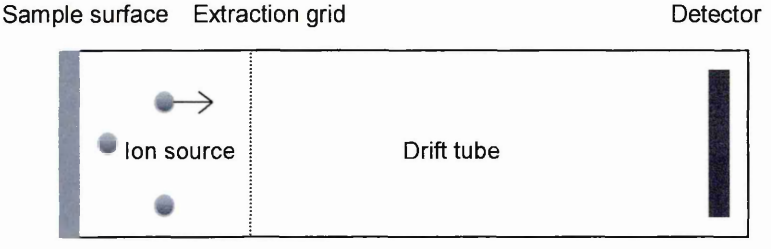

Figure 2-11. Basic schematic of a linear TOF mass spectrometer.

The main drawback of the linear TOF mass spectrometer, when used with continuous extraction, is the low resolving power achievable. Ions, depending on where they are formed, experience different acceleration potentials, and hence have slightly different kinetic energies. As a consequence poor mass resolution in the recorded mass spectra is obtained, since there is no energy focusing on a linear TOF mass spectrometer.

Improvements in resolution as well as mass accuracy have been obtained in two ways: the introduction of delayed extraction (DE) in a linear TOF instrument by Brown and Lennon [136] in 1995 and the use of reflectron TOF mass spectrometers, which was in fact first described by Mamyrin *et al.* in the 1970s [137].

Delayed Extraction (DE). In this technique, a calculated time delay in the order of several nanoseconds is introduced after the laser has fired and before the extraction pulse is applied to the backing plate or the intermediate grid in a two-stage extraction source. This ensures that ions of particular energies are focussed when they are accelerated into the drift region, as shown in **Figure 2-12.**

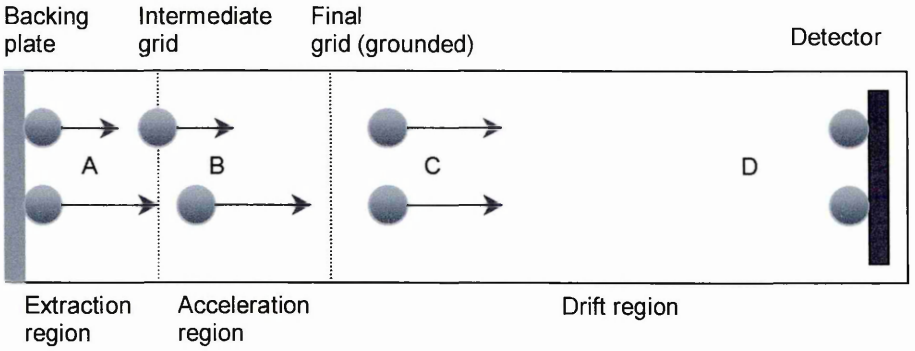

Figure 2-12. Schematic representation of delayed extraction in a two-stage ion source: (A) Ions are formed with different kinetic energies after the laser pulse, (B) the difference in their initial kinetic energies results in different velocities, as presented in the spatial spread. (C) After the time delay the extraction field is switched on and the ions gain different acceleration potentials, so that (D) the ions will reach the detector at the same time.

During the time delay, the ions rearrange their positions according to their initial velocities, so that those with larger velocities, travelling in the direction of the drift region, will gain less energy from the extraction field when it is switched on. The only disadvantage of DE is its mass dependency: the optimum time delay is different with each mass. Thus, the optimisation of resolution across the entire mass range of relevance is not possible. In practice, the time delay is set to focus on the highest mass of interest, since lighter ions have got a smaller velocity spread.

Reflectron TOF mass spectrometer. The reflectron was introduced into TOF instruments to improve energy focusing and hence to significantly enhance the mass resolution. At the end of the drift region an "ion mirror" is introduced, over which an

electric field is applied. Minor differences in kinetic energy of ions with the same m/z values are corrected, since ions of greater energy penetrate deeper in the reflectron than less energetic ions. This ensures that ions with different kinetic energies, but the same m/z values, arrive at the detector at the same time. The reflectron TOF mass spectrometer can also be used to gain structural information of the analytes using post-source decay, as described below.

Post-Source Decay (PSD). Since a large portion of the molecular ions undergo intensive metastable decay in the drift region, before they enter the mass analyser, structural elucidation can be performed in a reflectron TOF mass spectrometer. Intact molecular ions, which gained sufficient internal energy during the desorption process, can release their excess energy by dissociation during their flight, i.e. metastable decomposition. This process is called post-source decay (PSD) [138-143] and its principles are illustrated in **Figure 2-13**. The product ions formed by PSD continue to travel through the drift region with the same velocity, but with a range of kinetic energies, since their mass has changed. Ions with a higher kinetic energy (i.e. heavier ions) penetrate deeper into the "ion mirror" than lighter ions and hence mass to charge separation of the non-dissociated precursor ion and the product ions is achieved.

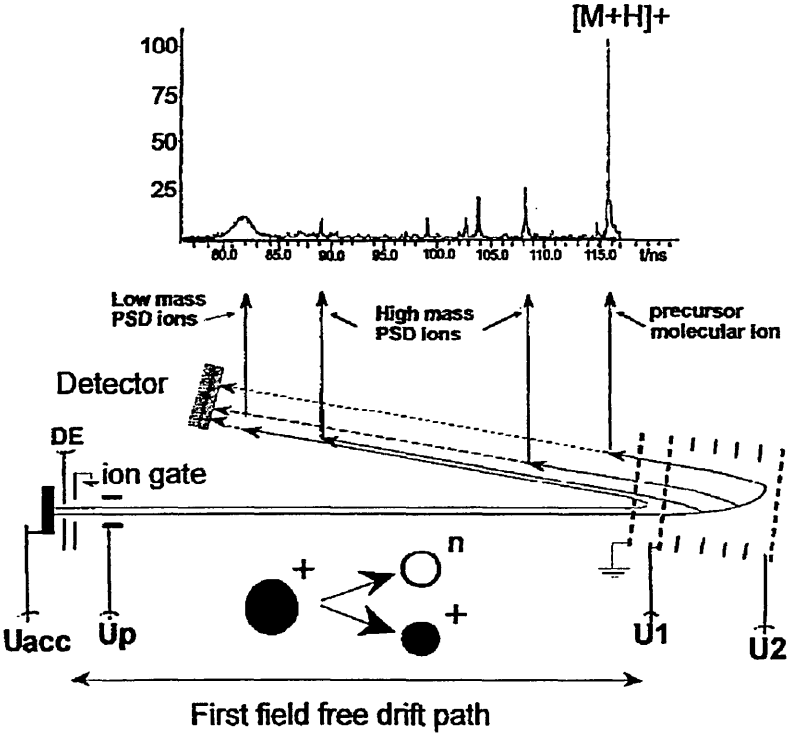

Figure 2-13. Principles of PSD-MALDI TOF mass spectrometry [142].

Conventional reflectrons require several experiments to be performed with the "ion mirror" voltage optimised for each small sequence of the product ion spectrum. A complete PSD ion spectrum can be obtained by combining the small sequences together. This is not required when a curved-field reflectron is employed. This type of reflectron is able to disperse all PSD ions over the acquired spectrum with the same "ion mirror" voltage applied. A complete product ion spectrum can thus be acquired within a single step [144]. The use of this technique in combination with TLC-MALDI MS is described in chapter 8 of this thesis.

Latest developments. In the last 14 years MALDI has been coupled to both linear and reflectron TOF systems, operated with or without delayed extraction. The latest results report the successful coupling of MALDI sources with quadrupole-TOF systems (q-TOF) using orthogonal extraction conditions [145-146, reviewed in 147] and with TOF-TOF systems [148]. Both instruments allow tandem mass spectrometry (MS-MS) experiments to be carried out on MALDI generated ions. The precursor selection occurs in the first analyser (quadrupole or TOF) and the product ions produced by collision-induced dissociation (CID) are mass analysed in the second analyser, the TOF analyser. Using a TOF analyser as the precursor ion mass filter has the advantage of a high duty cycle and the disadvantage of transmitting high energy ions into the collision cell, thus obtaining product ion mass spectra, which can provide less structural information, than those obtainable from low energy collisions. Other mass analysers, such as fourier transform (FT) cells [149-150] and ion trap (IT) mass spectrometers [151-152] have been successfully coupled to external MALDI ion sources. High mass resolution spectra from a range of fragile biomolecules, such as oligonucleotides [149], gangliosides [150], peptides [151], and oligosaccharides [152] could thus be recorded. In MALDI-FT-MS fragmentation and metastable decay of labile ions is a limiting factor, due to the relatively long time between the ionisation and detection (in the range of a few seconds instead of microseconds, normally obtained in MALDI-TOF-MS). Cooling of the hot ions with a high pressure gas [149-150] has been used to circumvent this disadvantage. In atmospheric pressure (AP)-MALDI-IT-MS the gas stream also assists in guiding the ions from the external source to the inlet system of the analyser [151-152].

2.3 References

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Chapter 3

Preparation of TLC Plates for MALDI MS Analysis

3.1 Introduction

The key step to a successful TLC-MALDI TOF MS analysis is the application of the MALDI matrix onto the developed TLC plate, prior to introduction into the mass spectrometer, as indicated in chapter 2 of this thesis. Several techniques for the deposition of the matrix have been described in the literature. The earliest method for plate preparation, developed by Gusev *et al.* [1], involves pressing a previously prepared layer of matrix crystals into the TLC plate. Bristow and Creaser [2] reported the TLC-MALDI determination of dyes, using plates prepared by spraying the matrix solution onto the plate with a TLC reagent sprayer. An air-spray method was developed by Haddleton *et al.* [3] as a sample preparation method for the MALDI MS analysis of polymers. Electrospray for sample preparation in MALDI was first suggested by Axelsson *et al.* [4] and independently, at about the same time, by Hensel *et al.* [5]. In this technique, a small amount of matrix-analyte mixture is electrosprayed from a HV-biased stainless steel or glass capillary onto a grounded metal sample plate, mounted 0.5 - 3.0 cm away from the tip of the capillary. A high degree of reproducibility was demonstrated for this sample preparation technique and it was shown to yield high quality quantitative data. Caprioli *et al.* [6] used electrospray matrix deposition to coat tissue samples during the MALDI based molecular imaging of peptides and proteins in biological samples.

In this chapter methods for the application of the matrix in TLC-MALDI TOF MS have been evaluated that allow the scanning of a TLC plate in order to obtain chromatographic as well as mass spectral data. The aim of this work was to develop a method for matrix deposition that is capable of being automated. This will allow routine use of the technique, and hence alternative approaches to the matrix pressing method [1] were tested. Preliminary work carried out showed that the use of a TLC reagent sprayer, [2] or an air-spray [3], to coat the TLC plate with matrix crystals, caused spreading of the TLC spots and would therefore not be suitable for the acquisition of chromatographic data. Since it was felt that the fine droplets produced by electrospray [4-6] would lead to minimal spreading, this technique was chosen for extensive investigation.

3.2 Experimental

3.2.1 Materials

The analyte tetracycline (compound A) was purchased from Sigma-Aldrich (Dorset, U.K.). Samples of known manufacturing impurities, epitetracycline (compound B), anhydrotetracycline (compound C) and compound D were supplied by Pfizer Global R&D (Sandwich, U.K.). The structures of these compounds are as shown in **Figure 3-1.** In all cases, the formula weight (FW) quoted is the molecular mass calculated using the isotope-averaged atomic masses.

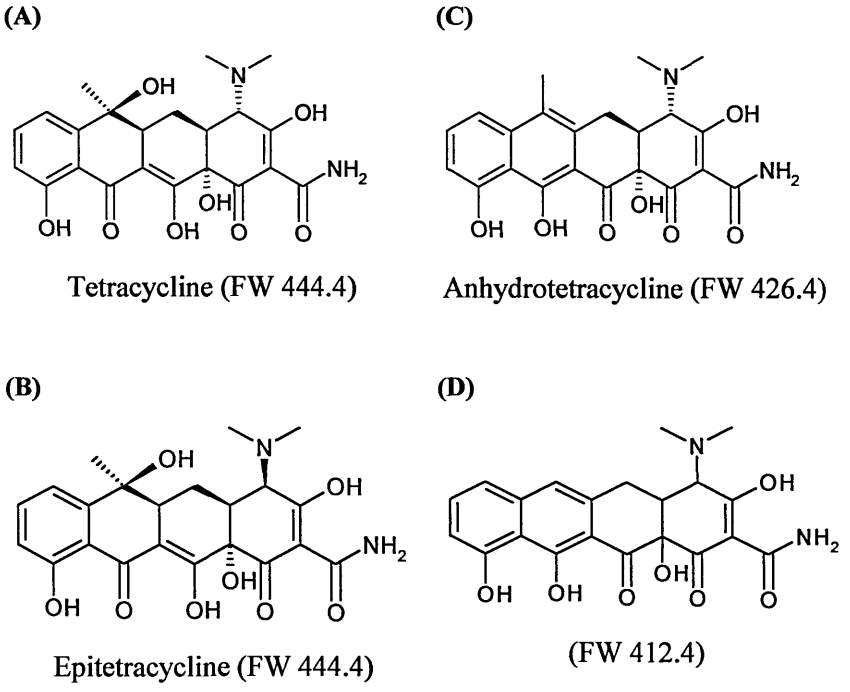


Figure 3-1. Structures of tetracycline and some of its impurities.

The following MALDI matrices were evaluated for TLC-MALDI analysis of these compounds: α-CHCA, HABA and DHB [7-8]. The matrices were purchased from Sigma-Aldrich (Dorset, U.K.). The solvents, acetone, methanol, ethanol and dichloromethane used, were all HPLC grade. Trifluoroacetic acid (TFA) (AR grade) was typically added at 0.1% v/v to the matrix solutions.

The TLC plates employed were silica gel 60 F_{254}, 0.2 mm thickness and aluminium backed (Riedel-de Haen, Germany).

3.2.2 Instrumentation

The time-of-flight (TOF) mass spectrometer employed in these investigations was a modified LaserTOF 1500 (SAI, Manchester, U.K.). This is a linear TOF instrument, equipped with a nitrogen laser (λ=337 nm) and delayed ion extraction. More information about the instrument and its performance can be found in the appendix (chapter 10) of this thesis. The sample holder of this instrument was redesigned to allow TLC strips of approximately 64 × 2 mm to be fitted into a recessed slot. The sample probe and software were also modified to allow free movement along the z (vertical) axis, and software was developed that allowed the construction of mass chromatograms from the acquired data sets. Only the positive ion mode was used in this study.

3.2.3 TLC Separation

Tetracycline and its impurities were separated to the procedure described by Weng *et al.* [9]. Before use, the silica gel plates were sprayed with an aqueous solution of disodium EDTA (10% w/w, adjusted to pH 9.0), dried horizontally at room temperature for 30 minutes, and then in an oven for another 30 minutes (120°C). The TLC development tank was lined with filter paper and saturated with the mobile phase dichloromethane/methanol/water (53/35/6, v/v) for at least an hour prior to use. Typically, a 10 µl aliquot of a sample solution (20 mg/ml in methanol) was applied to a plate with a disposable spotting pipette. The plate was developed to a distance of 7.0 cm, air dried at room temperature and the analyte spots were visualised under UV light (λ=254 nm).

3.2.4 Application Techniques of Matrix Material

Method 1: *Transfer of a previously prepared matrix crystal layer into the TLC plate.* A homogeneous matrix crystal layer was created on a 25 × 25 mm aluminium plate by depositing 50 µl aliquots of a saturated solution of α-CHCA, dissolved in acetone, on it. Sample spots, cut from the TLC plate, were wetted with an extraction solvent (ethanol or methanol/water 1:1, v/v). The previously prepared matrix layer on the aluminium plate was then pressed into the TLC plate for 2 minutes, using a small press. After

transfer, the aluminium plate was removed and thus the coated TLC spots were stuck to a MALDI target using double-sided tape.

Method 2: *Brushing of a supersaturated matrix solution.* A 50 × 2 mm strip of a developed (or uneluted) aluminium-backed TLC plate was mounted onto a MALDI target with double-sided tape. An extraction solvent (ethanol) was applied to the TLC spots. A hot supersaturated slurry of α-CHCA in acetone was then dabbed onto the TLC strip with a stiff brush. The prepared target was dried at room temperature for at least 30 minutes before analysis.

Method 3: *Electrospraying of a matrix solution.* For matrix application via electrospray, an electrospray deposition device was constructed, as illustrated in **Figure 3-2**. This electrospray deposition device consisted of a syringe pump (Harvard 11), fitted with a 25 µl syringe, which was connected via PTFE/PEEK tubing to a HPLC 'zero dead volume' fitting to a capillary steel needle, to which a high voltage (1-4 kV) was applied. The capillary needle was further threaded through a HPLC T-piece 'zero dead volume' fitting, through which dry nitrogen could be applied coaxial to the capillary tube. The TLC strip, which was attached to the MALDI target, was earthed and held horizontally during spraying and set at a distance of ca. 2-4 mm from the spray needle. The voltage and distance between the capillary needle tip and TLC plate were adjusted such that a stable Taylor cone was visible and remained stable.

A 50 x 2 mm strip of the developed (or uneluted) aluminium-backed TLC plate was stuck onto a MALDI target with double-sided tape. An extraction solvent (ethanol) was applied to the TLC spots, when required. Matrix concentrations were between 10-100 mg/ml, depending on the matrix solubility. Two solvent systems were tested: solvent system A, methanol containing 0.1% TFA and solvent system B, methanol containing 0.1% TFA/ water in the volume ratio 1:1. Typically, a 25 µl syringe was filled with matrix solution and electrosprayed at a rate of 150 µl/h while the TLC plate was moved at ca. 0.6 cm/min. The voltage applied to the capillary needle of the electrospray device was typically ~ 1.5 kV.

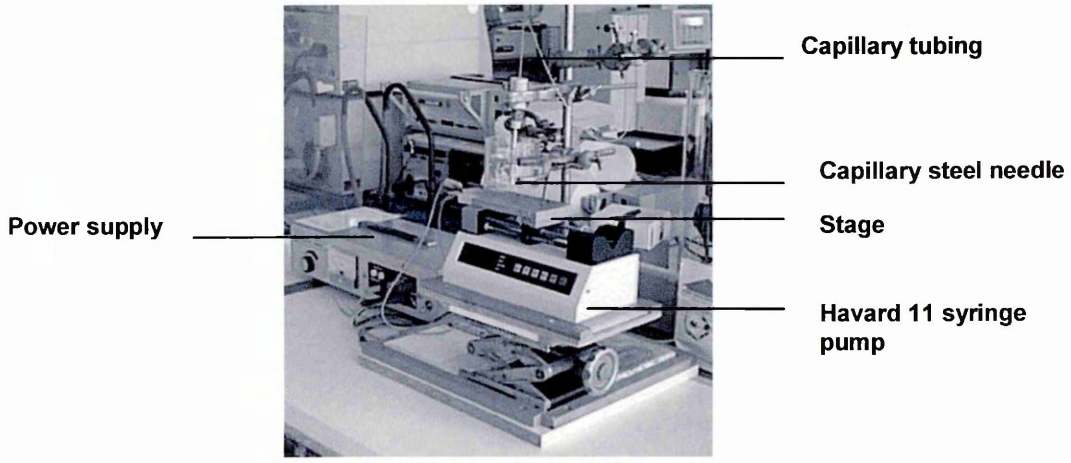


Figure 3-2. Electrospray deposition device.

3.3 Results and Discussion

3.3.1 Matrix Selection

The initial selection of matrix was based on examination of the analyte MS response observed from conventional stainless steel targets, during MALDI MS analysis [10-11]. A premixed solution of a matrix and sample solution (1μl), in a series of matrix to analyte ratios, was pipetted onto a stainless steel target. The matrix was selected on the following criteria: (i) solubility of matrix and analyte in similar solvents, (ii) absence of interfering peaks in the spectral regions of interest, and (iii) reproducible analyte signal intensity from laser spot to spot over the target (i.e. good crystal homogeneity). For tetracycline, all three matrices (α-CHCA, HABA and DHB) appeared suitable, based on the normal MALDI sample preparation procedure. However, the lower solubility of HABA (10 mg/ml in methanol) excludes its use for electrospray deposition. α-CHCA showed possible interfering peaks at m/z 423 $[2M-H+2Na]^+$ and 439 $[2M-H+Na+K]^+$, which made its use as matrix difficult, since mass spectral resolution appears to slightly deteriorate on TLC-MALDI coupling. This effect may be due to the uneven nature of the TLC plate surface, although it is not at present completely understood. (See chapter 4 of this thesis for a fuller explanation of this phenomenon.)

Optimum matrix to analyte (tetracycline) molar ratios varied from 5:1 (HABA), 100:1 (α-CHCA) to 700:1 (DHB). This small excess of matrix to analyte is a reflection of the low molecular mass of the analyte species, in contrast with the situation of large

molecular weight analytes, such as bioploymers, where very large matrix excess is used [12]. The mass spectrum obtained with the use of α-CHCA at a molar ratio to tetracycline of 100:1 showed considerable matrix suppression effects [13].

3.3.2 Comparison of Matrix Application Techniques

Method 1: *Pressing.* The indirect means of matrix deposition, by pressing a predeposited matrix layer into a wetted TLC plate, has been reported to work successfully by Gusev *et al.* [14]. This is not consistant with the studies presented here; the procedure was found to be extremely difficult, and only small sections of the silica gel plate could be handled. Therefore a developed TLC plate was cut up into small sections 1.5 × 1.5 cm. The silica gel plate was quite fragile and frequently cracked during the procedure. Mass spectrometric analysis of successfully prepared TLC spots showed areas of localised analyte-doped matrix crystals, but the number of points, from which analyte signals were obtained, was insufficient to allow chromatographic information to be obtained from a spot.

Method 2: *Brushing.* Deposition of matrix material by brushing a supersaturated solution of matrix into a TLC plate showed some incorporation of analyte-doped crystals and did yield analyte mass spectra. **Figures 3-3** to **3-6** show mass spectra from TLC spots obtained from the analysis of tetracycline and some of its known manufacturing impurities. The TLC procedure was as described in the experimental section. **Figure 3-3** shows the mass spectrum obtained from epitetracycline retardation factor R_f = 0.11. The $[M+H]^+$ ion can be clearly observed at m/z 444.8. **Figure 3-4** shows the mass spectrum of tetracycline, itself R_f = 0.25, the $[M+H]^+$ ion at m/z 445.3. Two unknown impurities at R_f values of 0.34 and 0.60 are shown in **Figures 3-5** and **3-6**, respectively. The pre-treatment of the TLC plate with disodium ethylenediaminetetraacetic acid solution was necessary for the improvement of the separation of tetracyclines from the impurities. Without the pre-treatment, streaking was observed on the plate, due to the interaction of the basic moieties of tetracycline with metallic impurities in the silica. However, this procedure frequently contributed to the enhancement of signals from sodium and potassium adducts of tetracycline (m/ 467 and 483, respectively) and its impurities, which complicates the interpretation of the mass spectra.

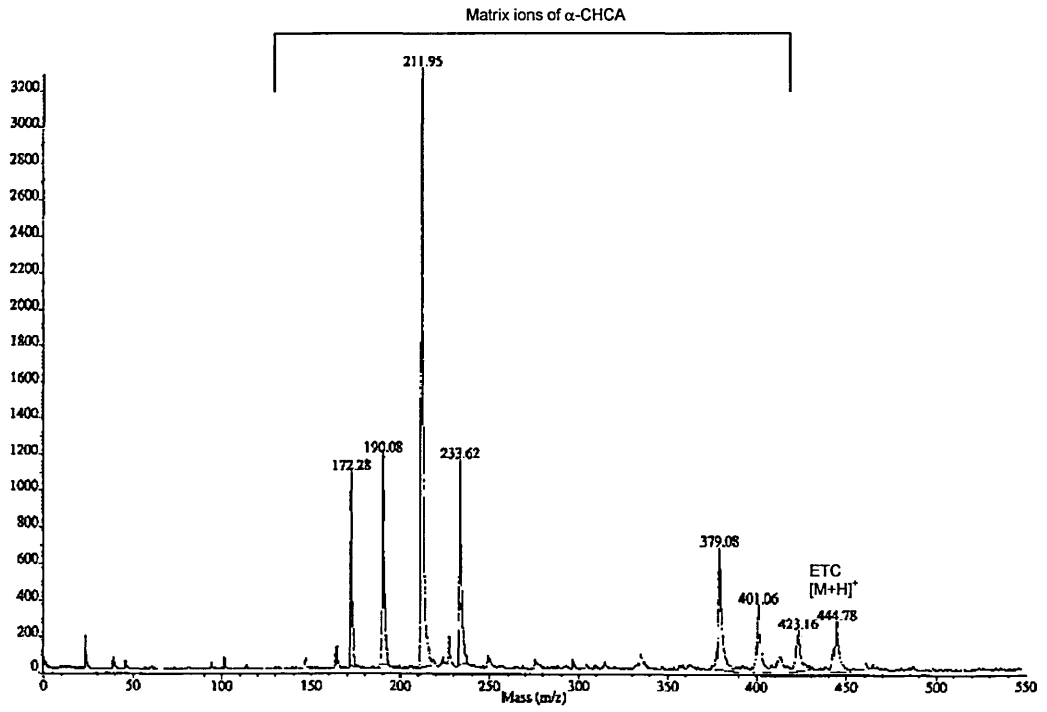


Figure 3-3. Mass spectrum of epitetracycline (ETC, R_f 0.11), from an α-CHCA matrix applied using the brushing method.

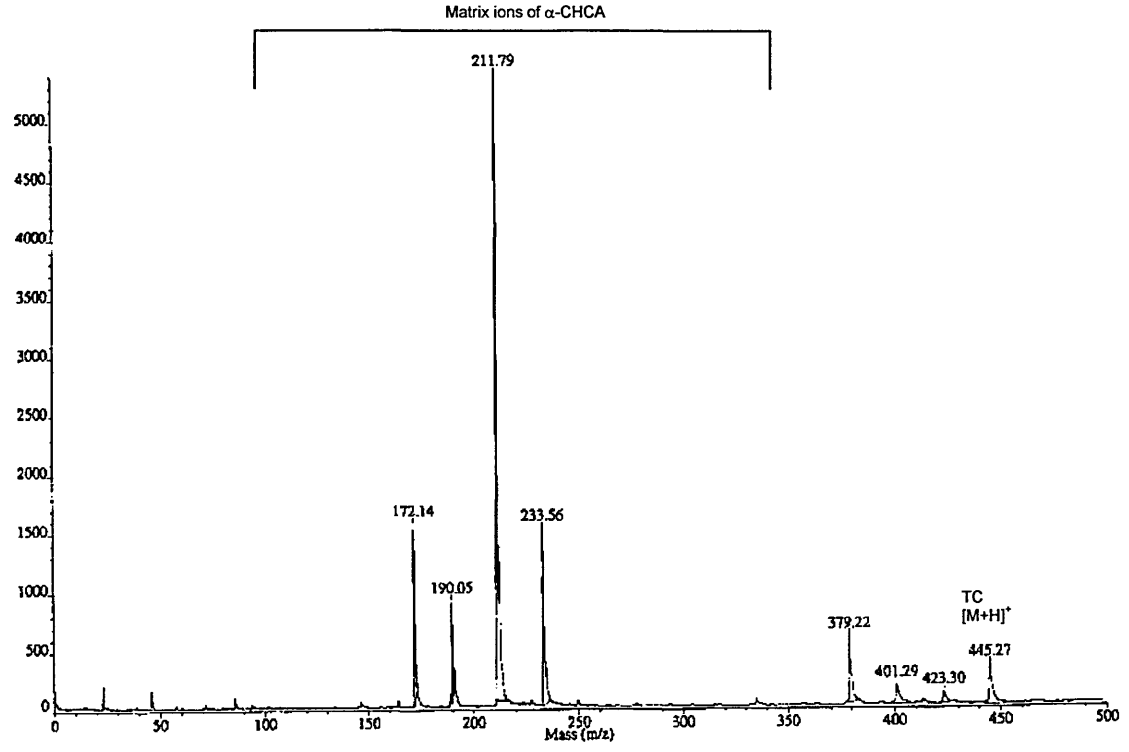


Figure 3-4. Mass spectrum of tetracycline (TC, R_f value 0.25), from an α-CHCA matrix applied using the brushing method.

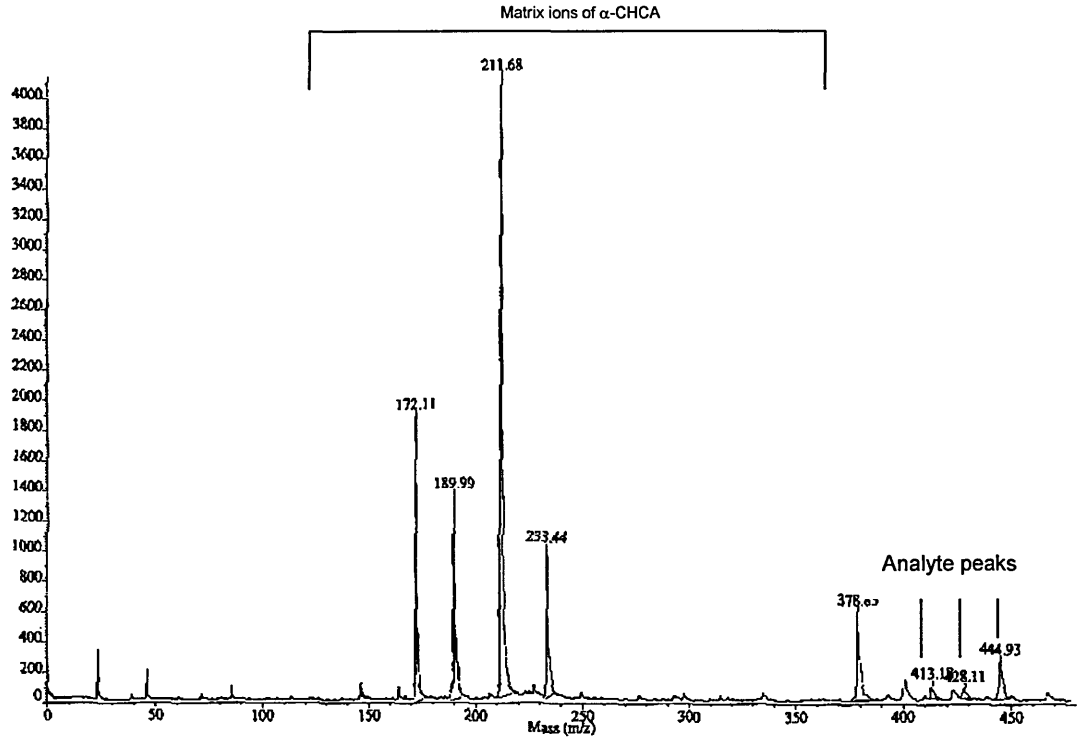


Figure 3-5. Mass spectrum of unknown impurity in tetracycline (R_f 0.34), from an α-CHCA matrix applied using the brushing method.

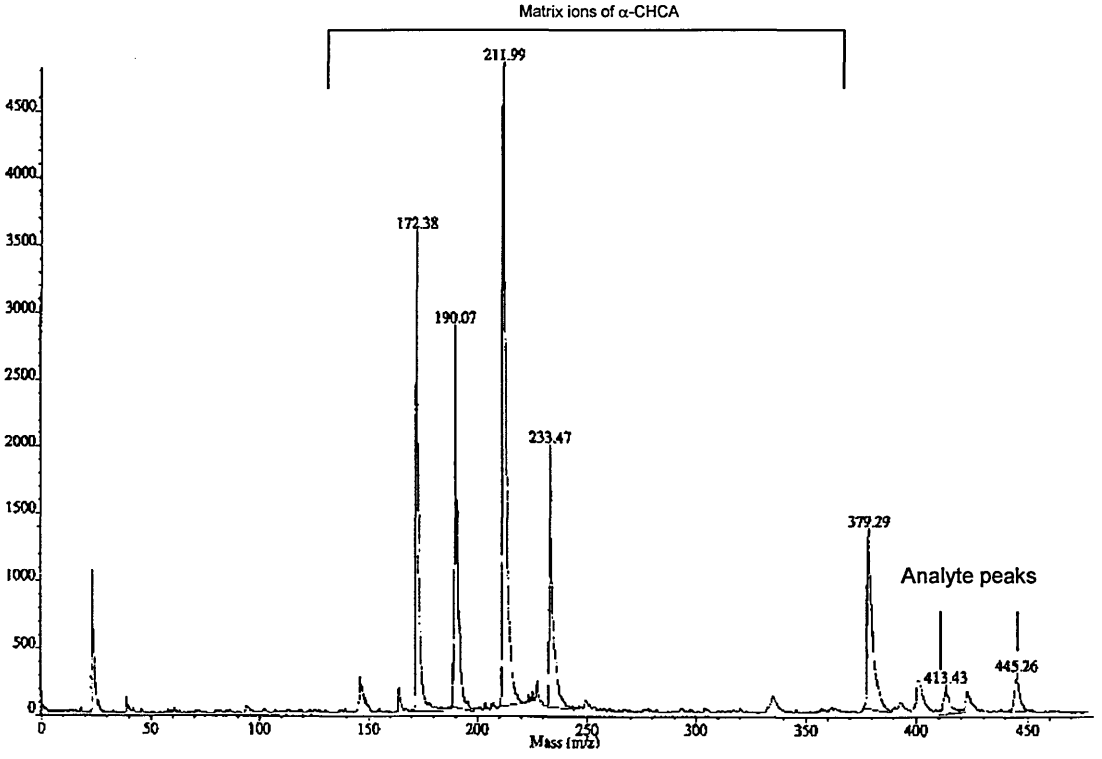


Figure 3-6. Mass spectrum of unknown impurity in tetracycline (R_f 0.60), from an α-CHCA matrix applied using the brushing method.

Because of the cation adducts, the close range of molecular weights of potential impurities, and deterioration of resolution on TLC-MALDI coupling, unambiguous identification of the impurities on the basic of assignment of the $[M+H]^+$ mass peaks proved challenging. In such a situation, only the use of good quality impurity reference standards would enable conclusive identification or the use of MS-MS techniques, as presented in chapter 8 of this thesis.

The procedure worked well only with volatile solvents, such as acetone, and in such cases lateral spreading of analyte species was minimal. MALDI MS analysis of targets prepared in this way showed analyte signals from localised areas on the TLC plate. However, it was difficult to produce consistent results in terms of signal intensity, since the procedure did not yield a consistent loading of matrix on the TLC plate. Since the analyte signals from the TLC plates prepared by the brushing method are localised, they are not obtained from a sufficient number of points on the TLC plate to generate chromatographic information from TLC spots. A degree of skill is needed to prepare a good plate, and the method does not seem to lent itself to automation. Therefore it was not investigated further.

Method 3: *Electrospraying.* For tetracycline the best data from plates prepared by electrospray were obtained using DHB as the matrix (100 mg/ml in solvent system B). It was found that a single spray pass was sufficient. A second pass showed either no improvement in signal intensity or a deterioration in the signal obtained, possibly because the initial layer of the crystals which is co-crystallised with analyte would be redissolved by the second pass of matrix solution.

Using the modified instrument, chromatographic data were successfully obtained from TLC plates, directly spotted with three replicated tetracycline spots, each of 100 and 1 μg, prepared using the electrospray method. **Figure 3-7** and **3-8** show summed mass chromatograms over the range m/z 440-460 for three tetracycline spots of 100 μg (**Figure 3-7**) and 1 μg (**Figure 3-8**). Typical mass spectra obtained for 100 and 1 μg tetracycline spots are shown in **Figure 3-9** and **3-10**, respectively. The ultimate limit of detection for tetracycline (localised signals) is however much lower at 1 ng spots (**Figure 3-11**), but mass chromatograms have not been successfully obtained at this level, since additional improvements in the sample preparation technique are required for this, as presented in chapter 5 of this thesis.

The data further illustrate the poor mass resolution, and hence mass measurement accuracy, obtained in these early TLC-MALDI TOF MS experiments. These appeared to be quite poor in the chromatographic acquisition experiments, possibly due to the movement of the TLC plate, which is in essence now the MALDI target, over several millimetres, during the acquisition of each spectrum. The small variations in flight time that would be cause these phenomena, arising from the uneven nature of the TLC plate surface, would obviously be greater the larger the area of TLC plate surface that data has been acquired from, in order to produce one spectrum.

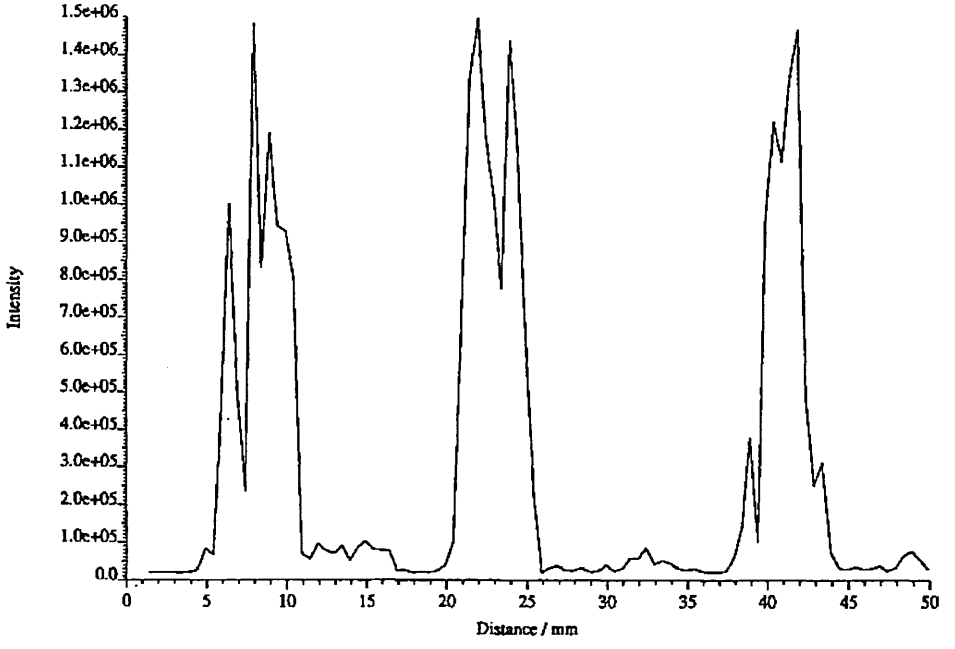


Figure 3-7. Mass chromatogram from m/z 440 to 460 for three replicate tetracycline spots of 100 µg on silica gel substrate.

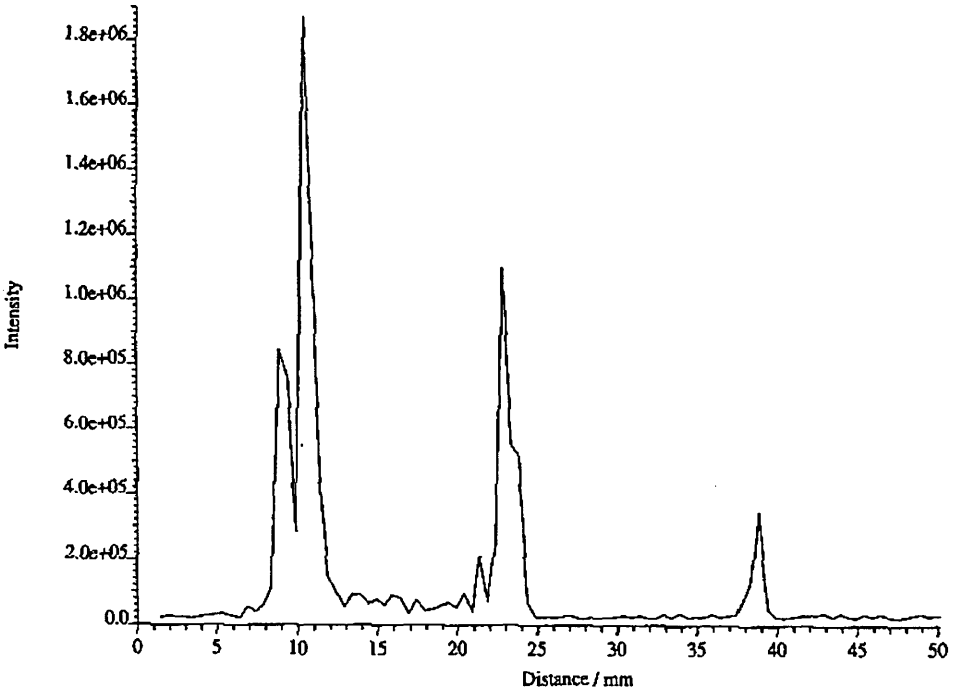


Figure 3-8. Mass chromatogram from m/z 440 to 460 for three replicate tetracycline spots of 1 µg on silica gel substrate.

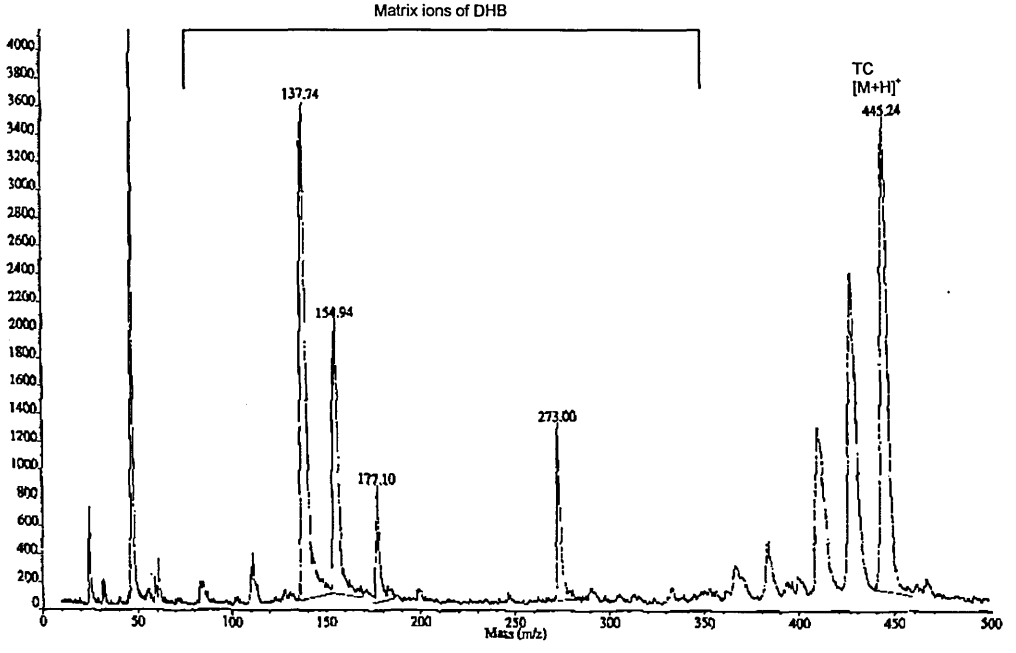


Figure 3-9. A typical mass spectrum obtained from scanning a 100 µg tetracycline (TC) spot. The matrix DHB was applied on the TLC plate using the electrospray deposition method.

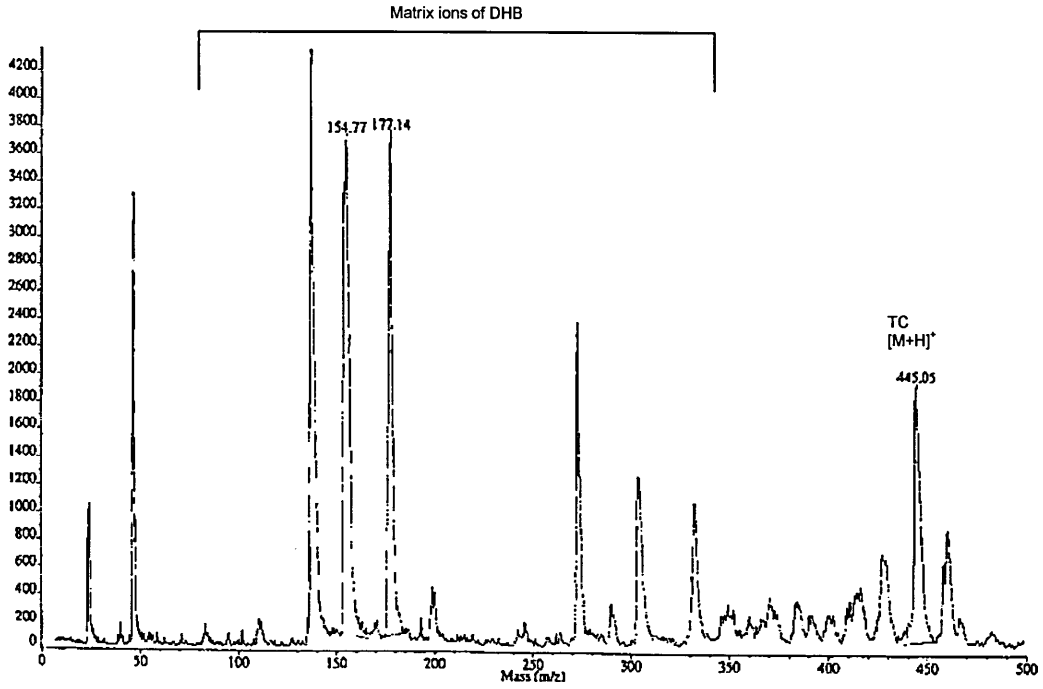
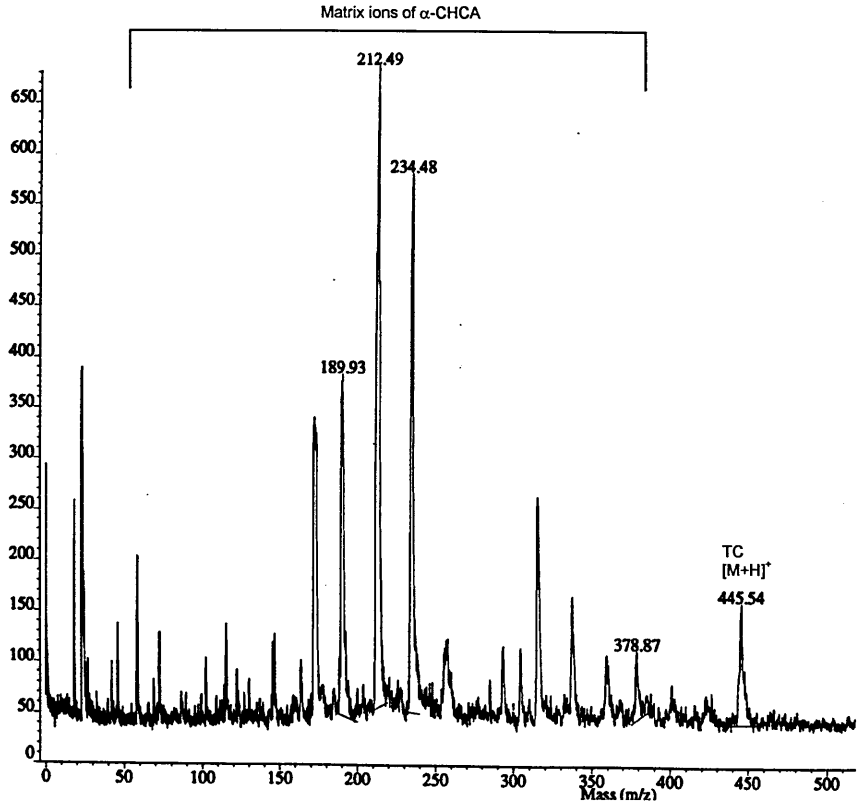


Figure 3-10. A typical mass spectrum obtained from scanning a 1 µg tetracycline (TC) spot. The matrix DHB was applied on the TLC plate using the electrospray deposition method.



3-11. Mass spectrum obtained from a 1 ng spot of tetracycline (TC). The matrix α-CHCA was applied on the TLC plate using the electrospray deposition method.

3.4 Conclusion

Three literature methods for TLC plate preparation for coupling to MALDI MS have been examined, i.e. pressing a previously prepared matrix layer into the TLC plate surface, the use of a reagent sprayer and an air-spray. Two new approaches have been also developed: brushing a supersaturated matrix solution and deposition of matrix material by electrospraying. All procedures gave TLC-MALDI targets, which on analysis produced mass spectra from analyte-doped matrix crystals. However, of the methods of matrix deposition into a TLC plate, only electrospray deposition has been shown to produce analyte signals from enough points on a sample spot to allow chromatographic information to be obtained from a TLC plate. By modifications of a commercial MALDI TOF mass spectrometer, and its control and acquisition software, chromatographic data from replicated spots of tetracycline at the 1 μg level could be obtained. The limit of detection is lower, about 1 ng (for localised signals). However, it is likely that a modified environment, which promotes more homogeneous crystallisation, is still needed in order to improve the ultimate limit of detection further, and hence allow the acquisition of chromatographic data at these lower levels. The data also highlighted problems with mass measurement accuracy and mass resolution obtained, when mass spectra were acquired directly from TLC plates.

3.5 References

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Chapter 4

Qualitative Determination of Pharmaceutical Compounds and Related Substances by TLC-MALDI TOF MS

4.1 Introduction

Some preliminary data was presented in chapter 3 on the development of methods for direct analysis of TLC plates by TLC-MALDI TOF MS. Electrospraying of the matrix solution onto the plate was described as a new method of preparing TLC plates for MALDI mass spectrometry. Mass spectra were directly obtained from plates, prepared in such a manner, and showed that modification of a commercial MALDI TOF mass spectrometer enabled chromatographic data to be obtained from such plates.

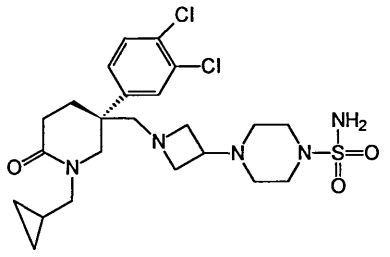
In this chapter the generation and characterisation of an impurity profile of a pharmaceutical compound by TLC-MALDI TOF MS is reported. UK-224,671 is a compound synthesised by Pfizer Global R&D, as part of a drug development program. A mixture of UK-224,671 and its known related substances was separated by TLC on silica gel, and the plate was prepared for TLC-MALDI by electrospraying the surface with a solution of α-CHCA. The identification of the main component and three major related substances was possible using mass chromatograms, recorded directly from the TLC plates. An increase in the signal intensities of the detected components in the recorded mass spectra was observed when each spot was wetted with methanol, before electrospray deposition of the matrix material. The mass spectra recorded contained mainly $[M+H]^+$ ions. The degradation in mass measurement accuracy and mass resolution, observed in the TLC-MALDI experiments, described in chapter 3, has been corrected by utilising a software that recalibrates each mass spectrum acquired during a run by using a matrix ion or other selected ions as "lock masses". Data acquired with, and without, the use of the "lock mass" are compared.

4.2 Experimental

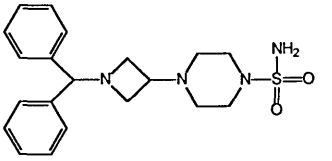
4.2.1 Materials

UK-224,671 ($C_{24}H_{35}Cl_2N_5O_3S$), a pharmaceutically active compound, and known related substances, UK-256,327 ($C_{11}H_{17}N_3O_2S$), UK-253,501 ($C_{20}H_{26}N_4O_2S$) and UK-260,489 ($C_{19}H_{23}Cl_2NO_3$), were supplied by Pfizer Global R&D (Sandwich, U.K.). The structures of these compounds are shown in **Figure 4-1**. In all cases, the formula weight

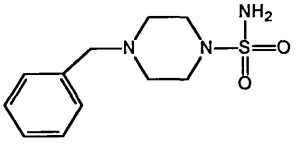
(FW) quoted is calculated using the isotopic-averaged atomic masses. A sample solution, containing 2.5 mg/ml of the pharmaceutically active compound and 2.5 mg/ml of each related substance, was prepared in methanol.



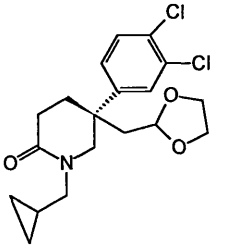
UK-224,671 (FW 544.6)



UK-253,501 (FW 386.5)



UK-256,327 (FW 255.3)



UK-260,489 (FW 384.3)

Figure 4-1. The structure of UK-224,671 ($C_{24}H_{35}Cl_2N_5O_3S$), a pharmaceutically active compound, and some of its known impurities, UK-256,327 ($C_{11}H_{17}N_3O_2S$), UK-253,501 ($C_{20}H_{26}N_4O_2S$) and UK-260,489 ($C_{19}H_{23}Cl_2NO_3$).

The suitability of the following organic MALDI matrices for TLC-MALDI coupling of these compounds was examined: α-CHCA, HABA, DHB, CA and 3-HPA. These organic matrices were purchased from Sigma-Aldrich (Dorset, U.K.).

All solvents used (methanol, ethanol, tetrahydrofuran and dichloromethane) were HPLC grade. Ammonia (35%) was prepared by dilution of a concentrated ammonia SG 0.88. The TLC plates employed were silica gel 60 F_{254}, 0.2 mm thickness and aluminium-backed (Merck, Germany).

4.2.2 TLC Separation

The TLC separation was carried out using the following procedure, which was adapted from an in-house method developed at Pfizer Global R&D. The silica gel plate was used

as supplied, without any pre-treatment. The TLC development tank was lined with filter paper and saturated with the development solvent dichloromethane/methanol/ammonia (35%) at a volume ratio of 60:10:1 over night, prior to use. An aliquot of sample solution (10 μl) was applied to the plate with a syringe. The plate was developed to a distance of 7.0 cm, air dried at room temperature and the TLC spots were visualised under UV light (λ=254 nm.).

4.2.3 Matrix Selection

For organic matrices, the analytes were dissolved in methanol (10 mg/ml). The organic matrices investigated (α-CHCA, HABA, DHB, CA and 3-HPA) were dissolved in a polar solvent, typically methanol at 10-100 mg/ml, depending on their solubility. TFA (AR grade) was added to the prepared matrix solution at a concentration of 0.1 % v/v. The analyte and matrix solutions were mixed in suitable ratios (typical ratios used were 1:1 or 1:10 v/v, respectively). Approximately 1 μl of the mixture was spotted onto a stainless steel MALDI target. The target was then analysed.

4.2.4 Matrix Application

The method used to prepare the TLC plates for MALDI analysis was to electrospray the matrix onto the TLC plate, as described in chapter 3.

4.2.5 Mass Spectrometry

The TOF mass spectrometer employed in these investigations was a modified LaserTOF 1500 (SAI, Manchester, U.K.). The modifications to the instrument are described in detail in chapter 3. In collaboration with SAI new software was developed to allow recalibration of each mass spectrum, during the scanning of a TLC plate, based on the measured m/z values of one or more peaks in the recorded mass spectra. Its use in these experiments is extensively described in the discussion section of this chapter. Only the positive ion mode was used in this study.

4.3 Results and Discussion

4.3.1 Matrix Selection

The selection of the organic matrix was based on an examination of their MALDI MS behaviour on stainless steel targets, as described in chapter 3. For UK-224,671 and related substances, α-CHCA was found to give the best results. Optimum matrix to analyte molar ratios were between 16:1 and 40:1.

4.3.2 Electrospraying MALDI Matrices onto TLC Plates

For UK-224,671 the best data from plates prepared by electrospray were obtained using α-CHCA as the matrix (20 mg/ml in methanol containing 0.1 % TFA). However, use of this matrix gave a mass spectral peak at m/z 379 $[2M+H]^+$, which could interfere with signals from UK-253,501 (FW 386.5) and UK-260,489 (FW 384.3), if adequate mass measurement stability/resolution were not achieved. However, instrumental resolution was adequate to separate the analyte and matrix related signals, as shown in **Figure 4-4 c-d** and **4-6 c-d**, respectively.

4.3.3 Acquisition of Mass Chromatograms and Mass Spectra

The TLC plate obtained from the separation of a mixture containing 25 μg of each of UK-224,671, UK-256,327, UK-253,501, and UK-260,489 is shown in **Figure 4-2**. The corresponding R_f values obtained by examination of the TLC plate under UV light are also presented in this figure. Chromatographic data have been successfully obtained from such a TLC plate, as shown in **Figure 4-3**. In these data the peaks, arising from the four TLC spots, are clearly visible. The software labels the x-axis with the distance, travelled from the origin by the spot, enabling R_f values to be readily calculated. From these data, the R_f values of UK-224,671, UK-256,327, UK-253,501 and UK-260,489 were calculated as 0.36, 0.51, 0.57 and 0.75, respectively and correspond to the R_f values obtained by UV detection (**Figure 4-2**). The mass chromatogram of the parent compound UK-224,671 comes from a different run. The corresponding mass spectra obtained for each spot are shown as **Figure 4-4**. These non-background subtracted

spectra show intense ions arising from the matrix, however the protonated molecular species for each analyte is clearly visible.

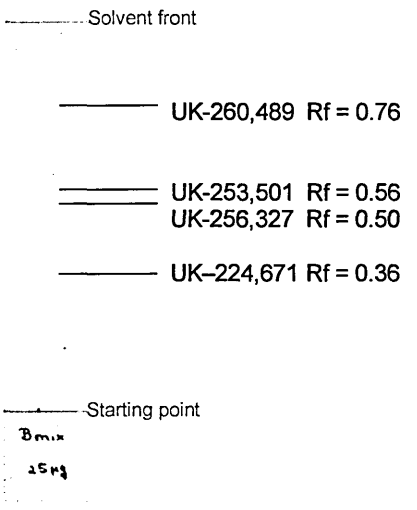


Figure 4-2. Typical TLC plate of a mixture of UK-224,671, UK-256,327, UK-253,501 and UK-260,489 (25 μg per component).

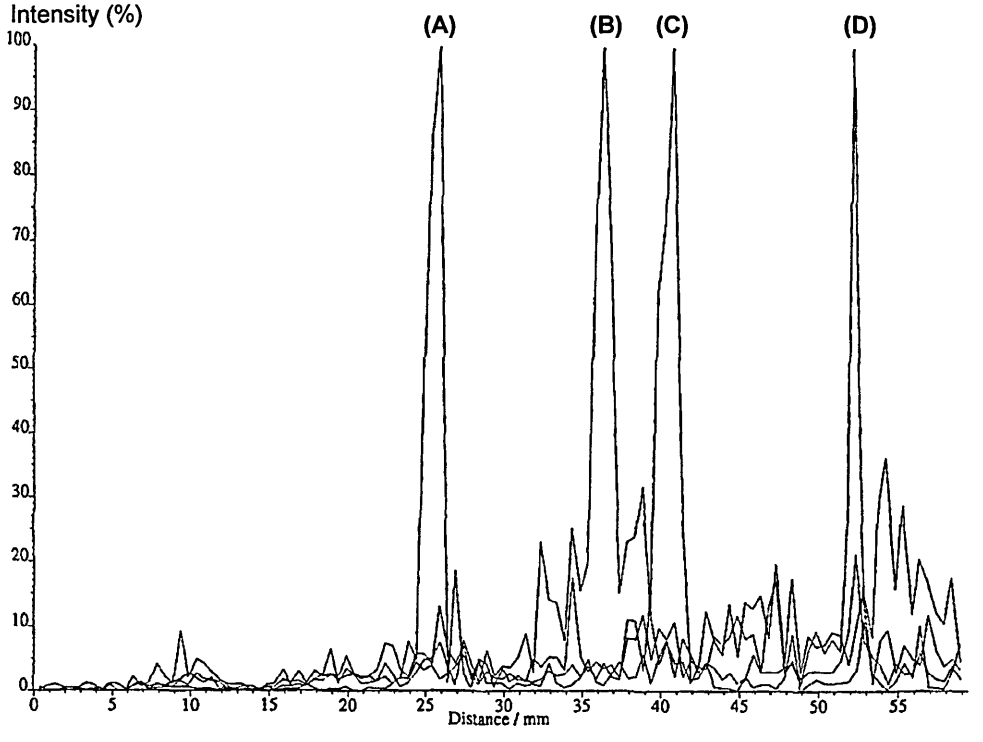


Figure 4-3. Overlaid mass chromatograms from the TLC-MALDI analysis of (A) UK–224,671 (m/z 545-547), (B) UK-256,327 (m/z 255-257), (C) UK-253,501 (m/z 385-388) and (D) UK-260,489 (m/z 382-385). 25 μg per component was separated on a TLC plate and the matrix α-CHCA was applied by electrospray deposition.

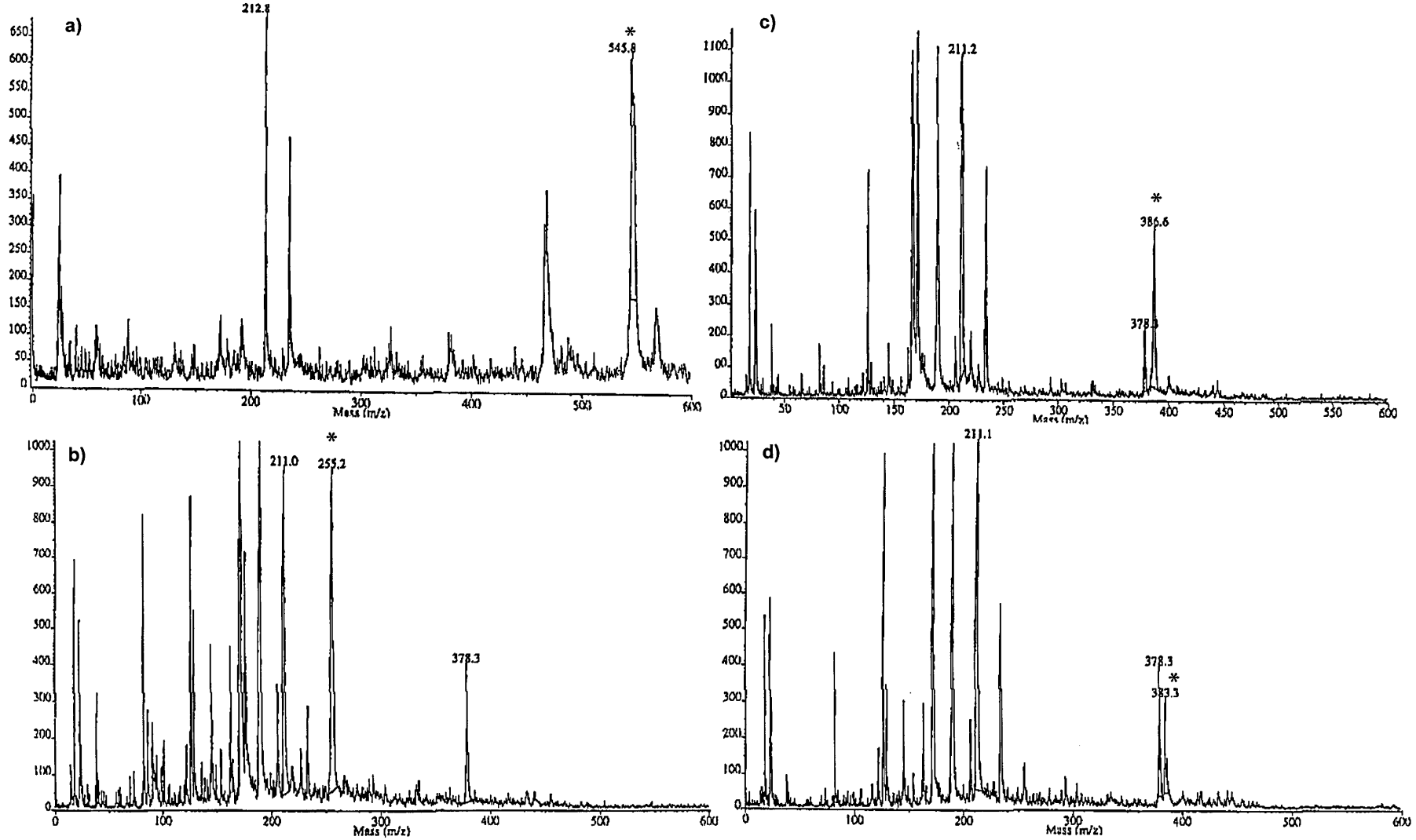


Figure 4-4. TLC-MALDI mass spectra of the peaks indicated in Figure 4-3, a) UK–224,671, b) UK-256,327, c) UK-253,501 and d) UK-260,489 (25 μg per component). The protonated molecular species of each component is labelled with an asterisk. The ions below 380 Da are α-CHCA matrix clusters. The mass spectra were acquired without "lock mass" corrections.

4.3.4 Extraction of Analytes from TLC plates

The analytes separated by TLC are presumably located up to 100-200 μm inside the plate [1]. To produce a MALDI MS signal, the analyte must be transferred from inside the silica gel to the surface, followed by electrospraying of matrix and crystallisation. The time for extracting an analyte from inside the TLC plate onto the surface is limited by the time of matrix crystallisation, which is less than ~ 10 minutes [1]. In this work, the extraction of analytes from the interior of the TLC plate was improved by the addition of an organic solvent, such as ethanol, tetrahydrofuran, methanol and methanol/water (1:1, v/v). The best results were obtained when methanol was applied onto each TLC spot. The addition of methanol may have increased the concentration of analyte molecules on the TLC surface, and/or led to a better co-crystallisation. An improved incorporation of analyte molecules into the matrix crystal layer was thus obtained. **Figure 4-5** shows the overlaid mass chromatograms from the TLC-MALDI MS analysis of a mixture containing 25 μg of each of UK-224,671, UK-256,327, UK-253,501, and UK-260,489, using the described approach. The jagged nature of the chromatographic peaks is typically for the use of methanol, which causes some analyte spreading in such a manner that less concentration of the analyte is obtained in the spot centre. The amount of extraction solvent, deposited on each TLC spot, is critical. On the one hand the amount should be as high as possible to increase the absolute quantity of analyte close to the surface, and on the other hand lateral spreading should be minimised, as far as possible. It has been found ca. 1 μl of methanol is appropriate. The lateral spreading was typically in the range of 1-5 mm depending on the analyte. UK-256,327 showed even planar diffusion along the plate, without the application of an extraction solvent (see **Figure 4-3**). **Figure 4-5** also illustrates that UK-256,327 and UK-253,501 could be found on the same position (37 mm from the starting point) due to the extraction procedure. The improvement of the sensitivity in the TLC-MALDI MS experiments carried out on UK-224,671 and related compounds by using methanol is demonstrated by: the increase in the ion intensity of the protonated molecular species by a factor of three for each substance observable in **Figure 4-6** and also by the fact that all four analytes could be detected in a single experiment.

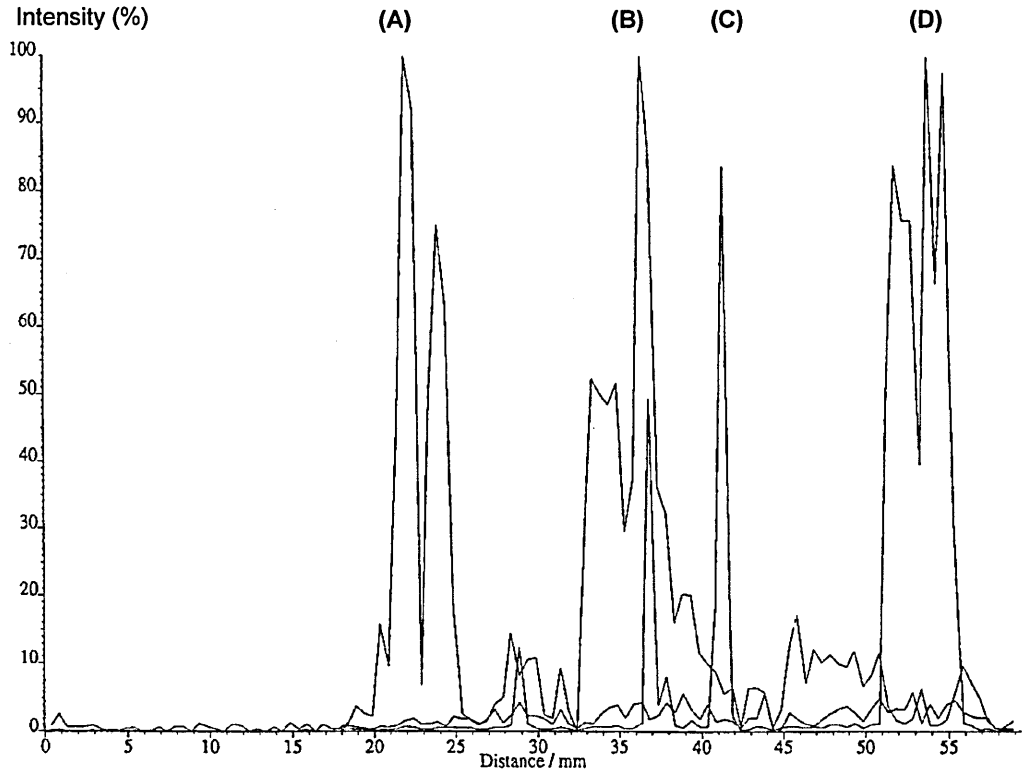


Figure 4-5. Overlaid mass chromatograms from the TLC-MALDI MS analysis of a mixture of (A) UK-224,671 (m/z 545-547), (B) UK-256,327 (m/z 255-257), (C) UK-253,501 (m/z 385-388) and (D) UK-260,489 (m/z 384-387). 25 μg per component was separated on a TLC plate and the matrix α-CHCA was applied by electrospray deposition. The data was acquired using methanol as an extraction solvent.

4.3.5 Recalibration of Mass Spectra

As shown in **Figure 4-4 d**, where the protonated molecular species of UK-260,489 (FW 384.3) was observed at m/z 383.3 rather than at 385, there is some degradation of mass spectral resolution and mass measurement accuracy observed, when mass spectra are recorded directly from TLC plates. This effect was described in the previous chapter of this thesis, and has also been observed by Guittard *et al.* [2], even when a reflectron based MALDI mass spectrometer (capable of much higher resolution than the instrument used in this work) was employed. The author of this thesis attributes the effect of poor mass accuracy and mass resolution to the uneven nature of the TLC plate surface, which leads to deviations in the ion flight time. Hence, the use of a software "lock mass" was investigated.

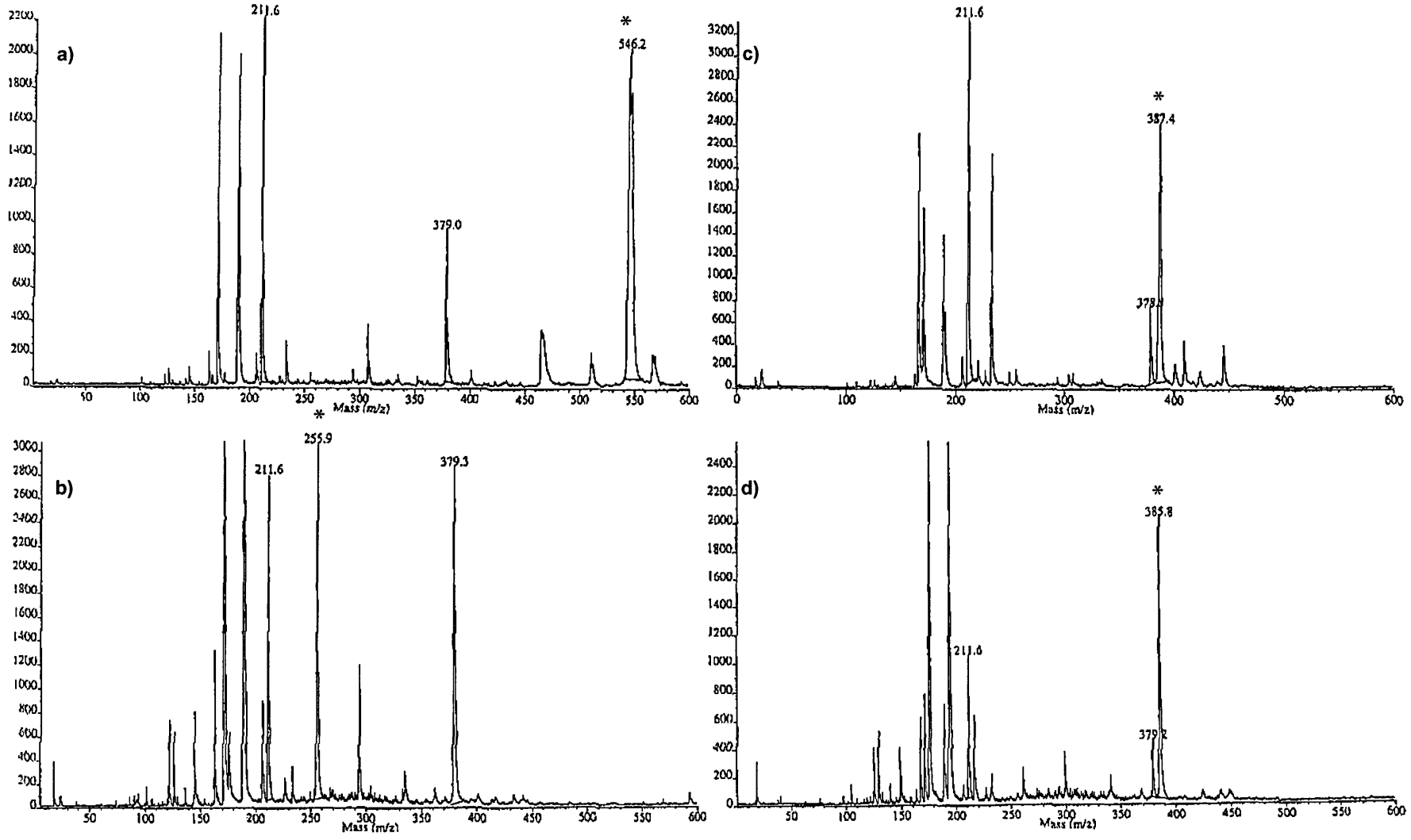


Figure 4-6. TLC-MALDI mass spectra of the peaks indicated in Figure 4-5, a) UK–224,671, b) UK-256,327, c) UK-253,501 and d) UK-260,489 (25 μg per component). The protonated molecular species of each component is labelled with an asterisk. The ions below 380 Da are α-CHCA matrix clusters. The mass spectra were acquired with "lock mass" corrections.

This software recalibrates each mass spectrum, acquired during the scanning of a TLC plate, based on the error in mass measurement observed, for one or more user defined m/z values. The "lock mass" correction is performed on individual spectra, before they are accumulated to enhance the signal-to-noise ratio. This has the effect of narrowing the peak width, and hence the resolution is significantly improved.

Initially two matrix ions were chosen for the recalibration, but it was found that one was sufficient. Thus, the $[M+Na]^+$ ion of α-CHCA was typically chosen. The mass spectra, shown in **Figure 4-6** were acquired using the "lock mass" software and illustrate that this approach presents a possible solution to the mass measurement accuracy issue. However, the mass spectral resolution achieved in the chromatographic acquisition experiments is still relatively poor compared to conventional MALDI applications. This is possibly due to the movement of the TLC plate over 0.125 mm during the acquisition of each spectrum.

4.4 Conclusion

Mass spectral and chromatographic data from a thin-layer chromatogram of a mixture of 25 μg quantities of a pharmaceutical compound and some of its related substances has been successfully demonstrated. This is, to the authors knowledge, the first time that the acquisition of genuine chromatographic data from a TLC plate by MALDI TOF MS has been reported.

The role of an extraction solvent has been investigated in detail. It has been demonstrated that its use results in a higher extraction efficiency and a better co-crystallisation and hence an increase in the intensity of ions from the compounds studied. It caused peak broadening in the chromatographic data obtained by TLC-MALDI MS. Therefore, in future TLC-MALDI experiments the solvent of the matrix will be selected carefully in order to improve the extraction of the analyte and its co-crystallisation with matrix , but avoiding extensive analyte spreading as presented here.

The degradation in mass measurement accuracy, reported previously for TLC-MALDI TOF MS experiments in chapter 3, has been successfully overcome by internal recalibration on selected matrix ions.

4.5 References

[1] A.I. Gusev, A. Proctor, V.I. Rabinovich, and D.M. Hercules, *Anal. Chem.* **67** (1995) 1805.

[2] J. Guittard, E. Mirgorodskaya, C. Costello, and W. Muehleeker, *Proceedings of the 47th ASMS Conference on Mass Spectrometry and Allied Topics*, Dallas, Texas, June 13-17 (1999).

Chapter 5

Detection of Related Substances at the 0.1% Level by TLC-MALDI TOF MS

5.1 Introduction

Monitoring of the presence of 'related substances' is required in the development of pharmaceutical compounds. The term 'related substance' describes compounds, structurally similar to the drug, including synthetic impurities, degradation products, or impurities, arising from storage or manufacturing [1-2]. The analysis of related substances is performed on both, the bulk drug and the finished drug product. Planar chromatography [reviewed in 3-8] is often used in the pharmaceutical industry as an analytical method for monitoring these substances [9-11] in order to prevent unknowns being missed due to their non-elution from a HPLC column [12]. TLC is easy to perform, fast and inexpensive, e.g. for the assay and purity tests of phospholipids a cost reduction by a factor of 2.5, compared to an existing HPLC method, was calculated [13]. In addition, other analytical methods can be carried out after the TLC separation by scraping off the purified parent compound from the stationary phase. However, TLC can lack sensitivity, since detection is often achieved by observing the absence of visible fluorescence at the excitation wavelength of 254 nm. Thus, it is necessary to apply large amounts of the parent compound onto the TLC plate to identify related substances at the 0.1% level. For example a sample of 400 μg of e.g. the bulk drug needs to be applied to detect ca. 0.4 μg of the related substances [14]. In this situation, TLC can be combined with mass spectrometry [15-16] to improve the sensitivity and to identify related substances by their molecular weight. Current methods of interfacing TLC to MS use the following soft ionisation techniques: FAB [17], LSI [18], MALDI [19], SALDI [20], and electrospray [21]. These techniques have been fully described in chapter 2. The work, described in chapter 3 of this thesis, indicates that TLC-MALDI has the advantages of minimal analyte spreading, compared to TLC-FAB and TLC-LSI, and a better sensitivity, compared to the early TLC-SALDI experiments.

The generation of an impurity profile of a pharmaceutical compound by TLC-MALDI was presented in chapter 4. 25 μg of each component was separated on a silica gel 60 F_{254} TLC plate, and mass spectra as well as mass chromatograms could be obtained from such a plate.

Improvements to the sample preparation procedure are reported in this chapter and were achieved by modifying a commercial robotic x-y-z axis motion system to act as an electrospray deposition device. This modified commercial device enabled the detection

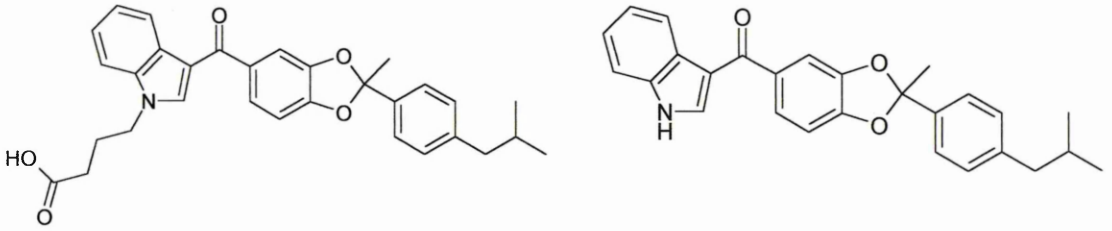
of related substances at the 0.1% level by TLC-MALDI MS. UK-137,457, a compound synthesised by Pfizer Global R&D as part of a drug development program, and UK-124,912, one of its related substances, were chosen as examples to demonstrate this. The new electrospray deposition device produced matrix crystals with a higher degree of porosity, compared to the electrospray deposition device, used in previous experiments (chapters 3 and 4). Thus, mass chromatograms of the related substance UK-124,912 could be constructed down to 2.4 pmol spots.

One limiting factor in the sensitivity, achievable by TLC-MALDI analysis, is that the analyte spot is not uniformly distributed in the silica gel layer [22]. The concentration of the analyte is increased on the surface and the extraction of the analyte is depending on its adsorbent strength to the silica gel. When the matrix solution is electrosprayed onto the TLC plate, the solvent acts as an extraction solvent to facilitate the movement of the analyte, located in the silica gel layer to the surface. By reducing the silica gel layer thickness from 200 µm to 100 µm an enhancement of the analyte signals detected by MALDI might be expected. Commercially available Si 60 F_{254} HPTLC plates with a layer thickness of 100 µm were tested, but difficulties encountered by cutting the developed plates to the required size of 60 × 2 mm excluded their use. Hence, a special Si 60 F_{254} HPTLC-MALDI target was developed in collaboration with Merck KGaA (Darmstadt, Germany) and enabled the detection of the related substance UK-124,912 in the high fmol range.

5.2 Experimental

5.2.1 Materials

UK-137,457 ($C_{31}H_{31}NO_5$), a pharmaceutically active compound, and one known related substance UK-124,912 ($C_{27}H_{25}NO_3$) were supplied by Pfizer Global R&D (Sandwich, U.K.). The structures of these compounds are shown in **Figure 5-1**. In both cases, the formula weight (FW) quoted is calculated using the averaged-isotopic atomic masses.



UK-137,457 (FW 497.6) UK-124,912 (FW 411.5)

Figure 5-1. Structures of UK-137,457 ($C_{31}H_{31}NO_5$) a pharmaceutical active compound and one of its known related substance UK-124,912 ($C_{27}H_{25}NO_3$).

The suitability of the following organic matrices for TLC-MALDI analysis of the two compounds was examined: α-CHCA, HABA, DHB, 3-HPA, and CA. These matrices were purchased from Sigma-Aldrich (Dorset, U.K.).

To enhance the sensitivity achievable in TLC-MALDI MS a special TLC-MALDI target was developed in collaboration with Merck KGaA (Darmstadt, Germany), as shown in **Figure 5-2.**

Figure 5-2. The Si 60 F_{254} HPTLC-MALDI target developed in collaboration with Merck KGaA.

A 100 μm thick layer of the silica gel 60 sorbent with a mean particle size of 5 μm was coated in the 60 × 2 mm recess of the MALDI target. Small amounts of a polymer binder and a fluorescence indicator were added to the sorbent. The adherence of the sorbent to the smooth stainless steel MALDI target was difficult and therefore thickness variations were obtained. Further improvements in the preparation of this layer are necessary, but it should be noted that the present work was undertaken to demonstrate the possibility of this approach.

5.2.2 TLC Separation

A method, developed at Pfizer Global R&D, was modified in order to improve the separation of both compounds. The mobile phase chloroform/methanol/glacial acetic acid at the volume ratio of 60:5:1 was saturated over night in a development chamber,

which was lined with filter paper. Aliquots of the sample solutions, dissolved in acetonitrile, were applied to silica gel 60 F_{254} aluminium backed TLC plates (10 × 10 cm) (Merck, Germany) and the plates were developed to a distance of 7.0 cm. After solvent evaporation at room temperature, the sample spots were visualised under UV light (λ=254 nm). (The replacement of chloroform with the less hazardous solvent dichloromethane in the mobile phase resulted in analyte streaking along the TLC plate.)

TLC separation of the analyte mixture on a Si 60 F_{254} HPTLC-MALDI target has not been carried out yet, since the experiments are at an early stage.

5.2.3 Matrix Application

Before the matrix was deposited on the silica gel surface, a strip of the developed TLC plate (60 × 2 mm) was attached to a modified MALDI target with double sided tape. The organic matrix was applied to the silica gel surface of the TLC plate by electrospray using an in-house modified commercial robotic x-y-z-axis motion system (PROBOT, BAI, Germany), as shown in **Figure 5-3**.

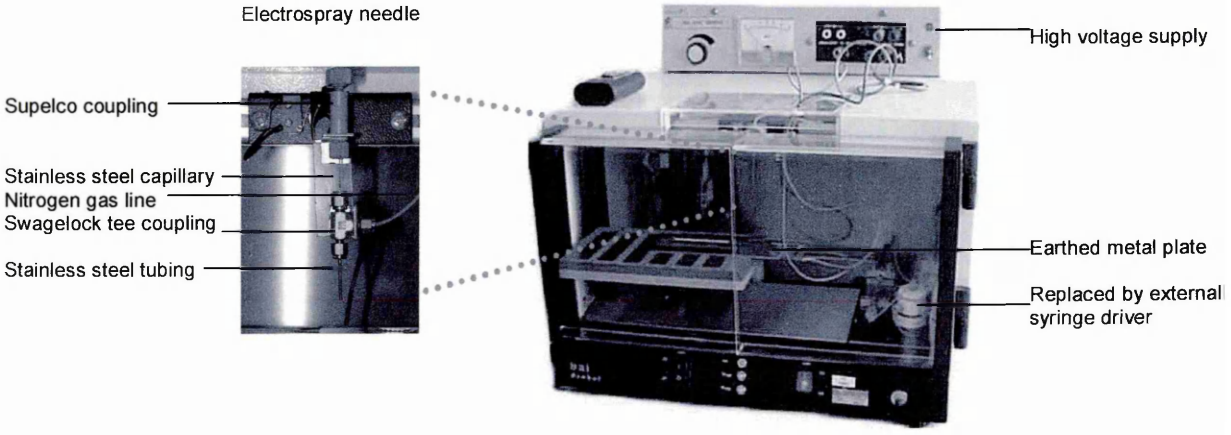


Figure 5-3. Commercial robotic x-y-z axis motion system (Probot, BAI GmbH), modified to act as an electrospray deposition device.

Modifications to the instrument were conducted in order to use the apparatus as an electrospray deposition device. The alterations were as follows: a PTFE tube was used to transfer matrix solution from a 100 µl syringe to a PEEK Supelco coupling (Cat. no.: Z227242). The electrospray needle consisted of a 94 mm length of stainless steel capillary (0.5 mm OD, 0.11 mm ID, ASH Instruments, U.K.) and the seal to the PTFE

tube was made within the PEEK Supelco coupling. The electrospray needle was threaded through a stainless steel Swagelock tee coupling (1/16" OD) and through a piece of stainless steel tubing (38 mm length, 1/16" OD, 0.75 mm ID, Jones Chromatography, U.K.). A gas line was attached to the tee to provide nitrogen (2 bar) coaxial to the spray to assist in gas nebulization if required. Nebulising gas was not used in these investigations, since rapid solvent evaporation occurred. This led to reduction in the extraction efficiency of the analytes from the silica gel layer. The syringe pump (Harvard microliter syringe pump) set at a flow rate of 0.6 ml/h delivered the matrix solution to the tip of the stainless steel capillary. To obtain a fine spray of the matrix solution ca. 2 kV was applied to the tee coupling using a high voltage supply. The metal plate (7 × 15 cm) was earthed and placed 2 mm from the end of the electrospray needle. The plate was then moved across in front of the spray at a rate of 25 mm/s. Typically an area of 60 × 2 mm on the TLC strip was covered with matrix crystals.

5.2.4 Mass Spectrometry

The time-of-flight (TOF) mass spectrometer employed in these investigations was a modified linear LaserTOF 1500 (SAI, Manchester, U.K.). The modifications to the instrument and its software have been extensively described in chapters 3 and 4. Only the positive ion mode was used in these investigations. 16 single shot mass spectra were averaged to give a composite mass spectrum. Typically, a data set of 120 mass spectra was acquired for each sample. The $[M+Na]^+$ ion of α-CHCA at the m/z value of 212 was used as "lock mass".

5.3 Results and Discussion

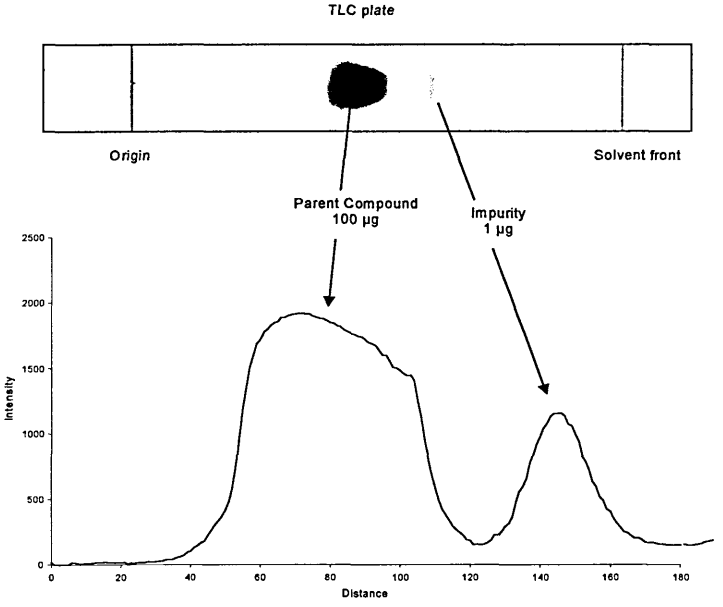
5.3.1 Matrix Selection

The selection of matrix was initially based on an examination of their MALDI MS behaviour on stainless steel targets, as described in chapter 3. CA as well as α-CHCA were found to give the best results, i.e. highest signal intensities of the compound studied and no interfering peaks arising from the matrix. However, electrospraying of CA (40 mg/ml in acetonitrile containing 0.1% TFA) did not yield a homogeneous

crystal layer. Changing of the solvent system to e.g. methanol decreased the sensitivity of the analytes detected on the TLC plate. The solvent of the matrix acts as an extraction solvent and is required to assist in releasing the analyte from the stationary phase and in this case both analytes have got the highest solubility in acetonitrile. The concentration of CA was finally increased by changing the solvent system to acetonitrile/water containing 0.1% TFA (70/30, v/v). At a concentration of 80 mg/ml still areas of the TLC plate with no matrix crystals could be detected, by examination under a high magnification microscope. By increasing the concentration to 100 mg/ml blocking of the capillary tubing was obtained. Hence, α-CHCA (20 mg/ml in acetonitrile/water containing 0.1% TFA, 70/30, v/v) was used for further experiments.

5.3.2 TLC-MALDI Analysis

The experiments were started at the 1% level of the related substance UK-124,912, since the compound was observable at this level (1 µg loading) by fluoresence quenching on the TLC plate, as shown in **Figure 5-4**. The R_f-values of the parent compound and the related substance were calculated from the TLC plate as 0.48 and 0.62, respectively.



Photographic scan of the TLC plate shown above, taken with an imaging system (Image Station 440CF, NEN Life Science Products).

Figure 5-4. TLC separation of UK-137,457 and UK-124,912 at the 1% level.

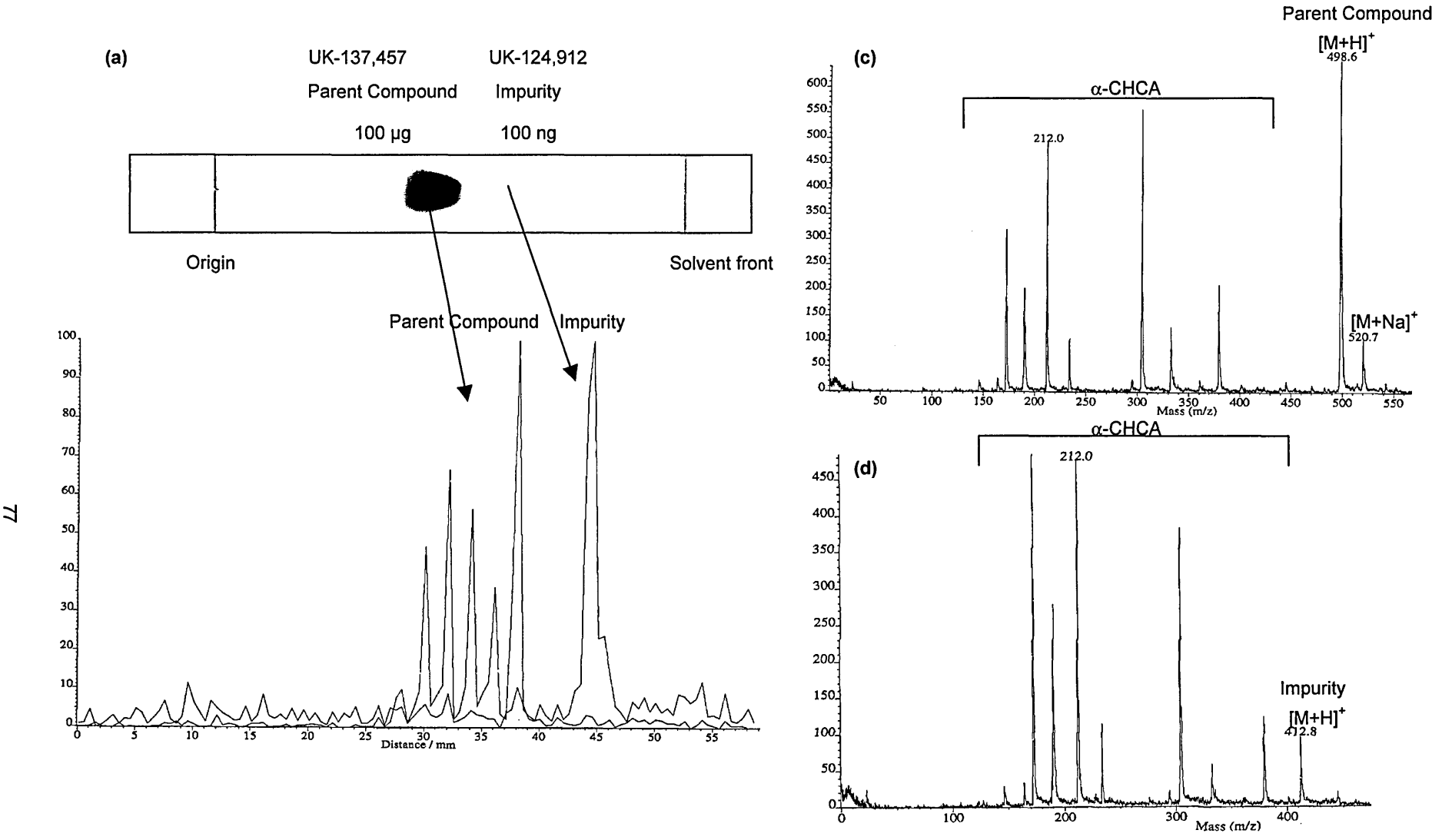


Figure 5-5. TLC-MALDI data obtained from the analysis of UK-137,457, containing UK-124,912 at the 0.1% level. The matrix α-CHCA was applied on the TLC plate with the new electrospray deposition device. (a) TLC separation. (b) Overlaid mass chromatograms obtained by MS scan of the TLC plate. The single mass chromatograms were constructed by use of the $[M+H]^+$ ion of UK-137,457 (m/z 498-499) and of UK-124,912 (m/z 412-413). (c) TLC-MALDI spectrum of the 100 µg spot of UK-137,457. (d) TLC-MALDI spectrum of the 100 ng spot of UK-124,912.

The photographic scan taken from the TLC plate (**Figure 5-4**) indicates that even with a loading of 100 μg of the parent compound the TLC plate is overloaded. **Figure 5-5 a-d** shows the data obtained from the analysis of UK-137,457 containing UK-124,912 at the 0.1% level. In these data, although UK-124,912 is not observable in the UV data (**Figure 5-5 a**), it can be clearly seen in the mass chromatogram of m/z 412-413 (**Figure 5-5 b**) at an R_f-value of 0.63. The protonated molecule at m/z 412.8 in the mass spectrum (**Figure 5-5 d**) correspond to the expected value of UK-124,912.

5.3.3 Improvements in Sensitivity

The sensitivity of the related substance UK-124,912 was assessed by spotting different amounts of it onto a TLC plate, followed by electrospraying of α-CHCA. The detection limit for the acquisition of mass chromatograms was determined as 1 ng (**Figure 5-6**). This is an improvement of a factor of 1000, when compared to the data for tetracycline described in chapter 3 of this thesis.

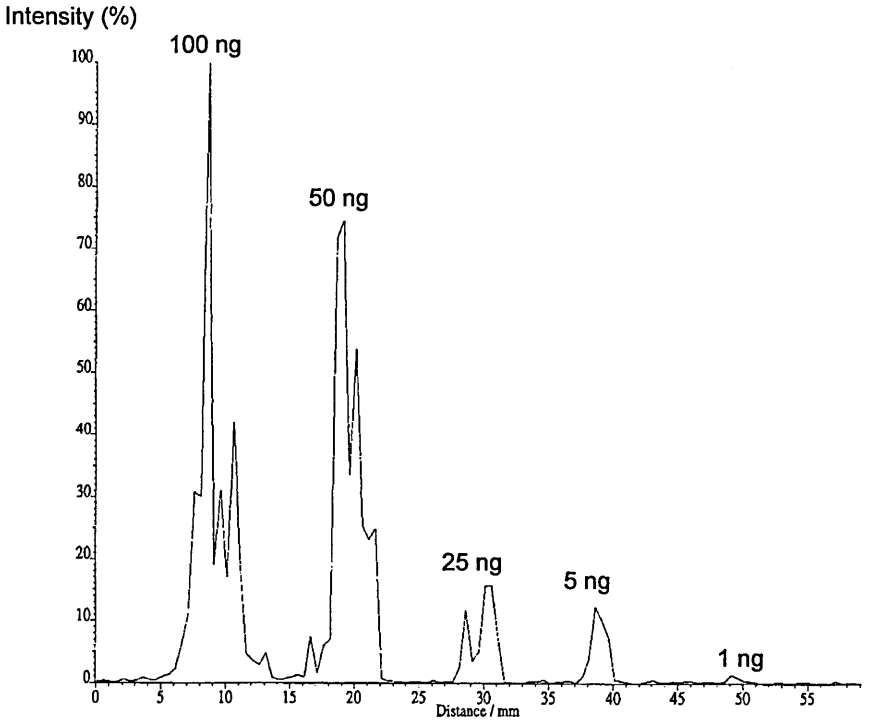


Figure 5-6. Assessment of the detection limit of the related substance UK-124,912. The mass chromatogram was constructed using the $[M+H]^+$ ion of the compound (m/z 412-413).

The question which arises is, if this improvement is only compound specific or is there any difference in the performance between the electrospray deposition device, described in chapters 3 and 4, and the new device. Initial examinations of the TLC plate surfaces by electron microscopy indicated that both devices gave similar good surface coverage of the MALDI matrix, as shown in **Figure 5-7**. However, by using a higher magnification, as shown in **Figure 5-8**, an obvious difference in the porosity of the matrix can be seen.

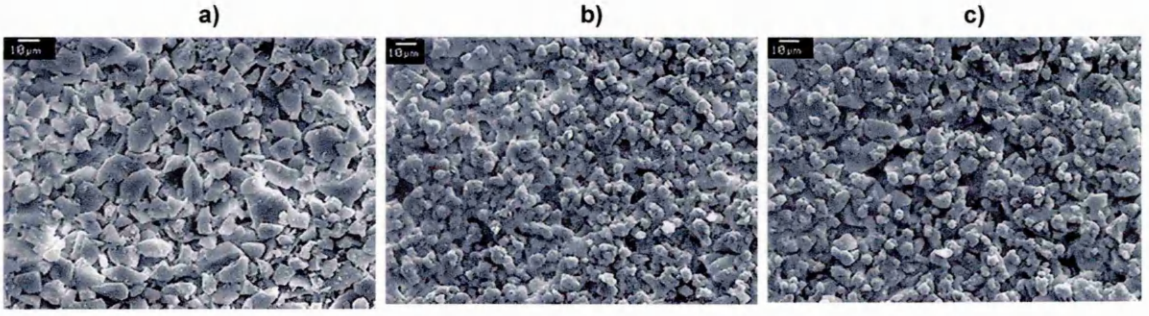


Figure 5-7. SEM pictures of a) blank silica gel TLC plate, and α-CHCA electrosprayed using b) the in-house build electrospray deposition device or c) the new modified commercial x-y-z axis motion system (magnification: 570).

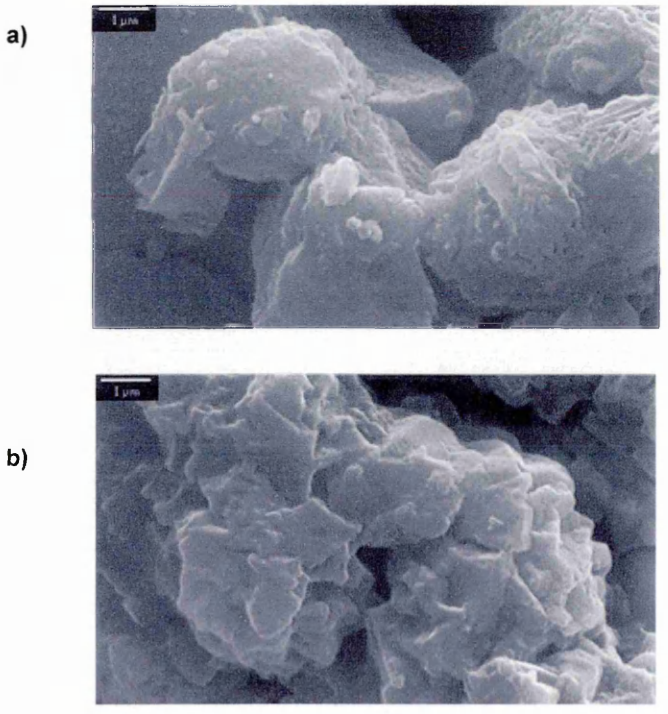


Figure 5-8. SEM pictures shown in Figure 5-7 b and c, but with a magnification of 11400.

The new electrospray deposition device produces crystals with a much higher degree of porosity. These results support the hypothesis that the adsorption model between analyte and matrix does not depend on crystallinity [23]. The solvent-free sample preparation method, introduced by Skelton *et al.* [24], and further investigated by Trimpin *et al.* [23], mixes matrix with analyte powder without the use of a solvent. This method does not require the co-crystallisation of analyte and matrix, which is commonly consider as the key factor for the success and quality of MALDI mass spectra. Trimpin *et al.* [23] concluded that the quality of the MALDI spectra is influenced by the homogeneity of the analyte/matrix mixture. The results presented here would imply that the porosity of the matrix plays an important role, when the matrix is electrosprayed. Whether the increase in the specific surface, obtained by the new electrospray deposition device, simply increases the number of accessible analyte molecules within the desorption area, or whether this also improves the analyte isolation on the surface, is not yet fully understood. Recently, Glückmann *et al.* [25] concluded their discussion of the results, obtained from the solvent-free sample preparation method of different biopolymers, in the same way by leaving this question open.

The possibility that the new electrospray deposition device produces smaller crystals can not be excluded. However a higher flow rate (600 µl/h) and a smaller distance between thetip of the electrospray capillary and TLC surface (2mm), compared to the electrospray deposition device, described in chapter 3 and 4, does not necessarly support this conclusion. Both parameters extend the evaporation time of the solvent.

Preliminary experiments, conducted with the Si 60 F_{254} HPTLC-MALDI target, showed further improvements in sensitivity. It was possible to detect 400 pg of UK-124,912, spotted onto the target, as shown in **Figure 5-9**. This is close to the limit of detection of the mass spectrometer, used in these investigations. The sensitivity lies, according to the instrument specifications, which can be found in the appendix of this thesis (chapter 10), in the sub picomol range. At this very low concentration level reproducibility was not obtained; UK-124,912 was detected in one of two spots. Furthermore, the plotted chromatogram did not show a recognisable chromatographic peak for the analyte due to high background noise.

The sensitivity presented here is poorer than the sensitivity reported by Gusev *et al.* [26-28]. Detection limits in the low nano- or picogram range were reported for several compound classes studied: e.g. angiotensin II 200 pg [26], rhodamine B 50 pg [26], the

pesticides avenage 4 pg [27] and glyodin 4 ng [27], and the nucleotide desoxyribonucleotide monophosphate 10 pg [28]. By using a hybrid TLC-MALDI plate detection limits in the range of 11 to 116 fmol for cyclic peptide spots were achieved by this group [29]. A matrix layer was created on the aluminium support of a 200 μm thick silica gel TLC plate by removing the silica gel layer on one side of the TLC plate.

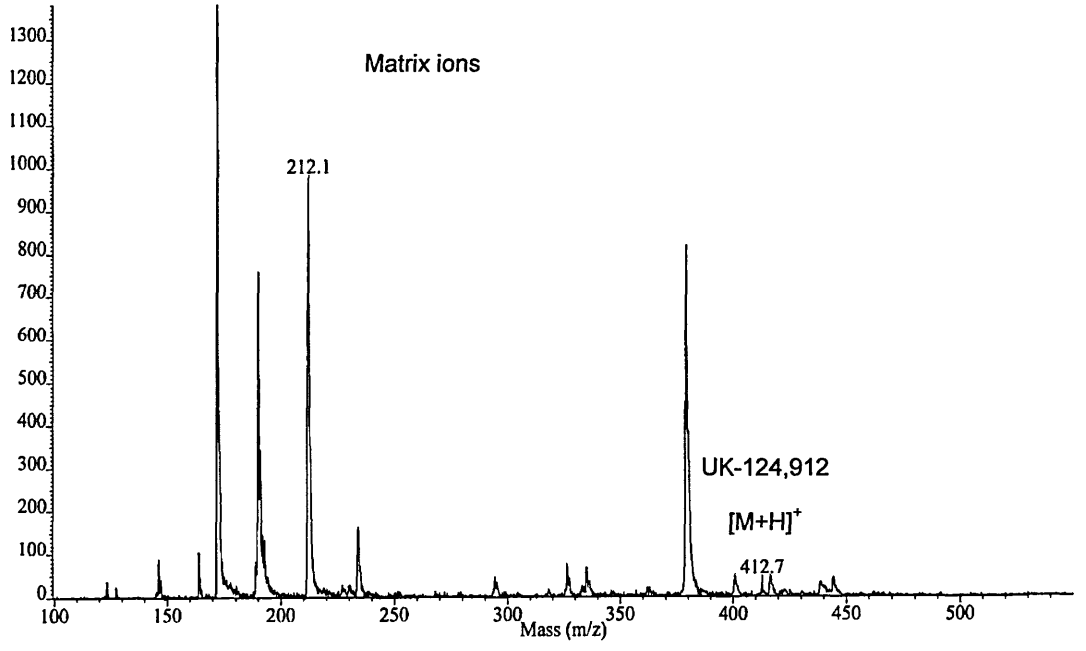


Figure 5-9. Mass spectrum of a 400 pg spot (972 fmol) of UK-124,912 in α-CHCA, recorded on a Si 60 F_{254} HPTLC-MALDI target. The ions of α-CHCA are visible between 120 and 405 Da.

The analyte spots were then eluted to the matrix layer via capillary action. This procedure refocused the analyte spots, enhancing the analyte signals detected by MALDI. However, the chromatographic information contained in the TLC plate is lost in this technique and it seemed to be very time consuming to scrape off the silica gel layer in order to create a MALDI layer on it. The use of a Si 60 F_{254} HPTLC-MALDI target, where the matrix has only to be electrosprayed on the silica gel layer seemed to be more favourable and further improvements in the sensitivity could be obtained by using a smaller sample volume spotted (typically 1 μl volume was used), which would reduce the diameter of the analyte spot and would act as an concentration step.

5.4 Conclusion

Using the modified commercial device mass spectra and chromatograms from a pharmaceutical compound and one of its related substance at the 0.1% level were successfully obtained. TLC-MALDI data down to 2.4 pmol (1 ng) spots were demonstrated and further improvements in the sensitivity were achieved by using a special Si 60 F_{254} HPTLC-MALDI target.

Furthermore, some aspects on the ion formation in MALDI were discussed, based on the TLC-MALDI findings. The porosity of the matrix might also be important, besides its crystallinity, which is commonly considered to be the key factor for a successful MALDI analysis. The role of the pores may be the separation of the analyte molecules from each other, which minimises their interactions, or maybe they simply increase the accessible surface area for analyte molecules to be desorbed by the laser pulse.

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Chapter 6

TLC-PALDI TOF MS of Tetracyclines

6.1 Introduction

Matrix-assisted laser desorption/ionisation (MALDI) is a powerful and widely used mass spectrometric method for the analysis of biopolymers [1], as described in chapter 2. 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 [2]. Mass spectra of various proteins, such as albumin (67 kDa), were recorded by mixing the analyte with nicotinic acid. Since then, different classes of organic acids with pK_a in the range of 2-4 have been considered as potential MALDI matrices [3-5]. Fitzgerald *et al.* [6] introduced a number of basic matrices derived from substituted pyrimidines, pyridines and anilines. Some of these matrices have proven to be excellent for observing non covalent complexes, because they allow samples to be prepared under non-denaturing conditions [7].

Substances other than solids, having a strong absorbance at the laser wavelength of 337 nm, have also been shown to function as UV-MALDI matrices. Liquids, such as 3-nitrobenzyl alcohol or nitrophenyloctylether, have been used in UV-MALDI [8-9], since they circumvent some of the problems associated with solids. The "sweet spot" phenomenon, known for solid matrices, can be largely eliminated, because liquid matrices continuously refresh their surfaces.

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.* [10] first demonstrated laser desorption/ionisation (LDI) spectra of proteins and polymers with molecular weights up to 25 kDa, using 30 nm diameter fine cobalt powder, suspended in a glycerol dispersant. Schürenberg *et al.* [11] 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.* [12] and Dale *et al.* [13-14] 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.* [12] to distinguish this technique from MALDI employing organic matrices. Ho and Fenselau [15] successfully analysed intact gram-negative and gram-positive bacteria by addition of a graphite or cobalt matrix, suspended in glycerol. Instead of using graphite particles, Kim *et al.* [16] demonstrated the use of a graphite plate to absorb and transfer UV energy for the analysis of low molecular weight polymers below 1000 Da. Kinumi *et al.* [17] 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.* [18] reported that fullerene C_{60}, with a diameter of a few micrometers, was a good matrix for protein analysis and Huang *et al.* [19] pursued this technique for the screening of diuretics in urine. More recently, laser desorption/ionisation (LDI) has been achieved without a matrix by depositing the analyte on a UV absorbing silicon substrate, a technique that has been called desorption/ionisation on porous silicon (DIOS) [20-23].

The coupling of thin-layer chromatography to mass spectrometry combines the simplicity of TLC with the selectivity and sensitivity of MS [24-25]. For the analysis of tetracycline antibiotics, as described in this chapter of this thesis, TLC-MS has the specific advantage that the non-volatile compound, disodium ethylene diamine tetra acetate, 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 [26]. FAB-MS was successfully employed by Oka *et al.* [27] 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 [28]. 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 by Chen *et al.* [29-30]. The appropriate zone of the developed TLC plate was coated with a suspension of carbon powder (graphite or activated carbon particles) in glycerol and analysed. Using this approach spectra were obtained for a variety of peptides (e.g. bradykinin and angiotensin II) and low molecular mass organic compounds (e.g. hydrochorothiazide, prometryn, cytosine and PEG 400). By addition of the surfactant p-toluenesulfonic acid the signals of the analytes studied (ATP and methylephedrine)

could be enhanced [31]. In their latest work [32], the graphite matrix is deposited onto the TLC plate before the analyte separation by using a pencil. Using this technique, improvements in the sensitivity of porphyrins were demonstrated. However, the porphyrin derivative meso-tetrakis(pentafluorophenyl)porphyrin is known to be a useful matrix for the analysis of small molecules [33]. Furthermore, even though enhancement in mass resolution were shown, it is still not comparable to the mass resolution, which is typically obtained by coupling TLC with MALDI TOF MS. The reasons for the reduced mass resolution were connected with the movement of the laser in order to obtain ions with a sufficient intensity and the possibly inhomogeneous distribution of the graphite matrix.

Limitations in analytical sensitivity and spectral quality led Han and Sunner [34] to create a carbon activated surface on the aluminium support of the TLC plate, so that the analyte could migrate towards the particle surface. The sensitivity as well as the mass resolution could be readily improved by this new methodology. A similar approach was used by Mehl and Hercules [35], 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 chapter, results for the analysis of tetracyclines by combining TLC with particle-assisted laser desorption/ionisation time-of-flight mass spectrometry (PALDI TOF MS) are reported. Suspensions of micro-particles as well as nano-particles were examined for their suitability for TLC-PALDI MS. The term PALDI was introduced to take into account that a variety of particle materials and sizes can be employed by this technique, in opposite to SALDI (surface-assisted laser desorption/ionisation) [29-34], in which only carbon-glycerol suspensions are used. Furthermore, the pore size seemed to be an important factor in PALDI, whereas in SALDI it is possible to obtain spectra from nonporous surfaces [36].

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

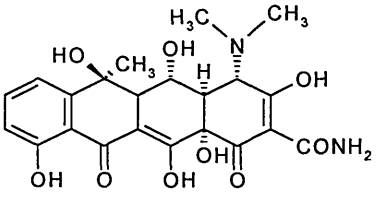
α-CHCA, were used.

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

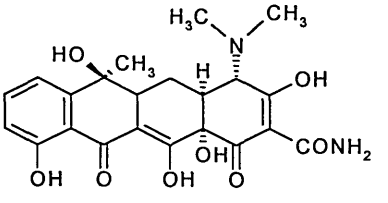
6.2 Experimental

6.2.1 Materials

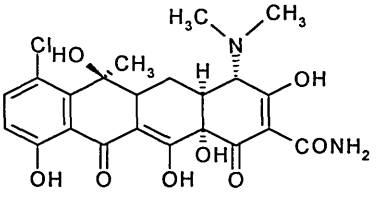
Oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC) and minocycline (MC) were purchased from Sigma-Aldrich (Dorset, U.K.). OTC was used as dihydrate, CTC and MC as hydrochloride. The structures of these compounds are shown in **Figure 6-1**. In all cases the formula weight (FW) quoted is calculated using the mono-isotopic atomic masses. The five tested nano- and micro-particle powders of different materials and particle diameters are listed in **Table 6-1**. All chemicals were used as purchased from commercial suppliers.



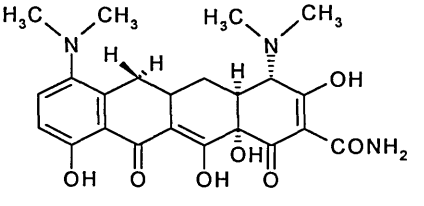
Oxytetracycline (OTC, FW 460)



Tetracycline (TC, FW 444)



Chlortetracycline (CTC, FW 478)



Minocycline (MC, FW 457)

Figure 6-1. Structures of tetracyclines.

Table 6-1. 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

6.2.2 TLC Separation

The tetracycline antibiotics were separated using the procedure described by Naidong *et al.* [37]. 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 minutes in a horizontal position and then activated in an oven (120 °C) for another 30 minutes. The mobile phase dichloromethane /methanol/water (59/35/8, v/v) was saturated for 2 hours prior use. The plates were eluted over a distance of 7.0 cm, air dried and visualised under UV light (λ=254 nm).

6.2.3 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 onto 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 minutes and then applied (30 µl) to the developed TLC strip using a 10 µl syringe.

Experiments with the crystalline chemical matrices DHB and 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 onto the TLC plates using the in-house modified

commercial robotic x-y-z-axis motion system (PROBOT, BAI, Germany), described in chapter 5. An area of 2 × 60 mm on the TLC strip was typically covered with matrix crystals. Good matrix coverage was obtained using the modified device, as shown in **Figure 6-2**. Note: Particle suspension matrices can not be successfully electrosprayed owing to capillary blockages.

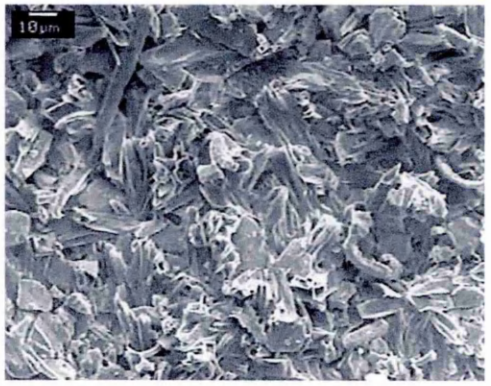


Figure 6-2. SEM image of the crystalline chemical matrix DHB, electrosprayed onto a silica gel TLC plate. The TLC-MALDI mass spectrum and mass chromatogram obtained from this sample are shown in Figure 6-9 and 6-10, respectively.

6.2.4 Mass Spectrometry

Mass spectra and mass chromatograms were recorded directly from the TLC plate with a modified linear LaserTOF 1500 mass spectrometer (SAI, Manchester, U.K.). The modifications to the instrument and its software for use in TLC-MALDI-MS have been described in chapters 3 and 4. 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 mass chromatograms were constructed. The mass chromatograms presented in this chapter are smoothed, since appropriate software was developed in collaboration with SAI.

6.3 Results and Discussion

6.3.1 Comparison of Particle Suspension Matrices with Organic Matrices

For the PALDI-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 PALDI mass spectra obtained for TC using nano-and micro-particles are presented in **Figure 6-3** and **6-4**, respectively. The inorganic matrices showed the following characteristics, compared to the crystalline organic matrices DHB and α-CHCA (**Figure 6-5**). No protonated molecule of TC could be observed, except when silicon powder was used, as shown in **Figure 6-4 c**. 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 (**Figure 6-5**). However the mass spectra were not dominated by complex signals at the lower mass range, as shown in **Figure 6-3** and **6-4**, respectively.

In order to get some idea of the relative sensitivity obtained from TLC-PALDI MS employing suspension matrices, in comparison to the sensitivity obtained from conventional targets, the same quantity of TC (10µg) was analysed on both, silica gel TLC plates and stainless steel targets . Three samples were analysed on each substrate and the results were 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 PALDI MS from conventional targets was two times better than that obtained by TLC PALDI MS.

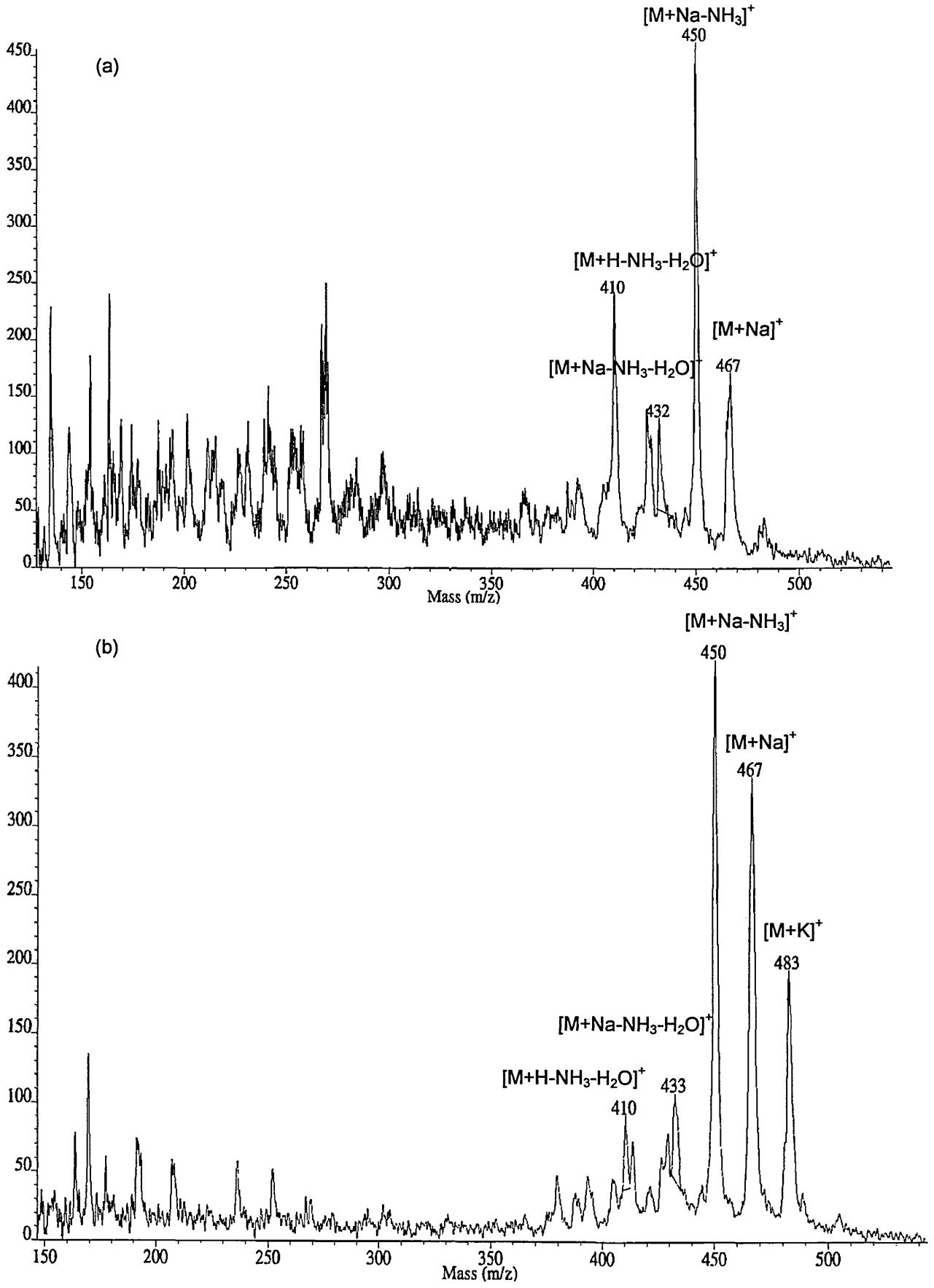


Figure 6-3. PALDI mass spectra of tetracycline using nano-particles, (a) Co-UFP, and (b) TiN.

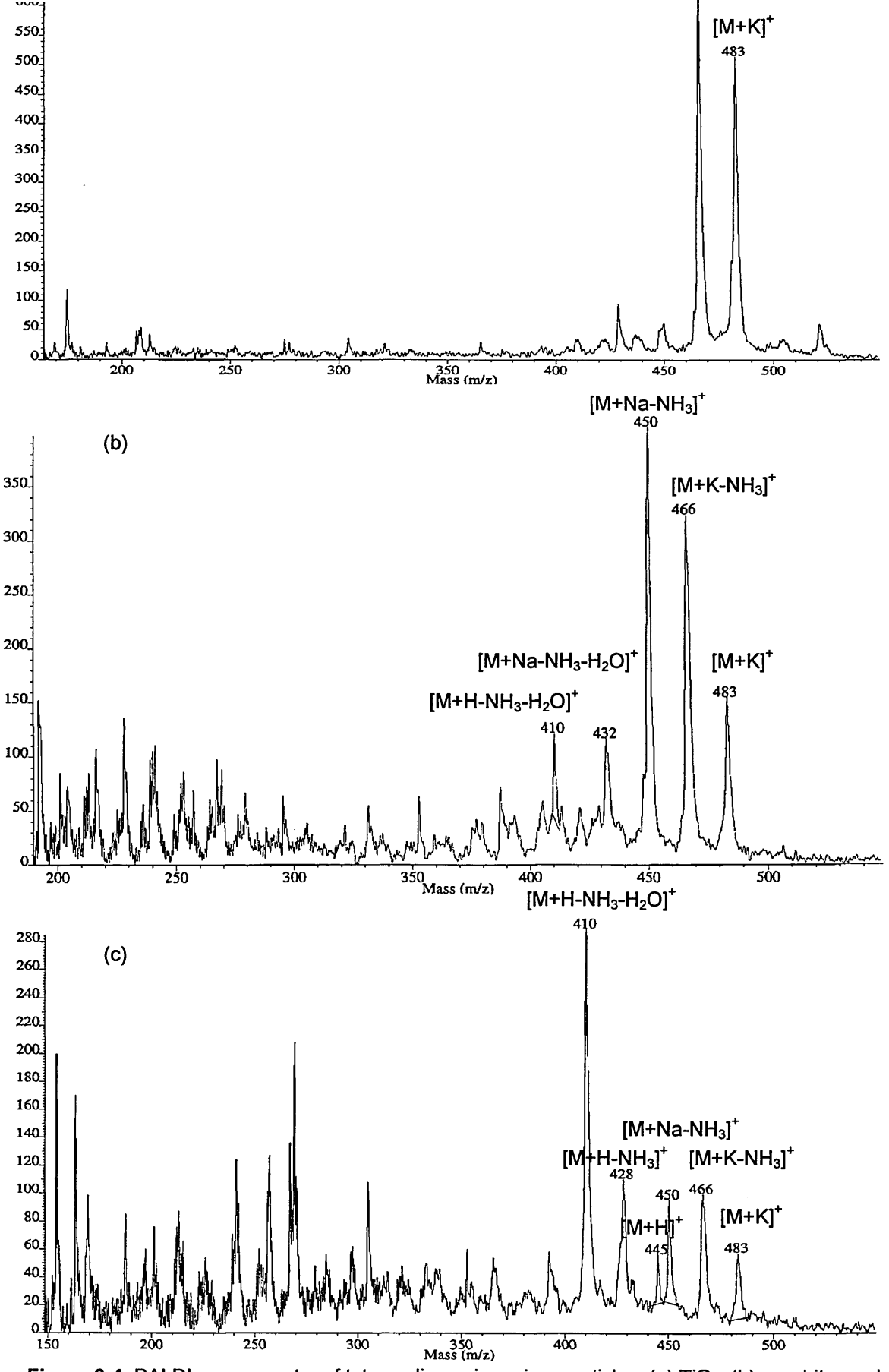


Figure 6-4. PALDI mass spectra of tetracycline using micro-particles, (a) TiO_2, (b) graphite, and (c) silicon.

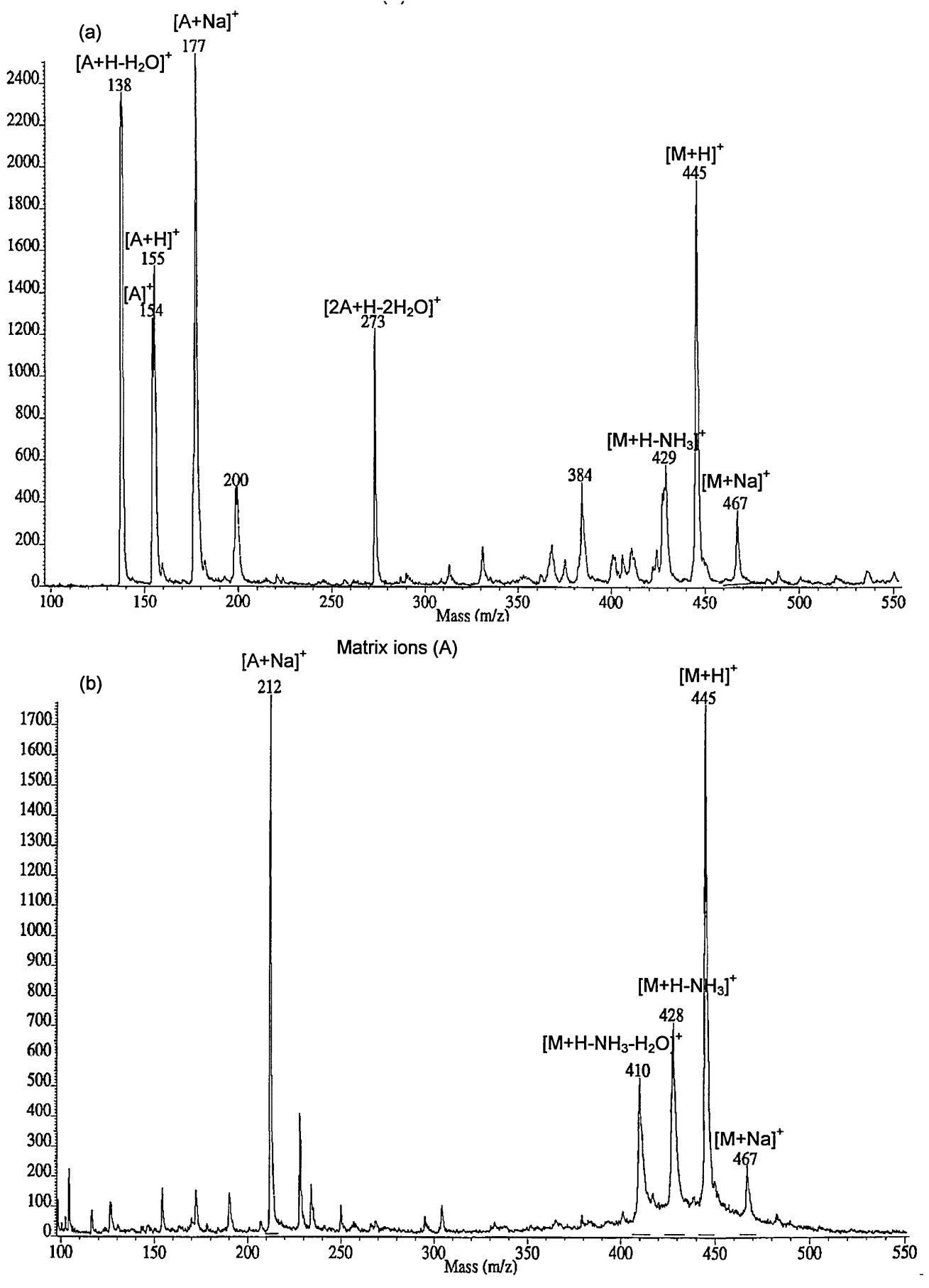


Figure 6-5. MALDI spectra of tetracycline using organic acids, (a) DHB, and (b) α-CHCA.

6.3.2 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 [10], ethylene glycol [38], and liquid paraffin [17]. The dispersant glycerol shows a characteristic background level in the low-mass region, and hence is in general not considered as a good choice for the analysis of low mass analytes [17]. Ethylene glycol showed in the studies presented here 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 phase transition is well documented in FAB and LSI. It is not clear if the function of glycerol is the same in PALDI. Dale *et al.* [13] 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 the initial studies of the work, presented in this chapter, it was found that the absence of dispersant in graphite (or any other particle material) caused the ion intensity 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 grid. As described earlier, typically no protonated signals of TC could be detected when particle/ethylene glycol systems were used (**Figure 6-3** and **6-4**). 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 mobilise the analyte by remaining liquid under vacuum.

6.3.3 Surface Morphology of Particle Suspension Matrices

No mass spectral peak of the antibiotic tetracycline could be obtained, when the analyte was spotted on the TLC plate and directly analysed without the addition of a matrix. This corresponds to the results reported by Wei *et al.* [20]. Obviously a pore size of 6 nm of the silica gel on the TLC plate is too small to give a significant analyte signal.

The importance of the pore size, when using silica gel particles as matrix, was recently reported by Zhang *et al.* [22]. Non-porous silica gel did not yield a mass spectral signal of the analyte. An increase in the pore size up to 30 nm showed an enhancement of the signal intensities of the analytes studied. The role of surface morphology in desorption/ionisation was recently studied for the relatively new technique DIOS [39]. The pore size as well as the overall porosity of the silicon wafers influenced the overall DIOS-MS performance.

The particle suspension matrices, employed in these investigations, showed pore sizes around 200 nm or greater. The surface morphology of Co-UFP and graphite, applied onto a silica gel TLC plate, are shown in **Figure 6-6 a** and **b**, respectively. These results imply that the pore size and moreover the overall porosity is an important factor in the desorption/ionisation process occurring at particle surfaces.

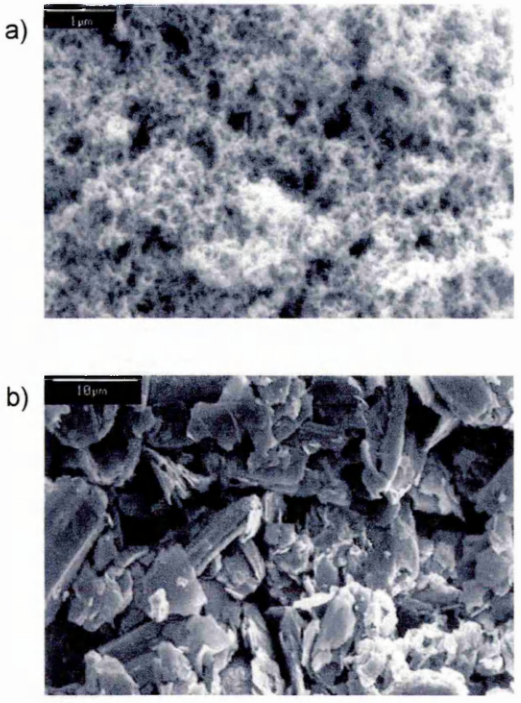


Figure 6-6. SEM images of particle suspension matrices (a) Co-UFP, and (b) graphite, applied onto a silica gel TLC plate.

6.3.4 TLC-PALDI TOF MS of Tetracycline

From the whole range of particles studied, the TLC-PALDI 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 (**Figure 6-9** and the corresponding mass chromatogram is shown in Figure **6-10**).

In TLC-PALDI 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 45 μm, the particles did not adhere to the silica gel layer after solvent evaporation. The addition of an additive, e.g. sucrose [29-32], 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.

Figure 6-7 shows the TLC-PALDI 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, as shown in **Figure 6-8**.

Since the absolute mass accuracy obtained was limited by thickness variations in the graphite film, an effect which has been observed by Zumbühl *et al.* [40], and which results in a mass inaccuracy of ±2 mass units, it was decided to employ a "lock mass" and to recalibrate each mass spectrum during acquisition. 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 signal response and no suppression of analyte signals, and hence was used in further experiments. Phe-Phe (3 mM in ethanol) was added to the graphite suspensions in order to recalibrate the TLC-PALDI 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 experiments

employing DHB (**Figure 6-9**).

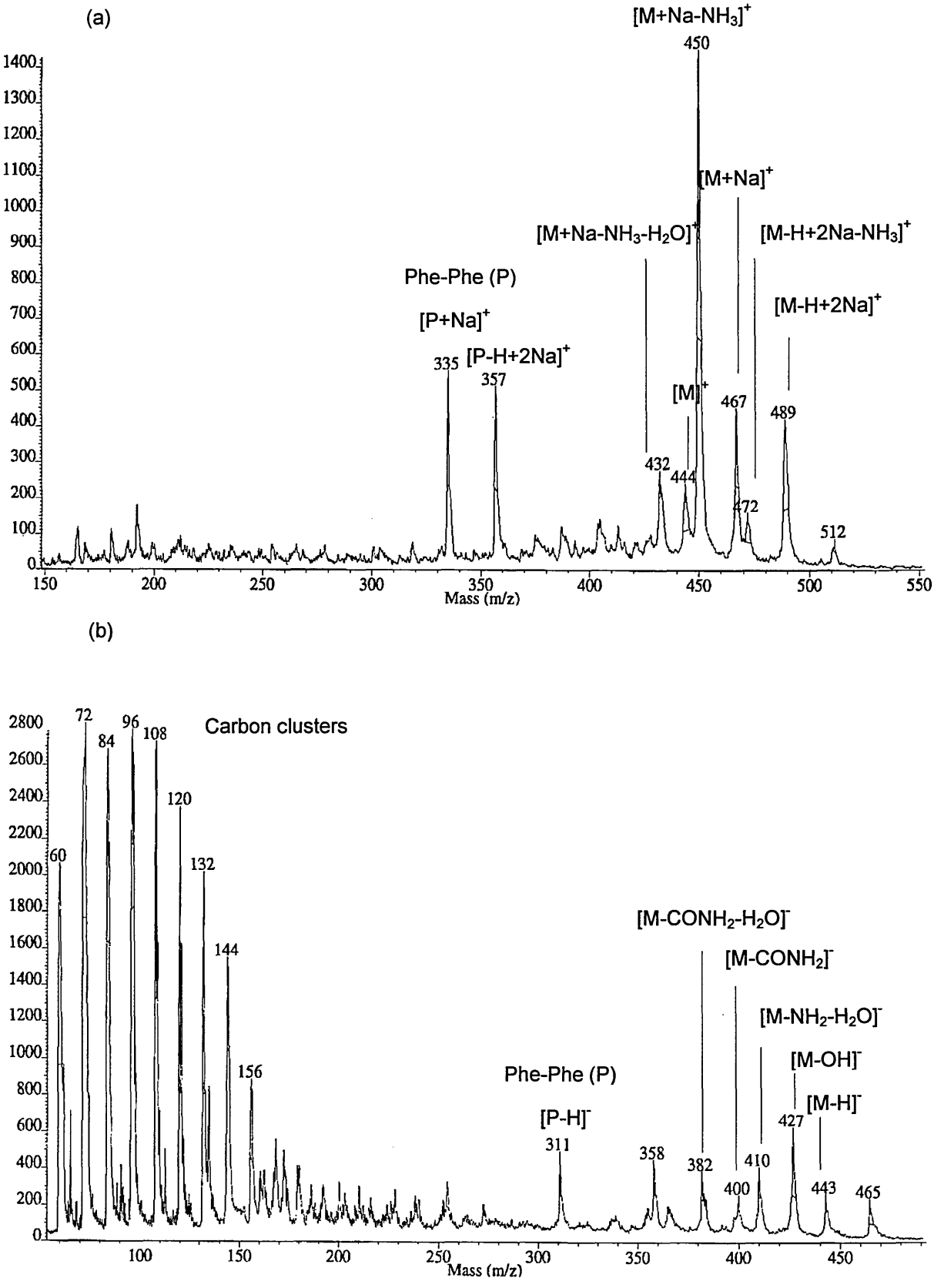


Figure 6-7. TLC-PALDI mass spectra of tetracycline spot (200 µg) using a graphite matrix in (a) positive, and (b) negative ion mode.

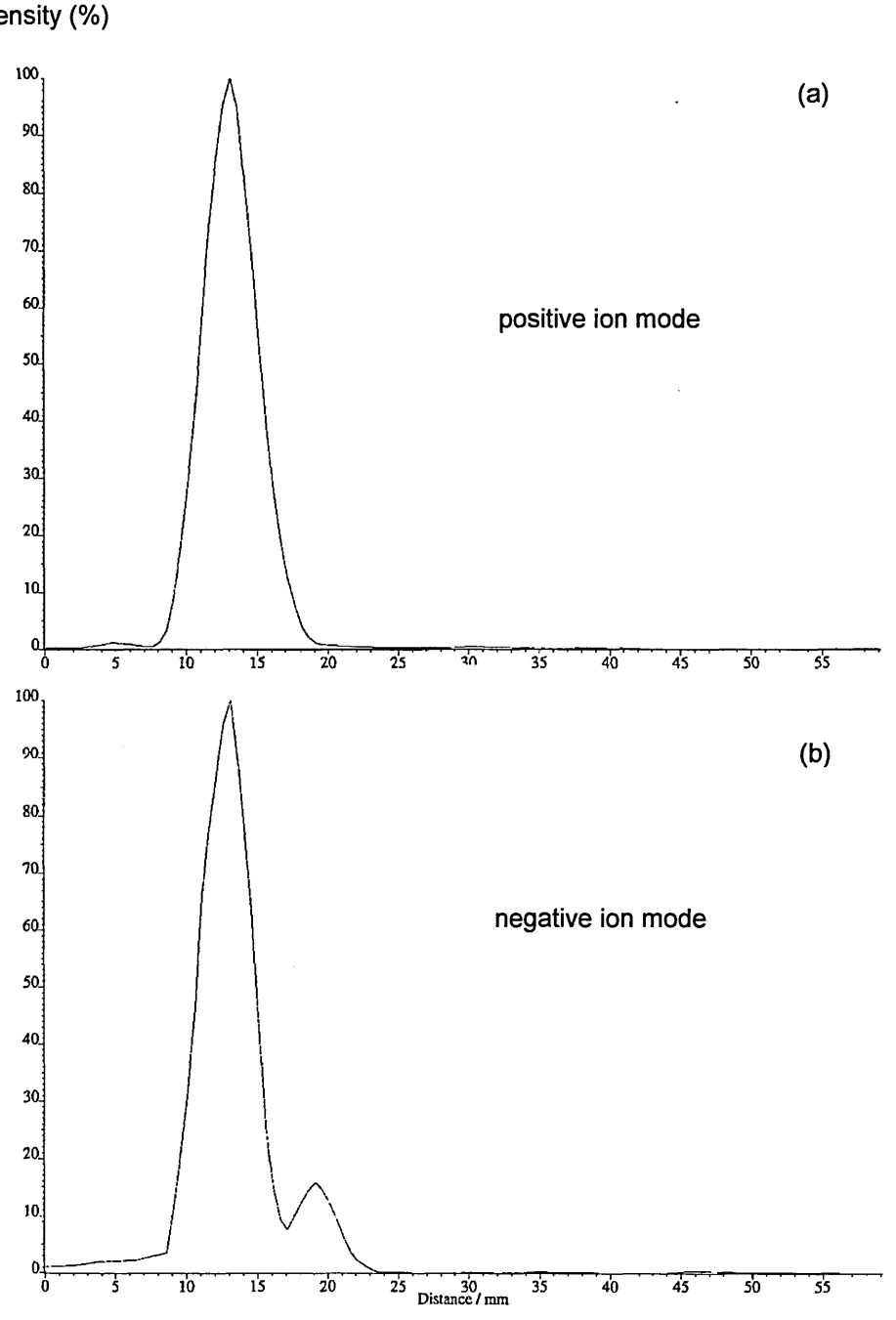


Figure 6-8. Smoothed mass chromatograms of tetracycline (200 µg spot), developed on a TLC plate, using a graphite matrix from the experiments shown in Figure 6-7 a and b. In the positive ion mode the $[M+Na-NH_3]^+$ ion (m/z 449-451) and in the negative ion mode the $[M-OH]^-$ ion (m/z 426-428) was used to construct the mass chromatograms.

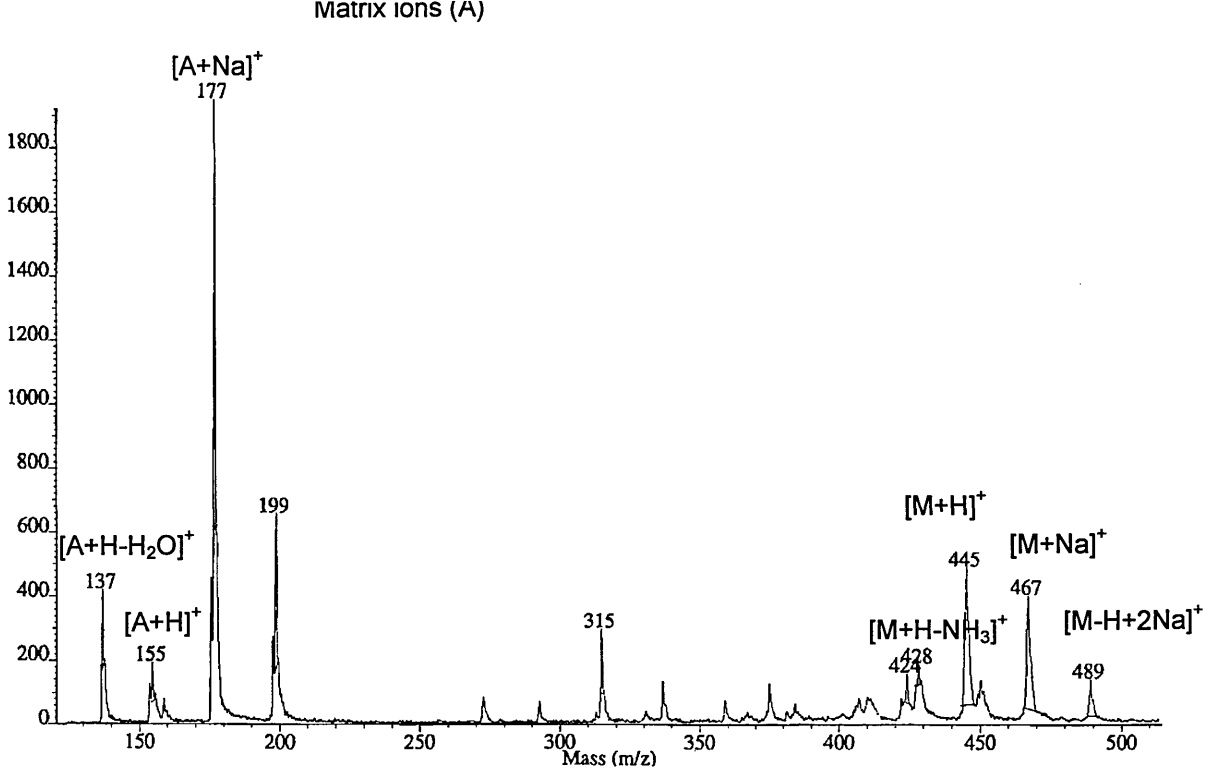


Figure 6-9. TLC-MALDI mass spectrum of tetracycline (200 µg spot) using DHB.

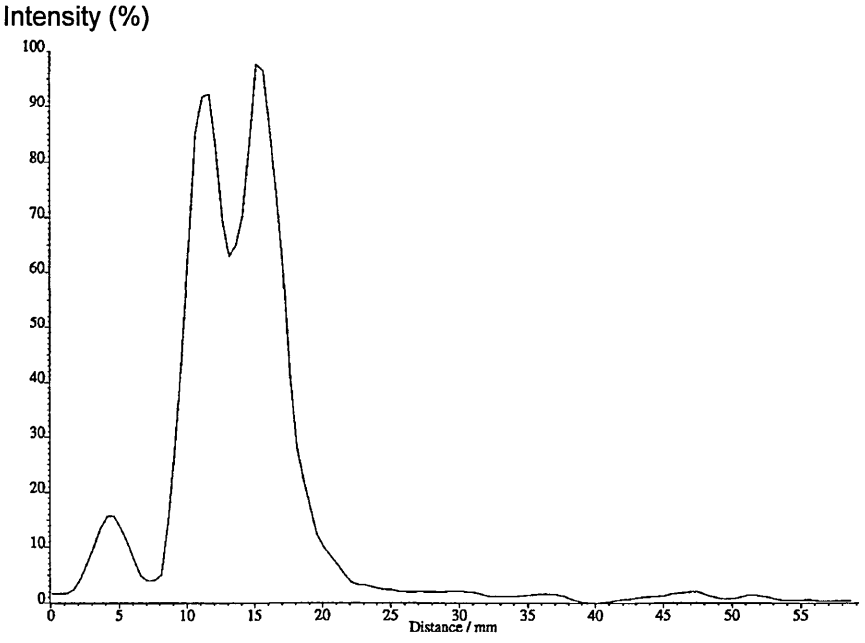


Figure 6-10. Smoothed mass chromatogram of tetracycline (200 µg spot) from the experiment shown in Figure 6-9. The $[M+H]^+$ ion (m/z 444-446) was used to construct the mass chromatogram.

6.3.5 TLC-PALDI 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 is shown in **Figure 6-11**. One unresolved spot for both CTC and TC ($R_f = 0.17$) was observed. From reference runs it was established that CTC has an R_f value of 0.16 and TC one of 0.18. The TLC-PALDI MS analysis of such a mixture confirmed this, as shown by the overlaid mass chromatograms in **Figure 6-12** and the single mass chromatograms in **Figure 6-13**.

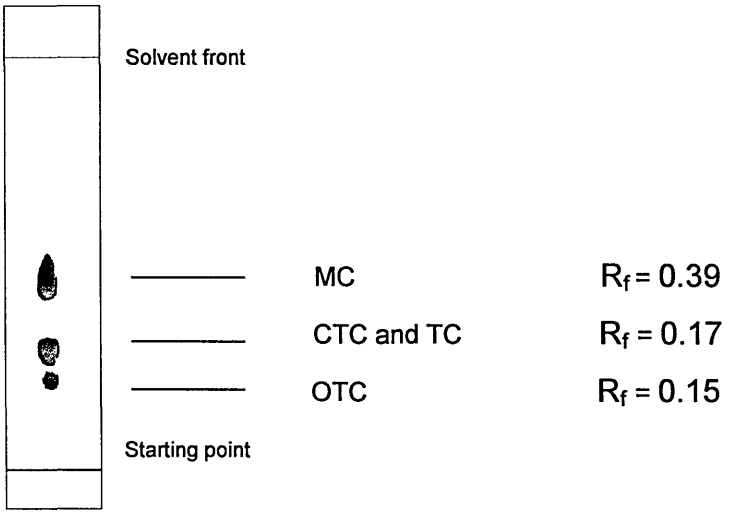


Figure 6-11. TLC separation of a mixture of OTC, CTC, TC and MC (10 μg per component).

This demonstrates the potential of TLC-PALDI 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 PALDI mass spectra recorded, as shown in **Figure 6-14**. Hence the $[M+Na-NH_3]^+$ was used for each tetracycline antibiotic to construct the corresponding mass chromatogram (**Figure 6-12** and **6-13**). The detection limit of tetracyclines in TLC-PALDI 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-PALDI TOF MS. It is anticipated that improvements in sensitivity and reproducibility could be achieved by finding procedures with higher and more consistent extraction efficiency. One possibility is to wet the TLC plate with an appropriate extraction solvent and then to blot the TLC plate

onto a graphite plate.

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 that higher peak temperatures are reached, when particles are employed. Zenobi and Knochenmuss [41] 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.* [11] has estimated that peak temperatures above 10 000 K are possible with 35 nm TiN particles. When a crystalline matrix is used, only temperatures of ca. 500 K are reached [42]. At this level, a thermal ionisation mechanism, is clearly not significant. Another factor, which leads to the consideration that thermal ionisation plays a key role in TLC-PALDI, is that higher laser fluences were required, compared to TLC-MALDI experiments, employing organic acid matrices.

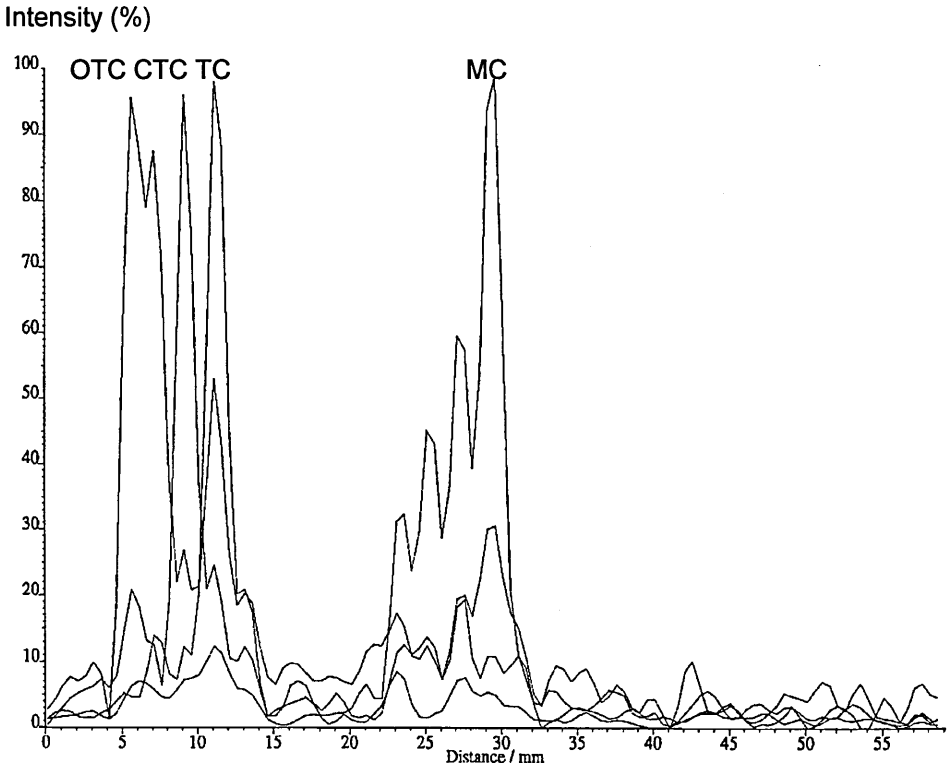


Figure 6-12. Smoothed overlaid mass chromatograms from the TLC-PALDI TOF MS analysis of OTC, CTC, TC, and MC (10 μg per component). The $[M+Na-NH_3]^+$ ion of each tetracycline antibiotic was used to construct the corresponding single mass chromatogram, as shown in Figure 6-13.

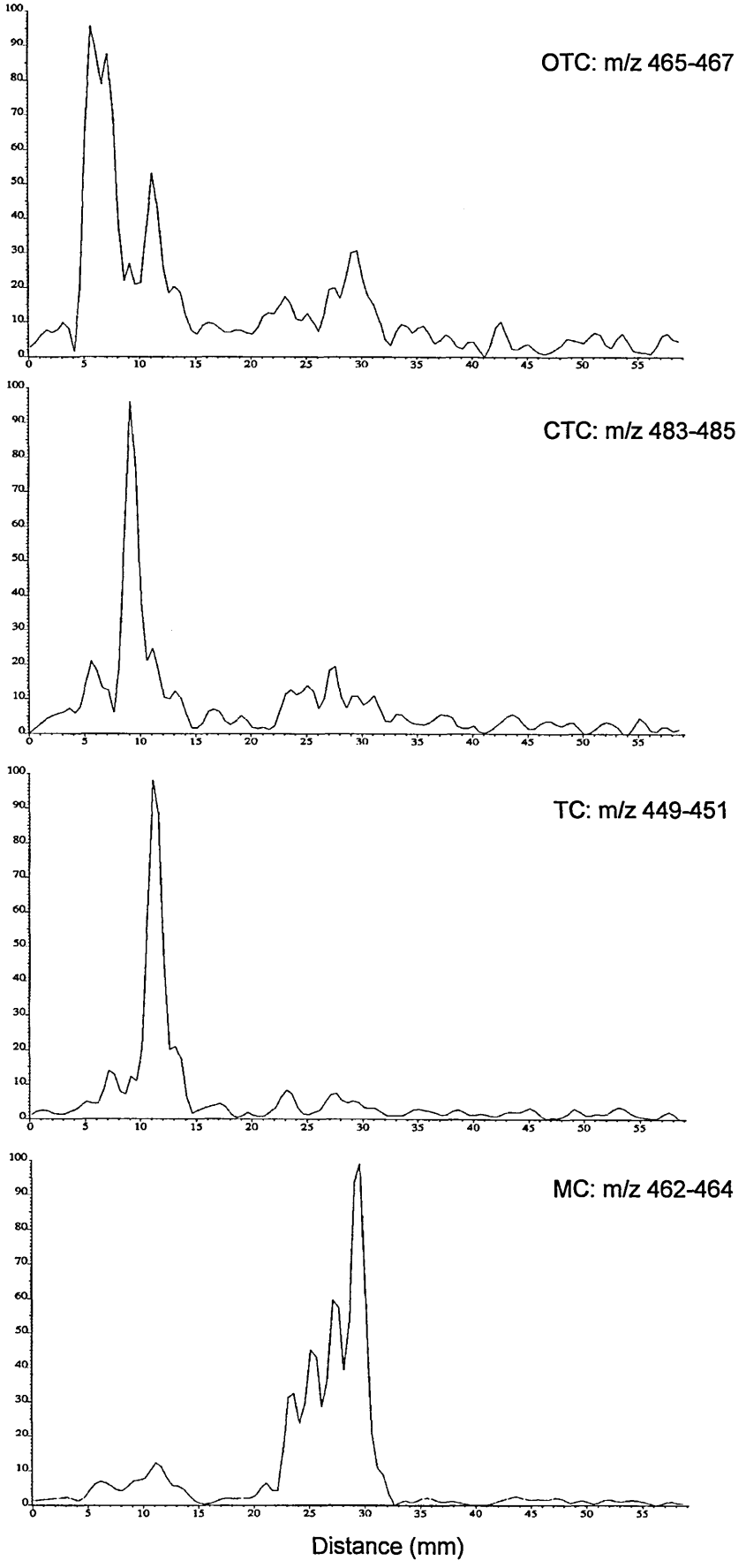


Figure 6-13. Smoothed single mass chromatograms from the TLC-PALDI TOF MS analysis of OTC, CTC, TC, and MC (10 µg per component). The single mass chromatograms were constructed using the $[M+Na-NH_3]^+$ ion of each tetracycline.

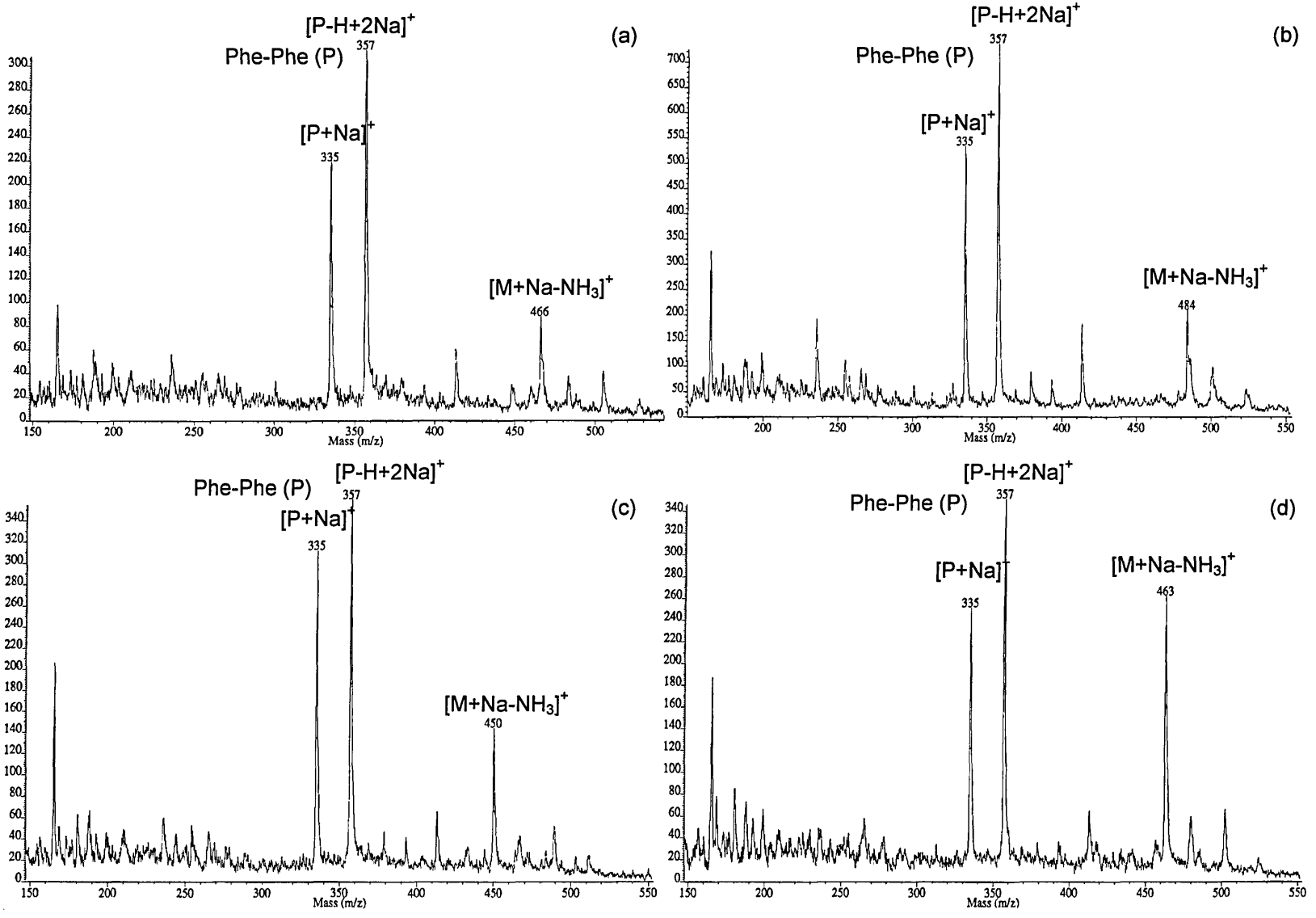


Figure 6-14. TLC-PALDI mass spectra from the peaks indicated in Figure 6-12 and 6-13, (a) OTC, (b) CTC, (c) TC, and (d) MC (10 µg per component).

6.4 Conclusion

The acquisition of chromatographic as well as mass spectral data from eluted TLC plates via TLC-PALDI 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-PALDI mass spectra.

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

6.5 References

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Chapter 7

Quantitative Determination of Piroxicam by TLC-MALDI TOF MS

7.1 Introduction

The quantitative determination of pharmaceuticals by TLC-MS has been the subject of several reports [1-6]. Caffeine was quantified by off-line TLC-SPE-APCI-MS in soft drinks [1]. On-line TLC-MS techniques, which have the advantage of allowing the generation of mass spectral as well as chromatographic information from the TLC spots, have been used for the quantification of the following drugs, pyridostigmine [2] and nicerogline [3] by TLC-LSI MS, the diuretic amiloride by TLC-FAB MS [4], imipragmine by TLC-two step laser mass spectrometry (L2 MS) [5], and cocaine by TLC-MALDI MS [6]. In most of the quantification experiments a chemical or stable isotope analogue was used as internal standard to enable quantification to be carried out directly on the separated TLC spots. TLC-FAB MS as well as TLC-LSI MS require a liquid matrix, which causes diffusion of the analyte after a certain time and hence makes the recording of a stable ion signal impossible. The two other soft ionisation techniques L2 MS and MALDI coupled to TLC show signal variations due to alterations from laser to laser pulse.

The non steroidal anti-inflammatory drug (NSAID) [7] piroxicam or 4-hydroxy-2-methyl-N-(2-pyridyl)-H-1,2-benzothiazine-3-carboxamide-1,1-di-oxide is widely used in the treatment of rheumatological disorders [8]. Several analytical methods have been described for the determination of piroxicam, including TLC [9-12], CE [13], spectrofluorometry [14], derivative spectrometry [15-17] and HPLC [18-21]. There appear to be no reports of the application of TLC-MALDI MS to the determination of this pharmaceutical. The main advantages, arising from the development of such a method, would be its simplicity, sensitivity and specificity.

Hence, the aim of the study, described in this chapter, was to develop and validate a quantitative TLC-MALDI TOF MS method for the analysis of piroxicam. Tenoxicam, a structural analogue of piroxicam, was used as internal standard to compensate for MALDI MS signal deviations and variations in the extraction efficiency of piroxicam from the TLC plate. To regulate the analyte signals, recorded directly from the TLC plate, the internal standard has to be located at the same positions as the analyte. Preliminary data, obtained by spotting mixtures, containing the internal standard and the analyte, on silica gel 60 F_{254} TLC plates proved that quantification of piroxicam with the chosen internal standard was possible. The next step was to find a suitable method

of incorporating the internal standard on the TLC plate so that the development of the TLC plate was possible. Since the internal standard did not show the same R_f value as the analyte in the TLC analysis, the following approaches were tested: development of the TLC plate in the mobile phase to which the internal standard was added, and electrospraying of a solution of tenoxicam or a mixture with the matrix onto the TLC plate. The best precision for the standard calibration curve, between 400 and 800 ng of piroxicam, was obtained by incorporating the internal standard in the mobile phase (15 to 63 % range errors, n = 4). The other methods investigated showed higher range errors above 86 % for the lowest point of the calibration curve, and hence were not considered as suitable.

7.2 Experimental

7.2.1 Materials

Piroxicam (PX) and tenoxicam (TX) were obtained from Sigma-Aldrich (Dorset, U.K.). Their chemical structures are shown in **Figure 7-1**. In both cases, the formula weight quoted is calculated using the mono-isotopic atomic masses. All chemicals were used without further purification, as supplied by commercial suppliers.

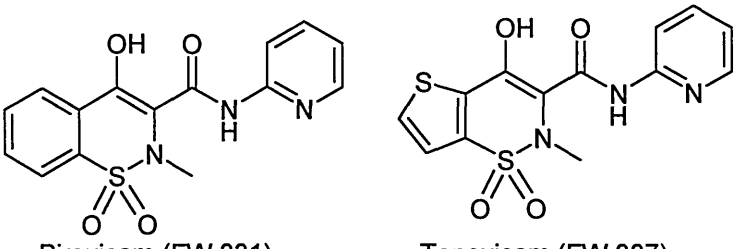

Figure 7-1. Chemical structures of analyte and internal standard.

7.2.2 Instrumentation and Conditions

For the application of the MALDI matrix on the silica gel surface of the TLC plate an in-house modified commercial robotic x-y-z-axis motion system (PROBOT, BAI,

Germany) was used. Modifications to the instrument have been described in chapter 5. Mass spectra were recorded directly from TLC strips with a modified linear LaserTOF 1500 mass spectrometer (SAI, Manchester, U.K.). The modifications to the instrument and its software have been extensively described in chapters 3 and 4. Experiments were carried out using a 10 kV extraction voltage. Only the positive ion mode was used 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 50 or 55 mm and mass spectra were recorded every 0.5 mm. A data set of 100 or 110 mass spectra was obtained for each TLC strip.

7.2.3 Quantification Methods

The section is divided into four sub-sections 1, 2, 3 and 4, which describe the different approaches for the incorporation of the internal standard, tenoxicam, into the TLC plate.

Method 1: Mixing of the internal standard to the analyte solution and spotting of the resulting mixture onto the TLC plate.

Method 2: Pre-development of the TLC plate in the mobile phase, to which the internal standard is added, followed by development of the analyte in the same mobile phase.

Method 3: Electrospraying of the internal standard prior matrix application.

Method 4: Mixing of the internal standard with the matrix solution and electrospraying of the resulting solution.

Calibration Standards

Method 1: Primary stock solutions of piroxicam (1 mg/ml) and tenoxicam (2 mg/ml) were prepared in hydrochloric acid (2.0 M), respectively. Five standard solutions of piroxicam (0.4, 0.5, 0.6, 0.7 and 0.8 mg/ml), with a constant concentration of tenoxicam (0.4 mg/ml), were obtained by combining aliquots of both primary solutions and diluting with HPLC-grade methanol.

Method 2: Primary stock solution of the analyte piroxicam (1 mg/ml) was prepared in HPLC-grade dichloromethane and five standard solutions (0.4, 0.5, 0.6, 0.7 and 0.8 mg/ml) were obtained by subsequent dilution of the primary stock solution.

Method 3 and 4: Five standard solutions of piroxicam (0.4, 0.5, 0.6, 0.7 and 0.8 mg/ml) were obtained by diluting the primary stock solution of piroxicam, prepared in method 1, with HPLC-grade methanol.

Sample Preparation

Method 1: Aliquots of standard solutions (1µl) were applied to an aluminium-backed TLC plate coated with a 0.2 mm layer of silica gel 60 F_{254} (Merck, Germany) using disposable spotting pipettes (Camag, Switzerland). A distance of 10 mm between the analyte spots was chosen to keep them separate in order to be able to detect 5 spots in a single experiment. After UV detection (λ = 254 nm) of the analyte spots, a strip of the TLC plate (2 × 50 mm) was cut out and attached to a modified MALDI target using double sided tape. The matrix α-CHCA (Sigma-Aldrich, Dorset, U.K.) was dissolved in HPLC-grade methanol containing 0.1% TFA. A solution of 20 mg/ml of α-CHCA was electrosprayed onto the silica gel TLC strip and the TLC sample was subjected to MALDI analysis after 30 minutes drying at room temperature.

Method 2: The TLC plate was pre-developed in 25.0 ml chloroform-methanol (9:1, v/v), in which tenoxicam was dissolved to a concentration of 0.4 mg/ml. The chamber was lined with filter paper and saturated for 30 minutes. The TLC plate was developed for 17 hours in the mobile phase to obtain a homogeneous surface coverage of tenoxicam. After air drying, 1µl aliquots of standard solutions were applied to the pre-developed TLC plate and the plate was developed again in the same mobile phase. The mobile phase was freshly prepared and saturated for one hour prior use. A typical TLC plate obtained by this procedure is shown in **Figure 7-2**. After the developed TLC plate was air dried, α-CHCA was electrosprayed onto the plate followed by MALDI-MS analysis, as described for method 1.

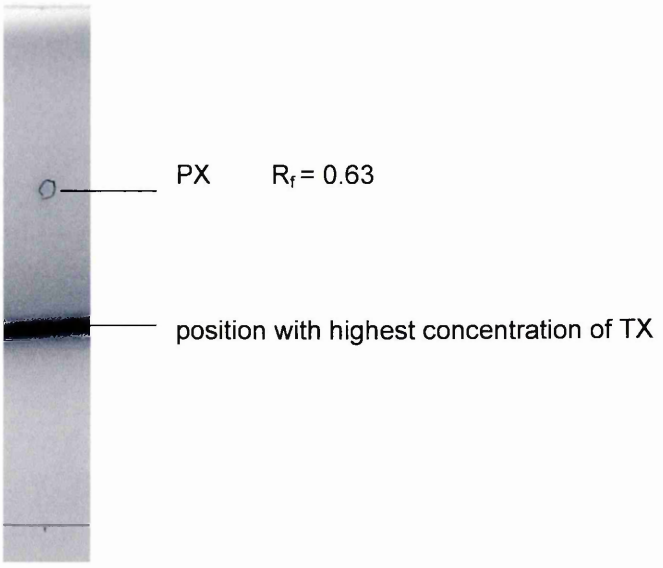

Figure 7-2. Typical TLC separation of 600 ng of piroxicam (PX), separated on a pre-coated TLC plate, according to method 2. The grey colour throughout the TLC plate represents the surface coverage of tenoxicam (TX).

Method 3: Aliquots of standard solutions (1µl) were applied to the TLC plate, as described for method 1, and a 0.25 mg/ml solution of tenoxicam, dissolved in acetone, was electrosprayed on the TLC plate, followed by electrospraying of the matrix solution.

Method 4: Aliquots of standard solutions (1µl) were applied to the TLC plate, as described for method 1. A 0.1 mg/ml solution of tenoxicam, dissolved in dichloromethane, was mixed to the matrix solution in the volume ratio 1:1 and electrosprayed on the analyte spots.

Calibration Curves

Standard curves were constructed for samples spots containing 400 to 800 ng of piroxicam for each method. Peak responses of piroxicam and tenoxicam were measured in each of the mass spectra recorded. From these data mass chromatograms of Piroxicam (PX) and tenoxicam (TX) were generated for each sample (**Figure 7-5**). The piroxicam-to-tenoxicam ratio for each single spot on the TLC strip was calculated using the software package Origin (OriginLab Corporation). For each spot, represented in the mass chromatograms as a peak, the piroxicam-to-tenoxicam ratio was obtained by dividing the integrated area of the piroxicam signals by the integrated area of the

tenoxicam signals at the same positions on the TLC plate. The piroxicam-to-tenoxicam ratios obtained by this methodology were plotted versus the corresponding piroxicam quantity. The calibration curves were determined by least-square linear regression analysis and the precision was evaluated on the range errors of the replicates. The calculated range errors are listed in the appendix of this thesis.

Method Validation

Method 1 and 2 were assessed by the following criteria [22]: accuracy, precision, specificity, limit of detection, limit of quantification, linearity and range of measurement. The robustness could not be determined, since the methodology is currently unique to this laboratory.

Accuracy and Precision: To evaluate the accuracy and precision of the proposed methods three quantities within the previously determined linear range (450, 600 and 750 ng piroxicam) were selected and analysed. From each quantity ten determinations (method 1) or five determinations (method 2) were performed. The accuracy of the method was defined as the amount of piroxicam determined by TLC-MALDI MS, expressed as a percentage of the "true" amount of piroxicam in the sample spot. To be acceptable, measures should be within ± 15% at all concentrations [23]. The precision of the method was expressed as the relative standard deviation (RSD) of replicate analyses, carried out within one working day. To be acceptable, measures should be lower than ± 15% at all concentrations [23].

Specificity: Six blank TLC plates were tested for the presence of interfering peaks, arising from the silica gel layer and the matrix.

Limit of Detection and Quantification: The limit of detection (LOD) was defined as the piroxicam amount, resulting in a peak area of three times the noise level. The limit of quantification (LOQ) was defined as the piroxicam amount, resulting in a peak area of ten times the noise level. The LOD and LOQ value were calculated from the constructed calibration curves [24], shown in **Figure 7-6a** and **b**.

Range: The range was determined by selecting three amounts of piroxicam (a lower, middle and upper amount) and demonstrating that the analyses could be obtained with a

suitable level of precision, accuracy and linearity.

7.3 Results and Discussion

7.3.1 Matrix Selection

The suitability of the following MALDI matrices, α-CHCA, DHB, 3-HPA, and HABA, was studied for the quantitative analysis of piroxicam. The selection of the matrix was based on examination of their MALDI-MS behaviour on stainless steel substrates. The matrix α-CHCA was found to give the best results, based on the following criteria, i.e. absence of interfering peaks in the spectral region of interest and reproducible analyte signal intensity from laser spot to spot over the target (good crystal homogeneity).

7.3.2 Internal Standard Selection

For quantitative MALDI analysis an internal standard is required to improve shot-to-shot and sample-to-sample reproducibility. The selection of an appropriate internal standard is critical for the success of such an analysis [25]. An isotopically labelled analogue is an ideal internal standard [26] in mass spectrometry, however these are seldom available and structural analogues are often employed, e.g. for the quantification of biomolecules [27] or small molecules [28] by MALDI mass spectrometry.

The selection of a suitable internal standard for quantification using TLC-MALDI was based on two important properties of the internal standard. Firstly, it needed to be chemically similar to the analyte so that similar extraction efficiencies out of the silica gel phase were achieved. Secondly, for these experiments a reasonable mass separation between analyte and internal standard signal was required since the mass spectrometer, employed in these investigations has a mass resolution of only ~425 (FWHM) for piroxicam. For these reasons tenoxicam, a structural analogue of piroxicam, was chosen. Tenoxicam is, as well as piroxicam, a member of the N-heterocyclic carboxamide derivatives of benzothiazine-1,2-dioxide and has been previously used as

internal standard for the quantitative HPLC analysis of piroxicam [21]. Their mass difference of 6 mass units gave adequate resolution of their protonated molecules (**Figure 7-3** and **7-4**, respectively).

7.3.3 TLC-MALDI Analysis

Method 1: Preliminary experiments, involving spotting mixtures containing 400 to 800 ng of piroxicam and the same amount of tenoxicam (400 ng) on untreated silica gel TLC plates, were carried out to prove that quantification of piroxicam with tenoxicam as internal standard is possible by TLC-MALDI TOF MS and to show that the method could be validated. Typical TLC-MALDI mass spectra, obtained from the blank TLC plate and a 800 ng spot of piroxicam containing 400 ng of tenoxicam, are shown in **Figure 7-3a** and **b**, respectively. Since the primary stock solutions of analyte and internal standard were prepared in hydrochloric acid and diluted with methanol to obtain the required concentrations, typically no sodium adduct of the matrix (m/z 212) was obtained at the location of the TLC spots (**Figure 7-3b**). The mass chromatograms, obtained for the analyte (m/z range: 331-333) and the internal standard (m/z range: 337-339), using this approach, are presented in **Figure 7-5a**. Piroxicam showed a higher signal response compared to tenoxicam and appeared to give a better MALDI response. Although piroxicam was increased in the spots from the left to the right side in **Figure 7-5a**, a steady increase in the recorded signal intensities was not obtained. Moreover, all five spots contained the same amount of tenoxicam, but the signal responses varied greatly. These variations are caused by local concentration changes of the compounds in the crystal structure [25]. The reason for these variations can be explained as follows. The solvent, in which the matrix is dissolved, acts as an extraction solvent of the compounds, spotted on the TLC plate (analyte and internal standard). The extraction efficiency of the solvent throughout the scanned TLC strip varies from position to position and causes these variations. This shows how important the use of an internal standard is to normalise MALDI signals from a TLC plate when attempting quantification.

An effect, where the signal from a less concentrated component is suppressed by that from a more concentrated one, has been described for MALDI-MS [25, 27, 29]. In the TLC-MALDI experiments presented here this effect lead to the limited linear range

(400 to 800 ng) obtained in the constructed calibration curve (**Figure 7-6a**).

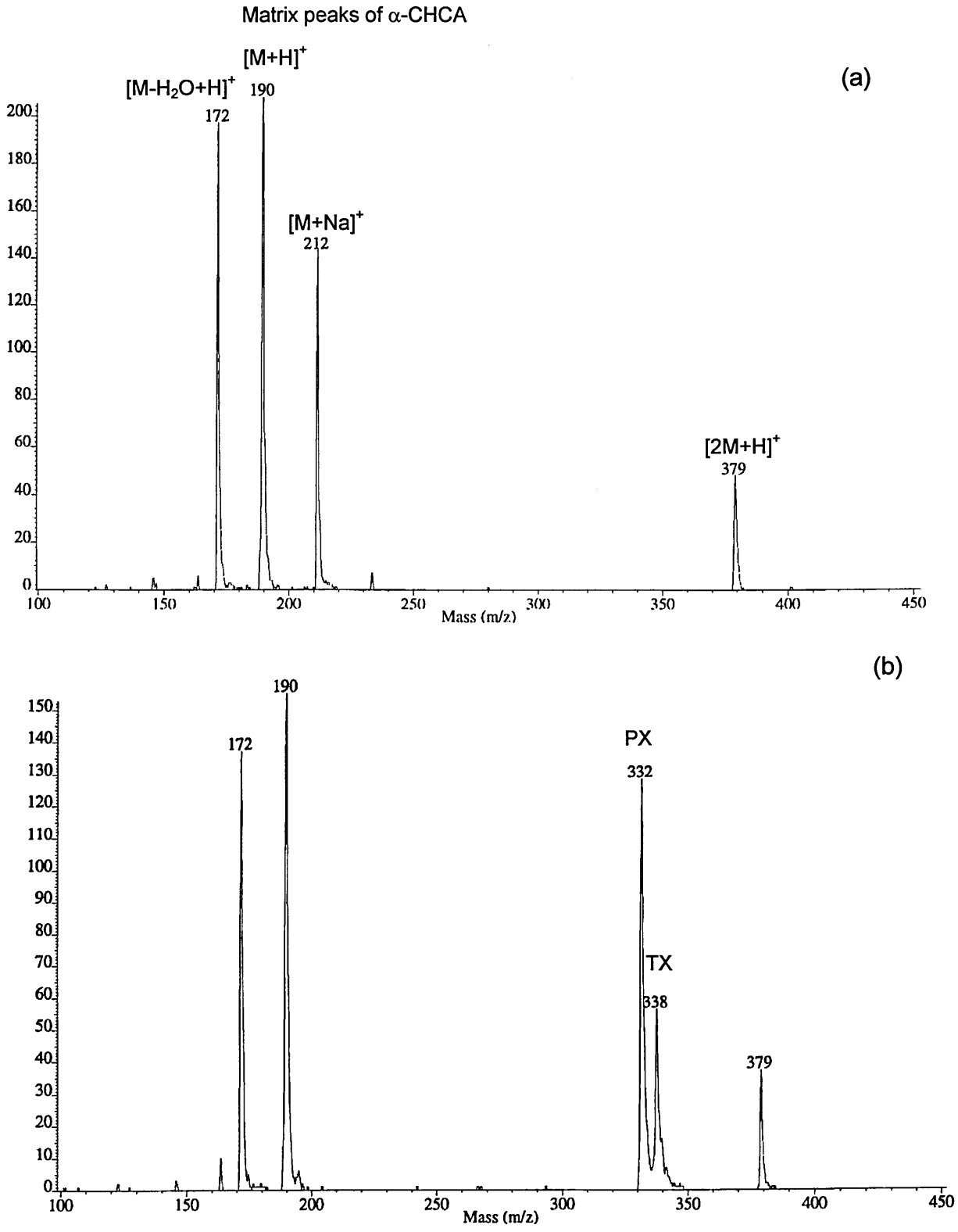

Figure 7-3. TLC-MALDI spectra of (a) blank sample, and (b) 800 ng of piroxicam (PX) containing 400 ng of tenoxicam (TX) (method 1).

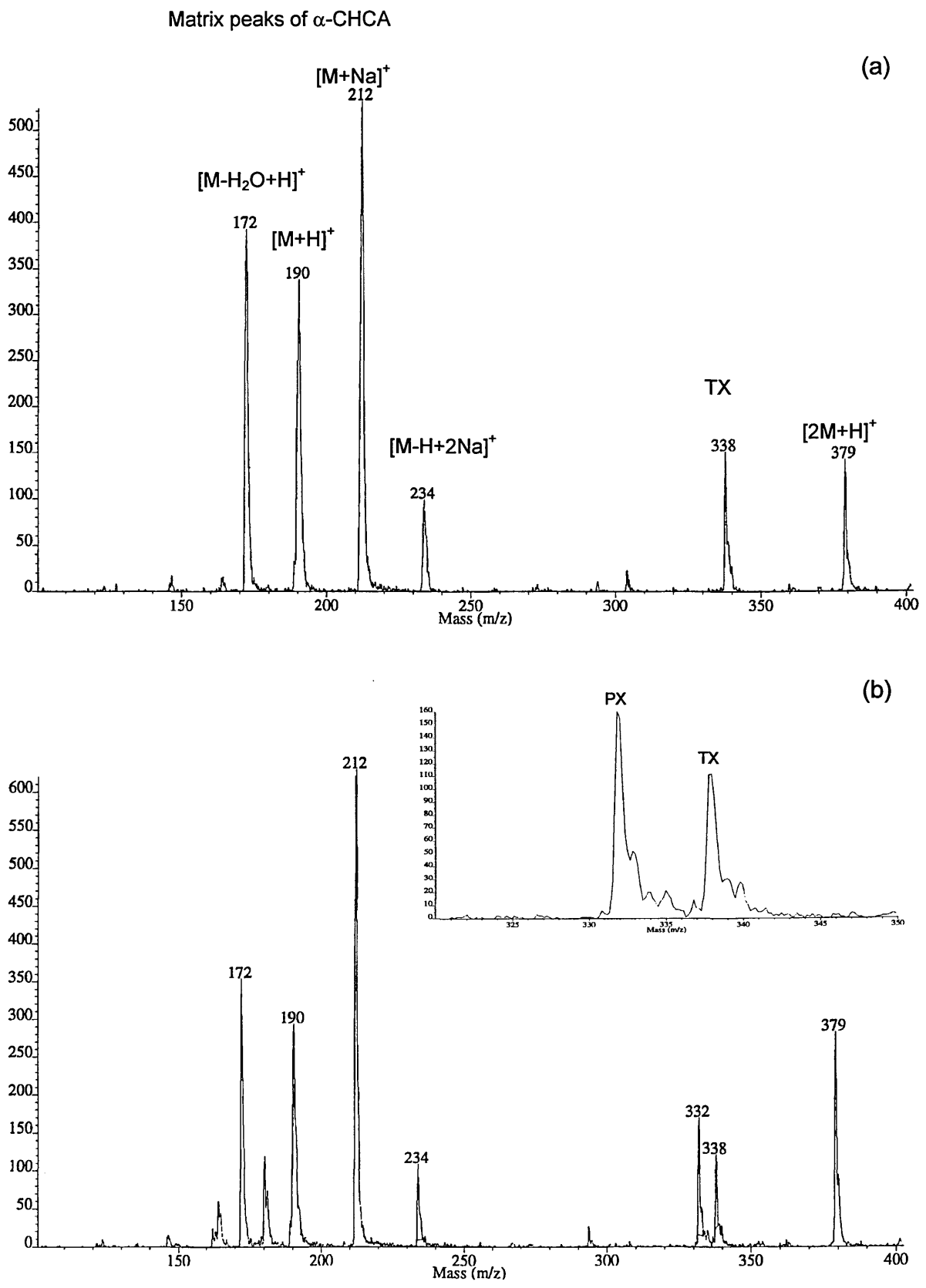

Figure 7-4. TLC-MALDI spectra of (a) blank sample, the internal standard tenoxicam (TX) is pre-developed on the TLC plate, and (b) 600 ng of piroxicam (PX) (method 2). The intercept shows the region around PX (m/z 332) and TX (m/z 338).

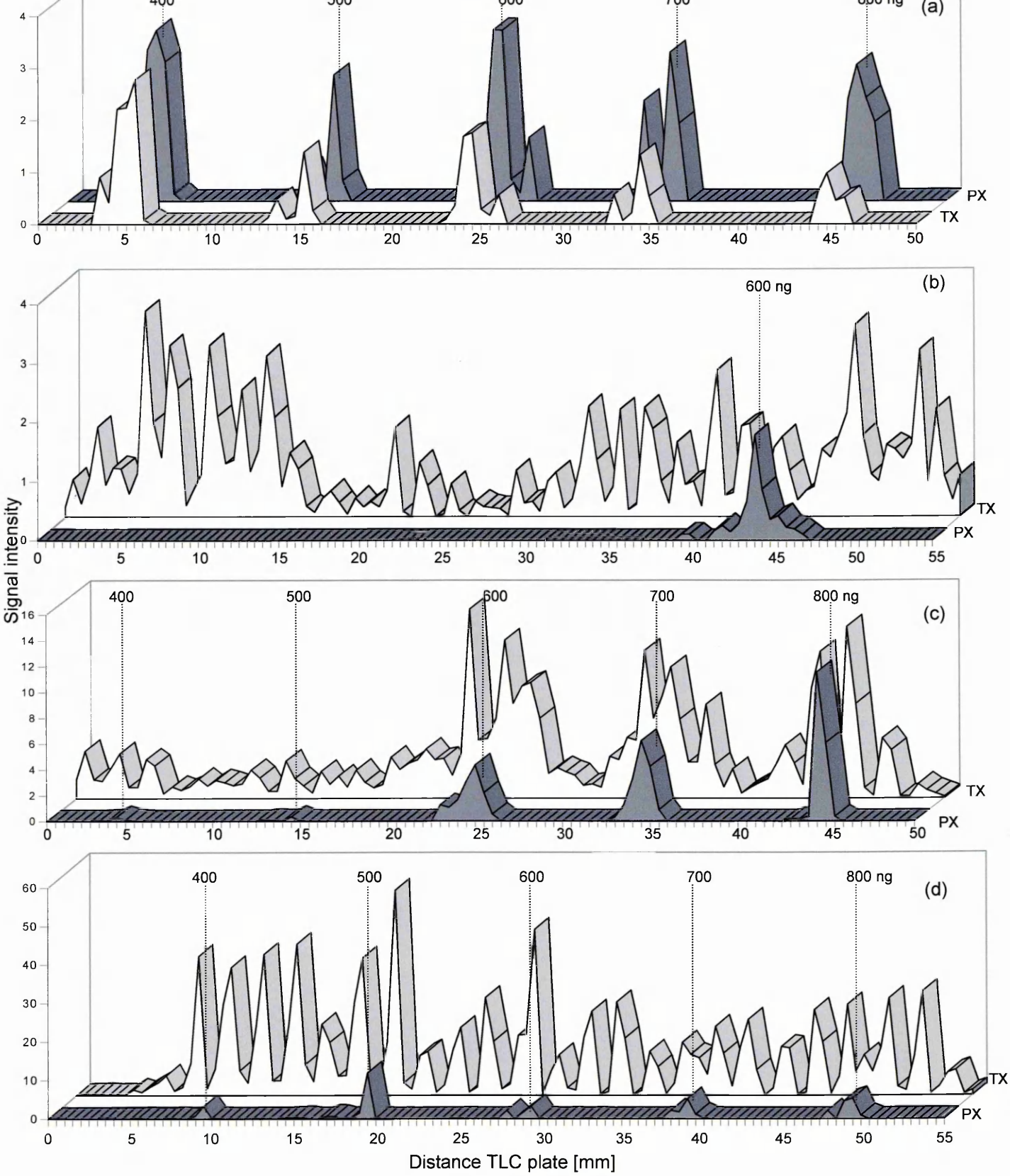

Figure 7-5. Mass chromatograms obtained by different approaches of incorporating the internal standard: (a) method 1, (b) method 2, (c) method 3, and (d) method 4. The various methods are explained in the experimental section. A range of quantities of piroxicam (400-800 ng) was spotted on the TLC plate in method 1, 3 and 4.

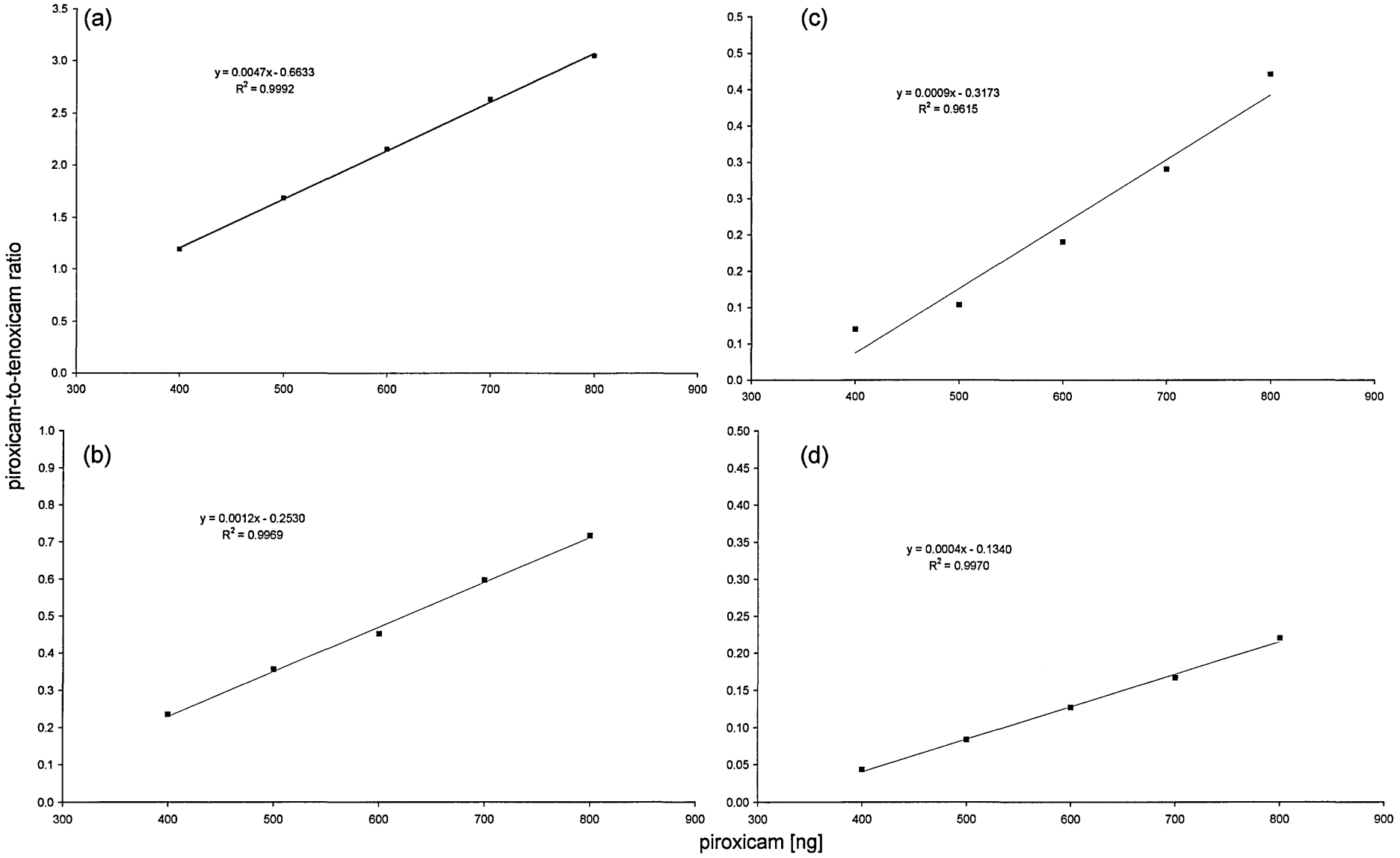

Figure 7-6. Standard calibration curves obtained for (a) method 1, (b) method 2, (c) method 3, and (d) method 4. The plotted data points are the arithmetic means of three experiments, except for method 2 (n = 4).

When a higher concentration than 0.4 mg/ml of the internal standard was used, suppression of the analyte occurred. The analyte signal caused also suppression of the internal standard signal, if a critical quantity was exceeded, e.g. a quantity of 3 μg of piroxicam suppressed 0.8 μg of tenoxicam.

Method 2: The mass chromatograms obtained by method 2 are presented in **Figure 7-5b**. Since tenoxicam has a smaller R_f value than piroxicam, pre-development of the silica gel TLC plate with the internal standard was necessary to achieve the same location for analyte and internal standard. The variation of the signal responses of the internal standard was 90% RSD from the averaged value, throughout the scanned TLC strip. However, only the surface coverage at the location of the piroxicam spot was of interest, and there the variation was in accordance to the one obtained by the analyte.

Even with the matrix electrospray deposition method, which has been shown to produce a homogenous layer compared to other methods of matrix application studied (chapter 3 of this thesis), variation of the matrix signals throughout the scanned TLC plate is obtained. The variation of the signals of the internal standard is in accordance to this, as revealed by plotting the signal intensities of the sodium adduct of α-CHCA (m/z 212) and the protonated molecular signal of tenoxicam (m/z 338) against the position on the TLC plate (**Figure 7-7**).

The resulting calibration curve obtained by method 2 (**Figure 7-6b**) shows a four times smaller slope value (b = 0.0012), in comparison to method 1(b = 0.0047) (**Figure 7-6a**).

Figure 7-4b shows the MALDI mass spectrum recorded at 45 mm of the scanned TLC plate with the $[M+H]^+$ ion of piroxicam clearly visible at m/z 332 and that of tenoxicam at m/z 338. Three matrix peaks of α-CHCA, $[M-H_2O+H]^+$ at m/z 172, $[M+H]^+$ at m/z 190, and $[2M+H]^+$ at m/z 379, were used as "lock masses" to overcome the degradation in mass measurement accuracy and mass resolution observed in the previous TLC-MALDI MS experiments, presented in chapters 3 and 4 of this thesis.

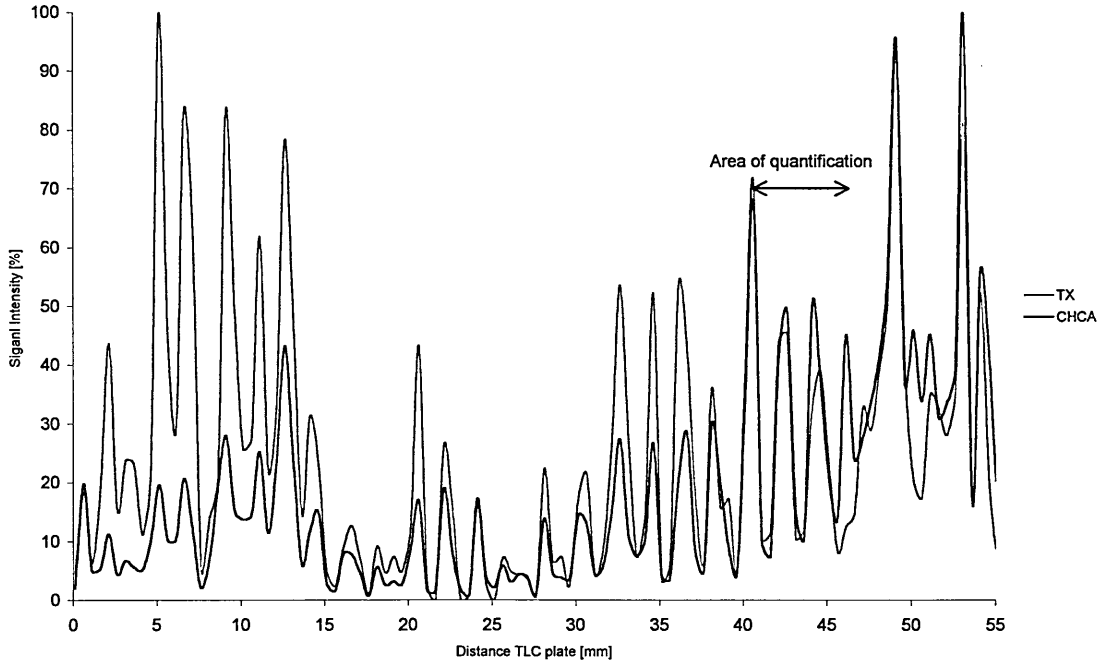

Figure 7-7. Plot of the signal intensities of the protonated molecular species of the internal standard tenoxicam (TX) (m/z 338) and the sodium adduct of the matrix α-CHCA (m/z 212) against the position on the TLC plate.

Method 3: When the internal standard is electrosprayed on the analyte spots, care has to be taken that minimal planar spreading of the analyte is caused. This can be achieved by using a non-polar solvent, in which the analyte is only sparingly soluble, but which has reasonable solvating properties for the internal standard. This second criteria is difficult to achieve, due to the chemical similarity of analyte and internal standard. It was found that acetone is a suitable candidate, since its polarity index is 5.4 [30] and the solubility of tenoxicam is 2 mg/ml in it [31]. The concentration of tenoxicam in acetone was reduced from 0.4 to 0.25 mg/ml, since analyte suppression occurred at higher concentrations.

In the mass chromatograms shown in **Figure 7-5c**, the increase in the internal standard response at the analyte spots containing 600, 700 and 800 ng of piroxicam is significant. A possible explanation could be as follows. The internal standard molecules are located at a layer above the analyte molecules, and when the solvent of the matrix solution is extracting the analyte molecules from the inner part of the silica gel layer to the surface, they have to pass through the layer of the internal standard molecules. An increase in

the concentration of the analyte molecules possibly causes an increase of internal standard molecules desorbed on the TLC plate surface.

The variation of the internal standard signals along the TLC strip varied around 102 % from the average value. The calibration curve (**Figure 7-6c**), showing an error range of 86 % for the lowest amount of piroxicam (400 ng), spotted on the TLC plate, and one of 32 % for the highest amount (800 ng), discounts this method for validation.

Method 4: Since electrospraying of the internal standard with the matrix solution caused a high degree of analyte suppression, the concentration of tenoxicam was reduced from 0.25 to 0.05 mg/ml in α-CHCA. Even at this level suppression effects could not be prevented (**Figure 7-5d** and slope value of calibration curve in **Figure 7-6d**). However, at lower levels the internal standard signal was not detected in a high number of mass spectra required, during the scan. The high variation of the internal standard signal in the recorded mass spectra is shown in **Figure 7-5d** (values vary from 0 to 53, which corresponds to an RSD of 111 %). This method, like method 3, was not considered as suitable for the quantitative analysis of piroxicam, since range error values of 130 % for the lowest and 55 % for the highest point of the calibration curve were obtained (n = 3) (**Figure 7-6d**).

Comparing all four methods of incorporating the internal standard into the TLC plate, the question arises why methods 3 and 4 are not suitable for the quantification of piroxicam by TLC-MALDI TOF MS. A possible explanation lies in the MALDI process itself. The incorporation of the analyte into the matrix crystals is not a requirement, but improves the overall MALDI performance [32]. In methods 3 and 4 the location of the internal standard and analyte molecules differ from each other, and hence the same incorporation process dos not occur. This highlights how important it is that both, the analyte and the internal standard, are applied on the TLC plate in the same manner, i.e. by spotting (method 1) or by TLC development (method 2).

7.3.4 Method Validation

Specificity: The specificity of method 1 and 2 was tested on six blank TLC-MALDI samples, respectively. The corresponding mass spectra (**Figure 7-3a** and **Figure 7-4a**)

showed no interfering peaks between m/z 330 and 340.

Linearity and range: Both methods exhibited linearity between the response (y) and the corresponding amount of piroxicam (x), over the range of 400 to 800 ng (**Figure 7-6a** and **b**). The mean correlation coefficient was 0.9992 (n = 3, method 1) or 0.9969 (n = 4, method 2), respectively.

Limit of detection (LOD) and limit of quantification (LOQ): The LOD for piroxicam was calculated as 16 ng and the LOQ as 53 ng (method 1). Method 2 showed two fold higher values, LOD = 39 ng, and LOQ = 131 ng. The latter values are similar to the ones, described for the quantitative HPTLC determination of piroxicam (LOD = 40 ng, and LOQ = 150 ng) [11].

Accuracy and precision: **Table 7-1** summaries the assay results for the analysis of three different quantities of piroxicam. Good accuracy and precision were obtained for both methods.

Table 7-1. Precision and accuracy of the TLC-MALDI methods for the determination of piroxicam.

Piroxicam [ng]	Method 1			Method 2		
	Detected [ng][a]	RSD [%]	Accuracy [%]	Detected [ng][b]	RSD [%]	Accuracy [%]
450	438 ± 28	6.4	2.7	457 ± 41	8.9	1.5
600	617 ± 32	5.2	2.8	586 ± 20	3.5	2.3
750	728 ± 23	3.2	2.9	745 ± 10	1.3	0.7

Values are mean of ten [a] or five [b] determinations ± S.D.

7.4 Conclusion

Four methods for quantification by TLC-MALDI TOF MS have been compared. The drug piroxicam could be successfully quantified by TLC-MALDI mass spectrometry,

when the internal standard was mixed into the mobile phase of the TLC analysis. The results of the method validation demonstrated that good accuracy, precision, linearity and sensitivity could all be obtained using this method.

The simplicity of pre-developing the TLC plate with an appropriate internal standard to perform quantification in TLC-MALDI MS makes this technique particularly attractive to the pharmaceutical industry, where TLC is a standard analytical method for quality control samples (QC samples).

7.5 References

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Chapter 8

TLC-PSD MALDI TOF MS of Small Drug Molecules

8.1 Introduction

The use of post-source decay (PSD) in combination with MALDI MS has become a widely used technique for peptide sequencing [1-5], since its introduction in the early 1990s [6]. Compared to the large amount of work that has been carried out using PSD-MALDI for the analysis of biomolecules, little work has been conducted on the structural elucidation of low molecular weight compounds. Recently, Cheng and Hercules [7] demonstrated the use of collision-induced dissociation, post-source decay matrix-assisted laser desorption/ionisation (CID-PSD MALDI) mass spectrometry for the analysis of pesticides. LeRiche *et al.* [8] compared product ion spectra of 4-quinolone antibiotics produced by PSD- and PSD-CID MALDI mass spectrometry with electrospray ion-trap MS^n. The possibility of recording PSD MALDI spectra directly from membranes was investigated by Guittard *et al.* [9]. In this work glycolipid samples were separated on a TLC plate followed by heat transfer to a membrane. More information about the latter technique can be found in chapter 2 of this thesis.

The structural analysis of small drug molecules by directly coupling TLC with PSD MALDI mass spectrometry is reported in this chapter. The applicability of this techniques is shown using two examples: (i) the TLC-PSD MALDI analysis of two representatives of non steroidal anti-inflammatory drugs (NSAIDs), tenoxicam and piroxicam, and (ii) the analysis of the pharmaceutically active compound UK-137,457 and one of its related substances UK-124,912. The matrices α-CHCA and graphite were used to investigate the effect of the precursor ion selection on the TLC-PSD MALDI spectra of the drug molecules studied. While α-CHCA enhances the $[M+H]^+$ ion formation, graphite produces in general only sodium adducts. Structural differentiation of tenoxicam and piroxicam was only possible by selecting the sodium adduct of both drug molecules as precursor ions. In the case of the TLC-PSD MALDI analysis of UK-137,457 and its related substance UK-124,912 at the 1% level, the PSD spectra obtained in α-CHCA, by selecting the protonated adduct of the small molecules as precursor ions, showed distinguishable dissociation patterns containing structurally significant information. Since α-CHCA showed matrix ions, which could not be eliminated from the PSD spectra in the case of tenoxicam and piroxicam due to the low resolution of the ion gate, the use of a quadrupole TOF instrument (q-TOF) was investigated for TLC-MS-MS experiments using piroxicam as model compound.

8.2 Experimental

8.2.1 Materials

The small drug molecules, tenoxicam ($C_{13}H_{11}N_3O_4S_2$) and piroxicam ($C_{15}H_{13}N_3O_4S$) were obtained from Sigma-Aldrich (Dorset, U.K.). UK-137,457 ($C_{31}H_{31}NO_5$), a pharmaceutically active compound, and one of its related substance UK-124,912 ($C_{27}H_{25}NO_3$) were supplied by Pfizer Global R&D (Sandwich, U.K.). The chemical structures of these compounds are shown in chapter 7 and 5, respectively. The matrices α-CHCA and graphite (1-2 μm) were purchased from Sigma-Aldrich (Dorset, U.K.). Methanol, acetonitrile, chloroform and dichloromethane were all HPLC grade.

8.2.2 Sample Preparation

Stock solutions of tenoxicam and piroxicam (2 mg/ml) were dissolved in dichloromethane. A mixture of both oxicam derivatives, with a concentration of 1 mg/ml of each component, was obtained by combining aliquots of both stock solutions.

Stock solutions of UK-137,457 (20 mg/ml) and UK-124,912 (1 mg/ml) were dissolved in acetonitrile. A mixture containing 10 mg/ml of UK-137,457 and 0.1 mg/ml of UK-124,912 was prepared by mixing both stock solutions in an appropriate ratio and diluting with acetonitrile to get the final concentrations.

8.2.3 TLC Separation

Appropriate aliquots of the mixture were applied to an aluminium-backed TLC plate (10 × 10 cm), coated with 0.2 mm layers of silica gel 60 F_{254} (Merck, Germany), using a 10 μl syringe. The development chamber was lined with filter paper and saturated with the corresponding mobile phase. For the separation of tenoxicam and piroxicam the mobile phase chloroform/methanol (9/1, v/v) [10] was used and saturated for one hour before development of the plate. UK-137,457 was separated from its related substance UK-124,912 employing the mobile phase chloroform/methanol/glacial acetic acid (60/5/1, v/v/v), which was saturated over night. The TLC plates were developed to a distance of 7.0 cm. After solvent evaporation at room temperature, the sample spots were visualised

by illumination with UV light at λ=254 nm.

8.2.4 Matrix Application

Before the matrix was deposited on the silica gel surface, a strip of the developed TLC plate (60 × 2 mm) was attached to a modified MALDI target with double sided tape. The organic matrix α-CHCA was applied to the silica gel surface of the TLC plate by using an in-house modified commercial robotic x-y-z-axis motion system (PROBOT, BAI, Germany), described in chapter 5.

The inorganic matrix suspension of graphite was prepared by dispersing 80 mg graphite in 1 ml of methanol/ethylene glycol (1000:1, v/v). The suspension was homogenised by sonication for 15 minutes and then applied to the developed TLC strip using a 10 µl syringe, as described in chapter 7 of this thesis. Typically 30 µl of the graphite suspension was used to cover an area of 60 × 2 mm.

8.2.5 Mass Spectrometry

A LT3 mass spectrometer (SAI, Manchester, U.K.), equipped with a nitrogen laser (λ=337 nm), was employed for this study. Further information about this instrument can be found in the appendix of this thesis (chapter 10). Modifications to the instrument and its software are the same as described for the linear LaserTOF 1500 mass spectrometer, which can be found in chapters 3 and 4 of this thesis.

TLC-MALDI analysis was performed in linear or reflectron positive ion mode with an accelerating voltage of 10 or 20 kV. 16 shots/spectrum were accumulated. The TLC strip was scanned over a distance of 60 mm and spectra were recorded every 0.5 mm. A data set of 120 mass spectra were obtained, from which mass chromatograms of the studied compounds were constructed. The mass chromatograms enabled the location of the highest analyte signals on the TLC strip ("sweet spots"). Note: The mass chromatograms presented here were smoothed.

TLC-PSD MALDI analysis was performed at these "sweet spots" using an accelerating voltage of 20 kV, and a focus mass applicable to the mass of the analyte. The gate was

set to include the analyte of interest +/- 2 Da. Since this instrument uses a curved-field reflectron [11] full PSD spectra could be recorded without the need to combine small spectra segments.

TLC-MALDI MS-MS analysis was performed on a Q-Star hybrid quadrupole TOF mass spectrometer, fitted with an orthogonal MALDI source (Applied Biosystems-MDS Sciex, Toronto, Canada) [12]. (The mass spectrometric analysis was not performed by the author of this thesis, since the laboratory was not equipped with this instrument, when this work was carried out.)

8.3 Results and Discussion

8.3.1 TLC-MALDI Analysis

Figure 8-1 a shows typical overlaid mass chromatograms of tenoxicam ($[M+H]^+$, m/z 338) and piroxicam ($[M+H]^+$, m/z 332), obtained from a TLC-MALDI experiment in reflectron positive ion mode with an acceleration voltage of 20 kV. Two µg of both drug compounds were separated on a silica gel TLC plate and the organic matrix α-CHCA (20 mg/ml in methanol containing 0.1% TFA) was electrosprayed on the eluted TLC plate.

The phenomenon of "donut shaped" analyte spots, normally occurs when polar solvents are used to apply the analyte spot on the TLC plate, prior to TLC analysis [13-14]. In TLC-MALDI analysis bimodal responses, as illustrated in **Figure 8-1 a**, were due to the electrospray deposition of the matrix, which was carried out in the polar solvent methanol. Both drug molecules have a solubility of < 1 mg/ml in methanol [15]. This is necessary to ensure sufficient analyte extraction. However, solubility sufficient to give good analyte extraction from the silica gel layer also causes some analyte spreading, indicated as peak broadening in **Figure 8-1 a**. Although the limit of detection of piroxicam is 39 ng when α-CHCA is employed as MALDI matrix, as determined in chapter 7 of this thesis, 2 µg quantities of both drug compounds were chosen in order to allow scanning of the TLC plate and PSD analysis to be carried out on the same sample. A high analyte concentration was particularly required when graphite was used as

matrix since fast signal decrease occurred when the laser was targeted on the same TLC position.

The generation of an impurity profile of UK-137,357 is presented in **Figure 8-1 b**. The overlaid mass chromatograms of the parent compound UK-137,457 ($[M+H]^+$, m/z 498) and its related substance UK-124,912 ($[M+H]^+$, m/z 412) at the 1% level were constructed from a TLC-MALDI analysis in linear positive ion mode using an acceleration voltage of 10 kV. One hundred μg of the parent compound was separated from 1 μg of the related substance on a silica gel TLC plate and the organic matrix α-CHCA (20 mg/ml in acetonitrile/water containing 0.1% TFA in the volume ratio 7:3) was electrosprayed onto the eluted TLC plate. To obtain a sufficient signal of the related substance for the following PSD analysis a 1% level was chosen, even though the sensitivity of the related substance is below 1 ng, as presented in chapter 5 of this thesis. With a quantity of 100 μg of the parent compound the TLC plate is overloaded, as indicated in **Figure 8-1 b**.

8.3.2 TLC-PSD MALDI Analysis

The mass chromatograms obtained from the TLC-MALDI analysis (**Figure 8-1**) enabled the location of the highest analyte signals on the TLC strip, and TLC-PSD MALDI analyses were performed at these positions.

Two matrices were chosen for the TLC-PSD MALDI analysis of tenoxicam and piroxicam, the organic matrix α-CHCA and the particle suspension matrix graphite. α-CHCA is classified as a "hot" matrix in MALDI, because of the high dissociation level induced [16]. The use of graphite for PSD experiments has not been previously reported. However, previous studies of graphite for the TLC-PALDI analysis of tetracyclines showed high in-source fragmentation rates in the linear positive and negative ion mode, as described in chapter 6 of this thesis.

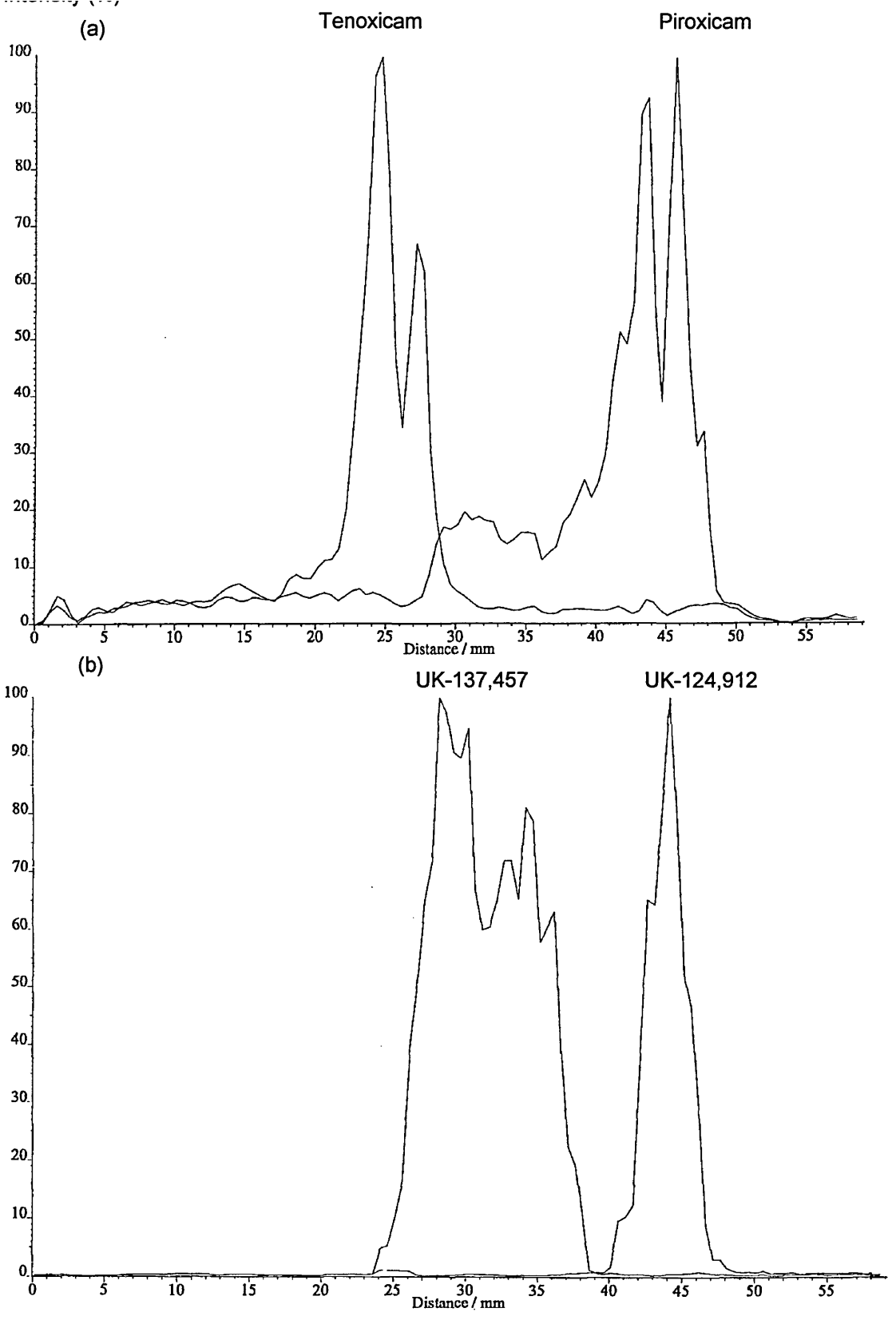


Figure 8-1. Overlaid, smoothed mass chromatograms from the TLC-MALDI determination of (a) tenoxicam ($[M+H]^+$, m/z 338) and piroxicam ($[M+H]^+$, m/z 332) (2 µg of each component), and (b) UK-137,457 ($[M+H]^+$, m/z 498) (100 µg) and its known related substance UK-124,912 ($[M+H]^+$, m/z 412) at the 1% level. In both analyses the small molecules were separated on a silica gel TLC plate and α-CHCA was used as matrix, as described in the experimental section.

The recorded TLC-PSD-MALDI spectrum of tenoxicam in α-CHCA is presented in **Figure 8-2 a**. The protonated molecular species of tenoxicam at m/z 338 was selected as precursor ion. An interference peak of α-CHCA, indicated as M* in the spectrum, is observed at m/z 335 . α-CHCA with a molecular weight of 189 Da, forms a dimer [2M-CO_2+H]$^+$ at m/z 335 [17], which passes through the ion gate and enters the mass analyser. For piroxicam the protonated molecular species at m/z 332 was chosen as precursor ion. The corresponding TLC-PSD MALDI spectrum of piroxicam in α-CHCA is shown in **Figure 8-2 b**. The interfering peak of the matrix (indicated as M*) is observed in low abundance. To ensure that the detected product ions in **Figure 8-2 a** and **b** are not arising from dissociated ions of α-CHCA, TLC-PSD MALDI analysis was carried out on TLC plate positions, at which no analyte was located, i.e. matrix only was present in this position. No product ions with m/z values shown in **Figure 8-2 a** and **b** were detected.

The oxicam derivatives tenoxicam and piroxicam form mainly sodium adduct ions in TLC-MALDI analysis using graphite as matrix, since sodium is believed to be a major contaminant in graphite. Hence, TLC-PSD MALDI analysis in graphite was performed on the [M+Na]$^+$ ion of tenoxicam at m/z 360 and of piroxicam on the [M+Na]$^+$ at m/z 354. The resulting TLC-PSD MALDI spectra of both drug molecules are shown in **Figure 8-2 c** and **d**, respectively. A comparison of the TLC-PSD MALDI spectra of tenoxicam and piroxicam obtained in graphite (**Figure 8-2 c** and **d**) with α-CHCA (**Figure 8-2 a** and **b**), shows that a lower noise level is obtained when the latter matrix is employed.

To improve the mass accuracy obtained on a TLC plate, which is limited by the porosity of the silica gel surface (see chapters 3 and 4), the instrument was re-calibrated using the selected precursor ion in the positive reflectron ion mode at the TLC position from which the TLC-PSD MALDI spectra were recorded. Comparison of the m/z values of the product ions obtained on the silica gel surface with the normal stainless steel surface showed no differences.

Possible structures for the product ions of tenoxicam and piroxicam are proposed in **Table 8-1**. It could be clearly seen that the selection of the protonated species as precursor ion for tenoxicam and piroxicam in the organic matrix α-CHCA produces the same product ions for both drug molecules, since dissociation presumably occurs from

the thiophene or benzene-group, which represent the only structural difference between both molecules (**Table 8-1** column 1). In contrast, the selection of the sodium adduct as precursor ion for both oxicam derivatives, in experiments conducted using the particle suspension matrix graphite, generates distinguishable dissociation patterns. All product ions observed (**Table 8-1** column 2) are believed to be sodium adducts. Dissociation is believed to occur from both sides of the drug molecules, the thiophene or the benzene-group and the pyridine-group. The structural information obtained by selection of the sodium adduct as the precursor ion is complementary to the protonated adduct, and hence the combination of both precursor ions enables a more complete structural picture of the test analytes.

It is interesting to note that the PSD analysis of cationised peptides was reported to be less efficient than PSD of protonated species [5]. Cationised ions were found to be much more stable. This does obviously not apply to pharmaceutical compounds, such as tenoxicam and piroxicam.

The addition of sodium chloride to α-CHCA was tried in order to produce sodium adducts of the drug molecules. However, the generated sodium adducts of tenoxicam and piroxicam were of low abundance and the protonated ions were still the dominant species. Despite this, when the sodium adducts were selected as precursor ions for PSD analysis the same dissociation patterns were obtained as those when graphite was used as the matrix. The PSD spectrum of piroxicam is presented in **Figure 8-3**. The sodium adduct of piroxicam with the m/z value of 354 was produced by adding an appropriate aliquot of a 30 mg/ml solution of sodium chloride, dissolved in methanol/water (1/1, v/v), to the sample. These data prove that the degree of dissociation of the drug molecules studied does not depend on the choice of matrix, but rather on the selection of the precursor ion.

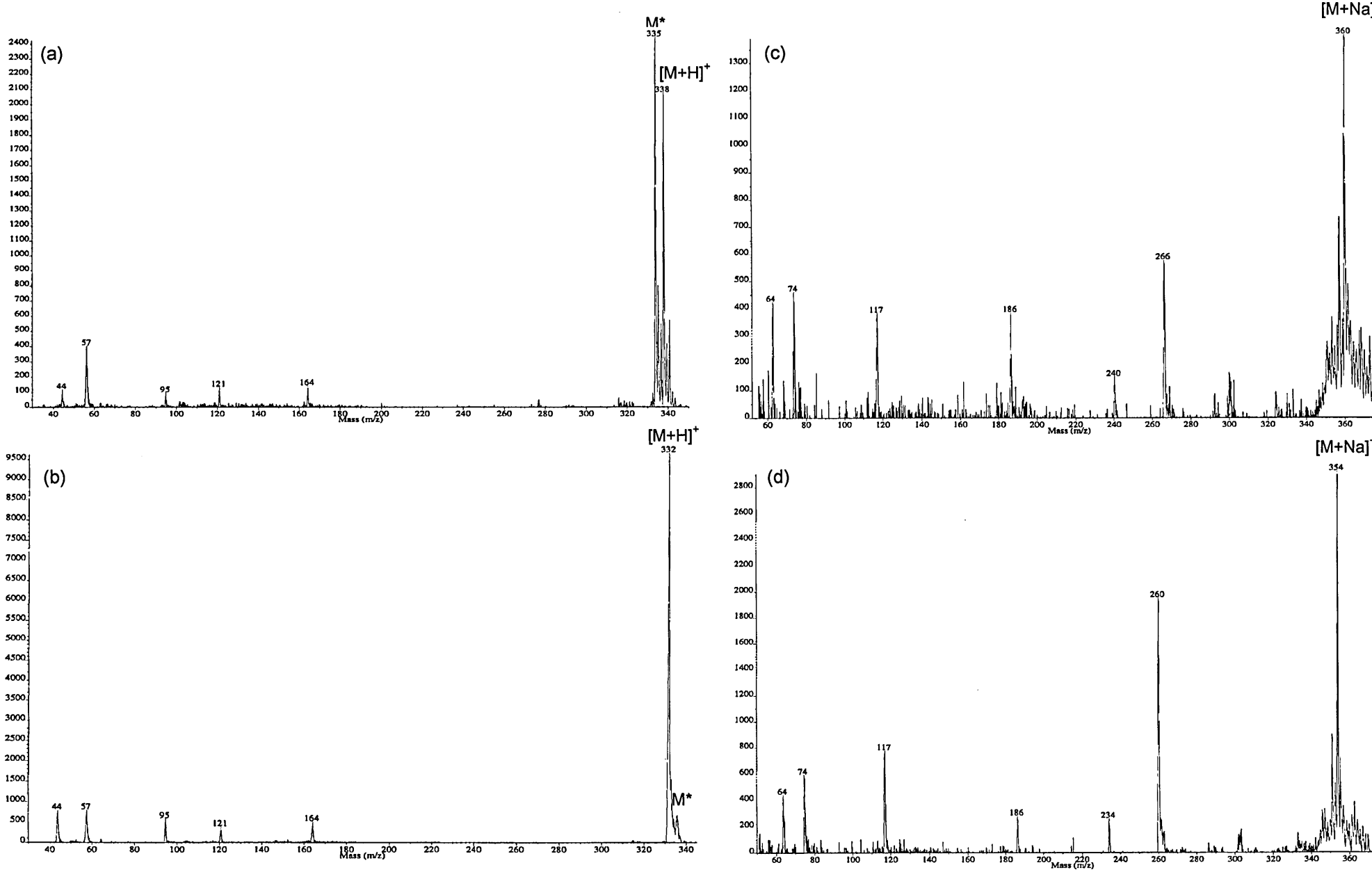


Figure 8-2. TLC-PSD MALDI spectra of tenoxicam (2 µg) and piroxicam (2 µg), separated on silica gel TLC plates using different matrices. Mass spectra of (a) tenoxicam and (b) piroxicam were acquired using α-CHCA as matrix, and mass spectra of (c) tenoxicam and (d) piroxicam were acquired using graphite as matrix.

when the $[M+Na]^+$ ion was used.

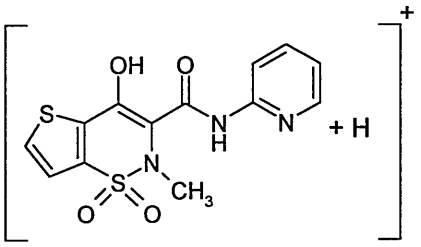
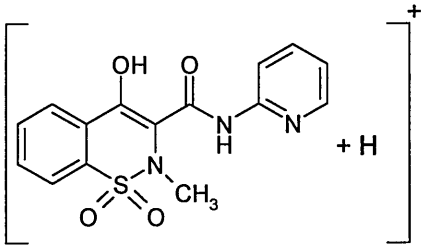
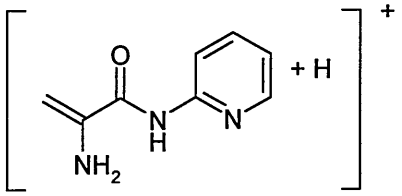
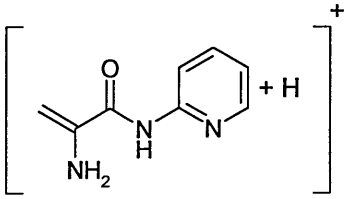
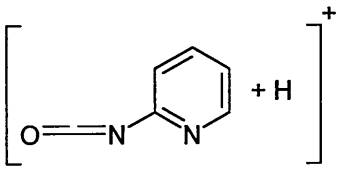
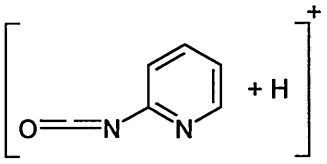
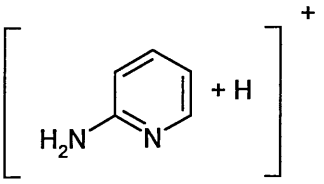
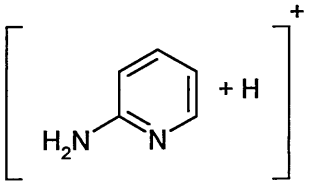
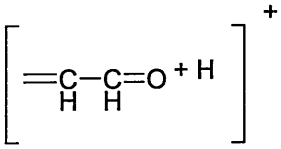
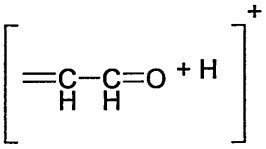
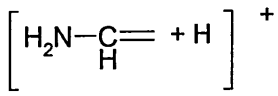
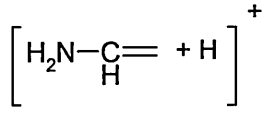
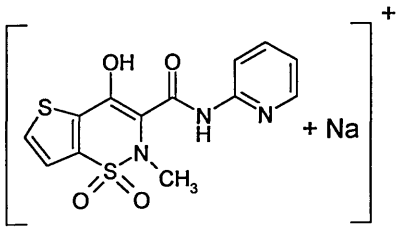
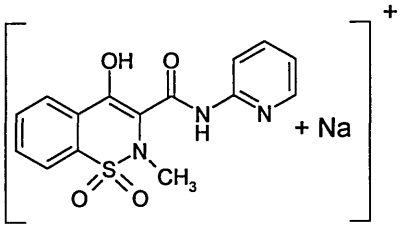
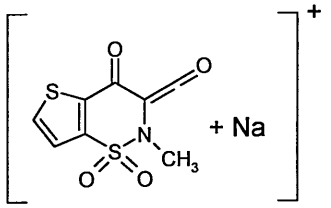
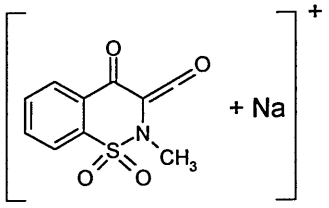
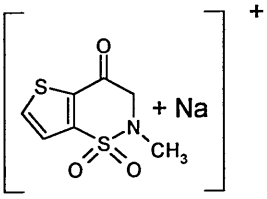
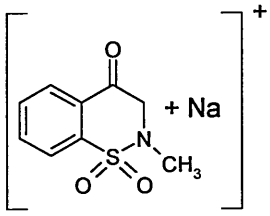
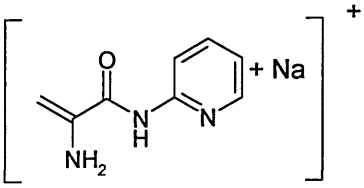
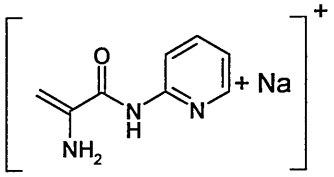
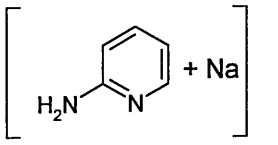
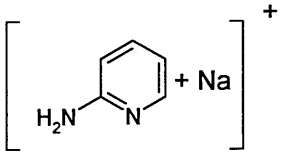
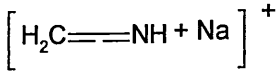
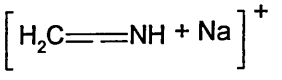
Column 1	
Tenoxicam	Piroxicam
	
(m/z 338)	(m/z 332)
	
(m/z 164)	(m/z 164)
	
(m/z 121)	(m/z 121)
	
(m/z 95)	(m/z 95)
	
(m/z 57)	(m/z 57)
	
(m/z 44)	(m/z 44)

Table 8-1. (continued)

Column 2	
Tenoxicam	Piroxicam
	
(m/z 360)	(m/z 354)
	
(m/z 266)	(m/z 260)
	
(m/z 240)	(m/z 234)
	
(m/z 186)	(m/z 186)
	
(m/z 117)	(m/z 117)
	
(m/z 64)	(m/z 64)

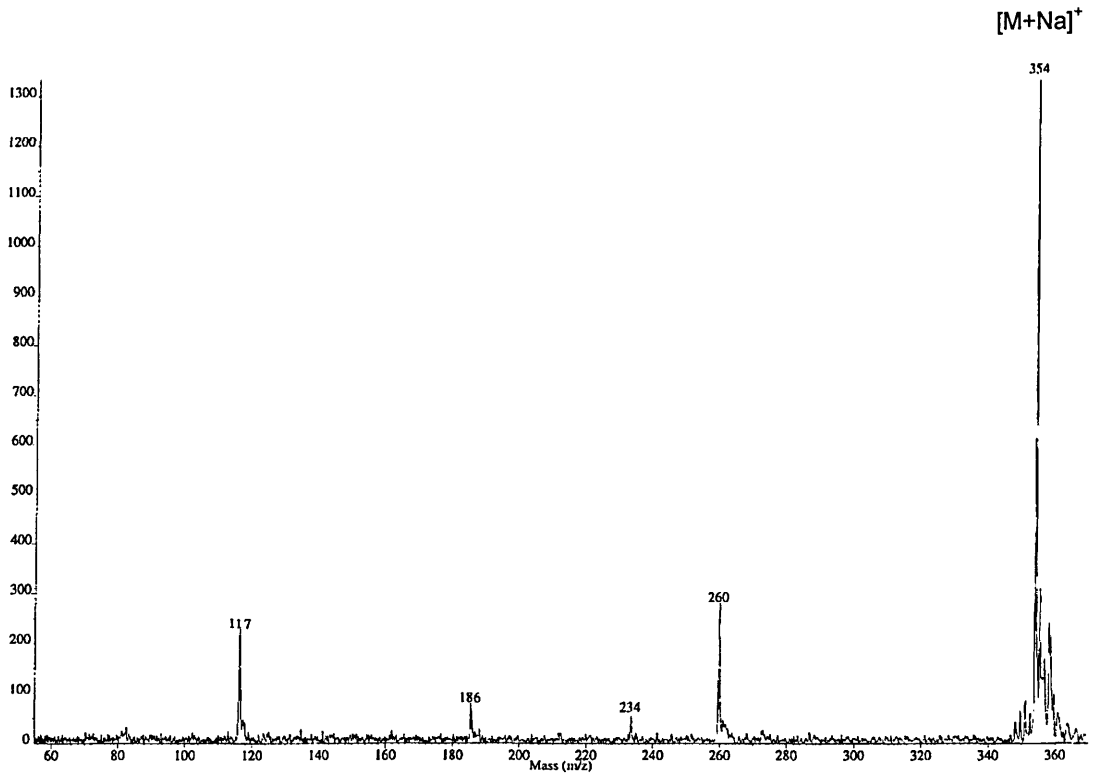


Figure 8-3. PSD-MALDI spectrum of piroxicam obtained in α-CHCA by addition of sodium chloride to produce the $[M+Na]^+$ ion at m/z 354.

For the TLC-PSD MALDI analysis of UK-137,457 in α-CHCA the protonated molecular species at m/z 498 was selected as the precursor ion. The corresponding PSD spectrum is shown in **Figure 8-4 a**. The $[M+H]^+$ ion of the related substance UK-124,912 was detected at m/z 412 and was used as precursor ion for PSD analysis (**Figure 8-4 b**). In both TLC-PSD MALDI spectra, α-CHCA does not show interfering peaks. Possible structures of the recorded product ions are summarised in **Table 8-2**. The important species are those of m/z 230 and 341 for UK-137,457 and m/z 144 and 255 for UK-124,912. As can be seen from **Table 8-2**, the difference in m/z value of 86 between corresponding ions is the RMM of the side chain group of the pyrrole ring of UK-137,457 and hence allows structural identification of UK-124,912.

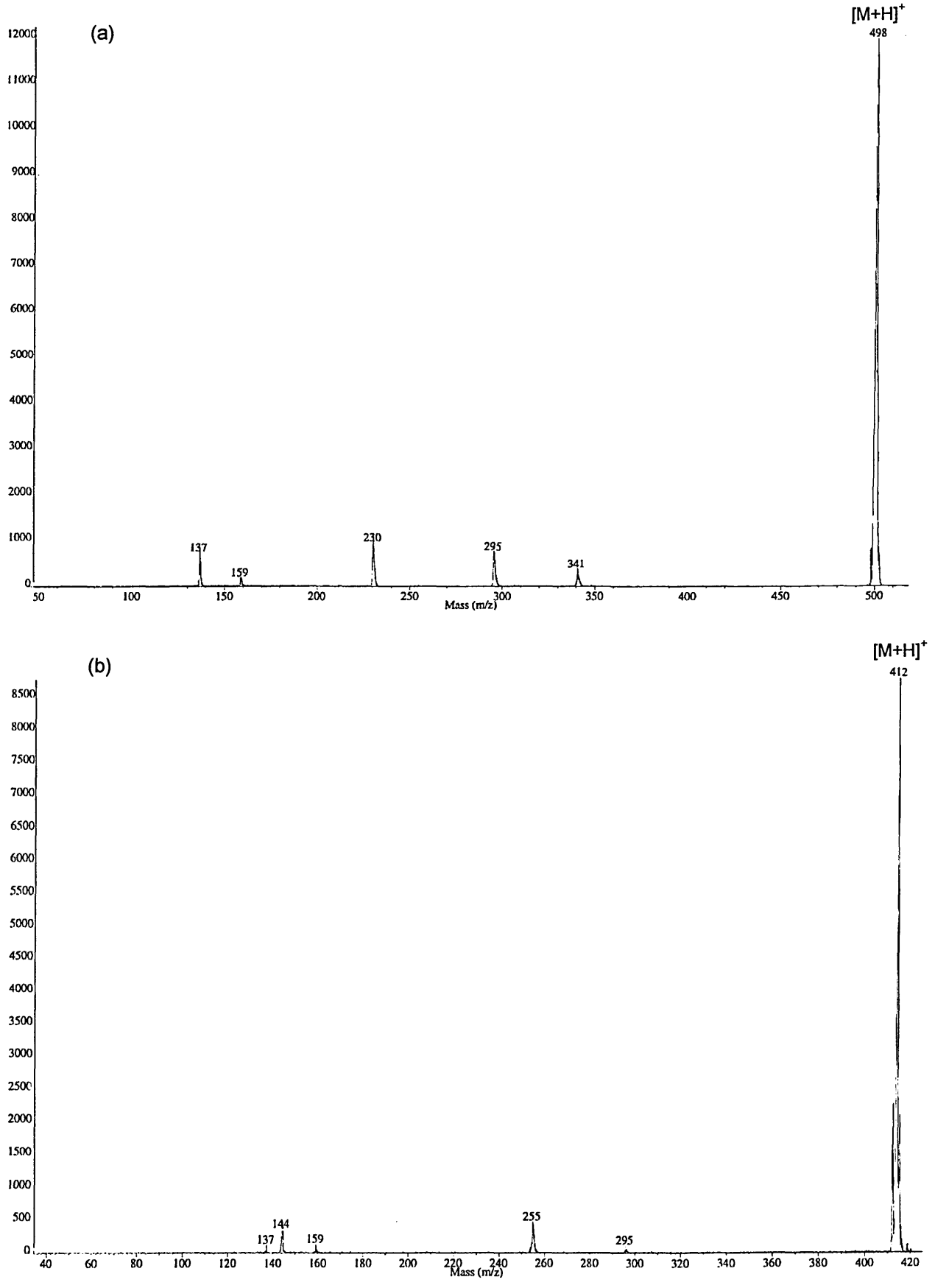
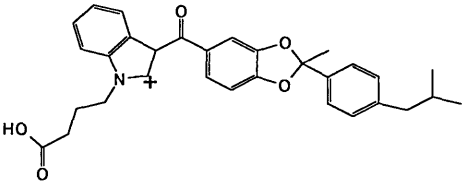
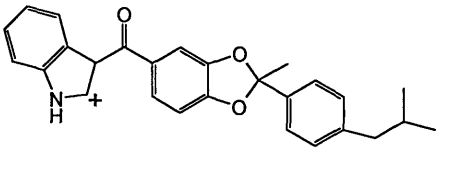
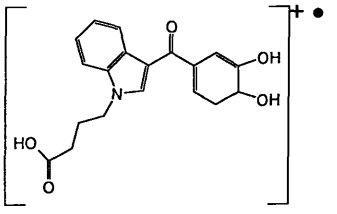
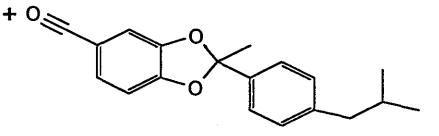
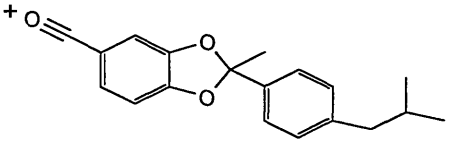
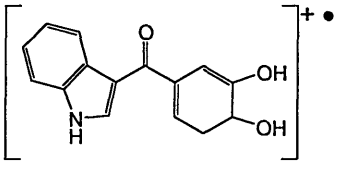
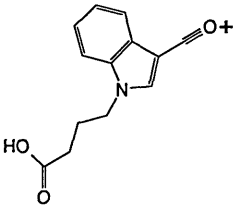
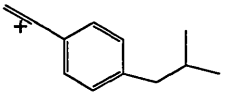
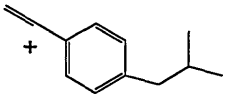
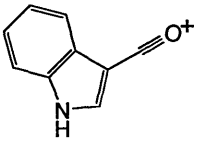
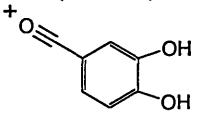
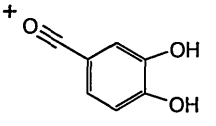


Figure 8-4. TLC-PSD MALDI spectra of (a) UK-137,457 (100 µg), and (b) its related substance UK-124,912 at the 1% level. The related substance UK-124,912 (1 µg) was separated on a silica gel TLC plate from the parent compound and α-CHCA was used as matrix.

Table 8-2. Proposed structures of the product ions observed in the TLC-PSD MALDI mass spectra of Figure 8-4.

UK-137,457	UK-124,912
 (m/z 498)	 (m/z 412)
 (m/z 341)	 (m/z 295)
 (m/z 295)	 (m/z 255)
 (m/z 230)	 (m/z 159)
 (m/z 159)	 (m/z 144)
 (m/z 137)	 (m/z 137)

8.3.3 TLC-MALDI MS-MS Analysis

The organic matrix α-CHCA shows one peak at m/z 335, as discussed under point 8.3.2 of this chapter, which could not be eliminated due to the low resolution of the ion gate in the reflectron TOF instrument. To compensate this, a q-TOF instrument was employed for tandem MS analysis. **Figure 8-5** shows the MS-MS spectrum of piroxicam obtained by spotting 800 ng of it on a silica gel TLC plate followed by electrospray deposition of α-CHCA. The mass accuracy and mass resolution were excellent using this instrument, as shown in **Figure 8-5**. Re-calibration of the precursor ion on the TLC spot was not necessary, since precursor ion selection was performed with a quadrupole mass filter.

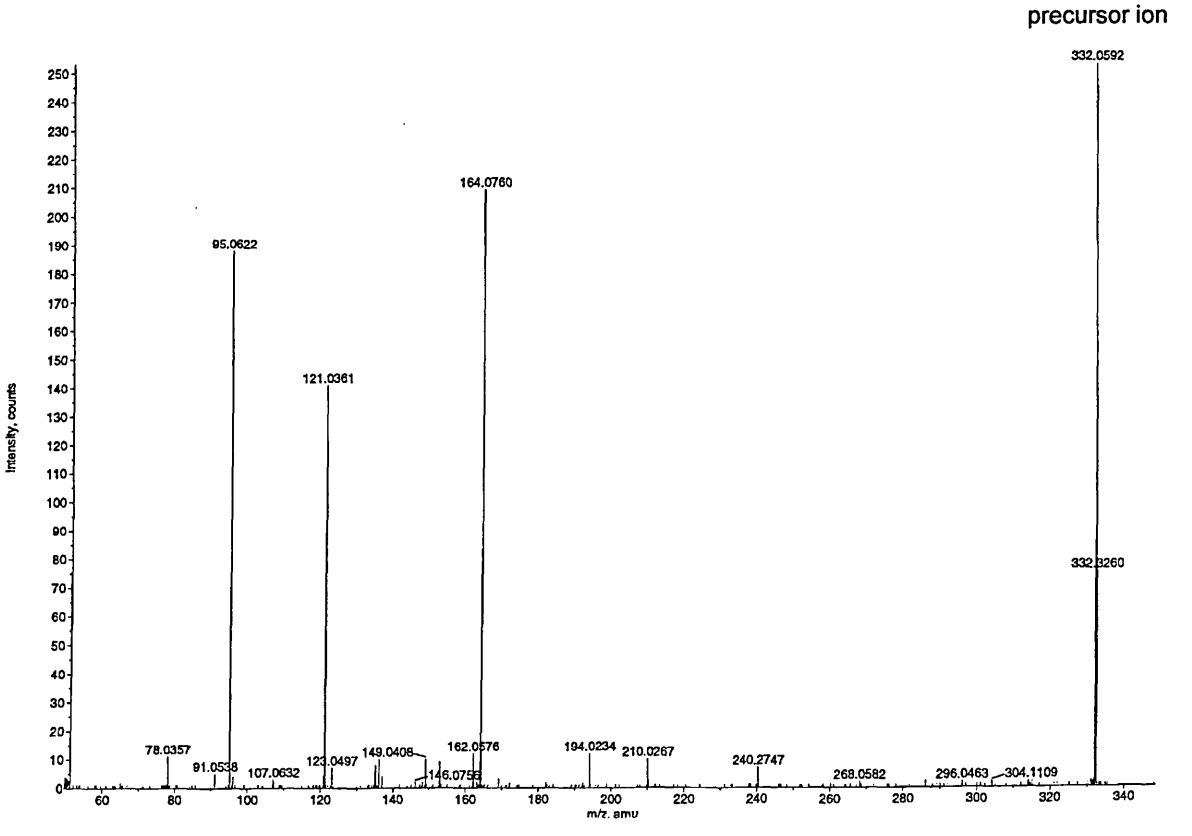


Figure 8-5. TLC-MALDI MS-MS spectrum of 800 ng of piroxicam using α-CHCA as matrix.

8.4 Conclusion

TLC-PSD MALDI spectra of the drug analogues tenoxicam and piroxicam and the pharmaceutically active compound UK-137,457 and its related substance UK-124,912 have been successfully obtained, and the effect of the precursor ion selection on this technique was evaluated. The extent of PSD of these small molecules was determined by the selection of the precursor ion. A high degree of PSD activation and distinguishable product ions were only obtained for tenoxicam and piroxicam when the sodium adduct was chosen as precursor ion. α-CHCA showed the disadvantage of generating matrix ions, which could not be eliminated from the PSD spectra due to the low resolution of the ion gate. Hence, the feasibility of employing a q-TOF instrument for TLC-MALDI MS-MS experiments was tested to overcome this matrix interference and to enhance the mass accuracy and mass resolution.

In the TLC-PSD MALDI analysis of UK-137,457 and its related substance UK-124,912 in α-CHCA, differentiation in the PSD spectra was achieved when the protonated ion of both molecules was selected. It was possible to obtain TLC-PSD MALDI data from UK-124,912 at the 1% level which allowed its structural characterisation.

8.5 References

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Chapter 9

Conclusions and Future Perspectives

In recent years has been an increased interest in the use of MALDI MS for the direct analysis of TLC plates [1]. However, there are several issues, which have to be addressed for successful TLC-MALDI coupling. The first is concerned with the method used for the deposition of the MALDI matrix onto the TLC plate. The matrix facilitates the ionisation of the analyte molecules separated on the TLC plate. The application of the matrix directly onto the TLC plate is crucial to the success of such analysis. The matrix application has to be carried out in such a manner that analyte spreading along the silica gel layer is avoided. This maintains the chromatographic integrity of the analyte spots. The second issue is the requirement for efficient extraction of the analyte from the interior of the silica gel layer to enhance sensitivity. The porosity of the silica gel layer, can also lead to problems, ions starting from different points in the surface can have a slight variation in their flight times and hence lead to a decrease in the mass resolution and mass accuracy of spectra recorded, if a TOF analyser is used. Finally, for the analysis of low molecular weight compounds, such as pharmaceuticals, strong matrix peaks can interfere and suppress analyte signals.

In this thesis the direct TLC-MALDI analysis of pharmaceutical compounds was evaluated. The issues described above for the direct coupling of TLC with MALDI mass spectrometry have been fully investigated and discussed. The study was started by comparing several approaches for the application of the matrix for TLC-MALDI coupling, including a newly developed electrospray matrix deposition (chapter 3). The electrospray method was found to be superior to the other techniques studied. It produced a stable signal, minimised analyte spreading and hence allowed the scanning of a TLC plate to obtain chromatographic as well as mass spectral data. To enhance the signals recorded directly from a TLC plate the use of an extraction solvent prior the matrix application was explored (chapter 4). Further improvements in sensitivity was obtained by alterations of the electrospray deposition step and by reducing the silica gel layer to a thickness of 0.1 mm (chapter 5). The mass measurement inaccuracies observed commonly in TLC-MALDI TOF MS could be corrected by internal recalibration on selected matrix ions, during the scanning of a TLC plate (chapter 4). Quantification in TLC-MALDI MS was achieved by pre-development of the TLC plate in the mobile phase to which an internal standard was added (chapter 7). Other work that has been described in this thesis was the use of PSD-MALDI to aid the identification of compound spots (chapter 8) and a study of the minimisation of matrix

interference by applying suspensions of particles of different materials and sizes (Co-UFP, TiN, TiO_2, graphite and silicon) onto eluted TLC plates (chapter 6).

For TLC-MALDI mass spectrometry to be more widely used, e.g. in industrial applications, future developments would be necessary to enhance sensitivity, mass resolution, reproducibility and to decrease the presence of abundant matrix-related ions in the low-mass range.

The reduction of the thickness of the silica gel layer below 100 μm would presumably improve the sensitivity, and hence would enable the detection of low femtomoler amounts of material. Another possible approach would be to employ an IR laser for the desorption and ionisation of analytes, separated on the TLC plate. At present the MALDI sensitivity, shown in this study, is limited to the confined penetration depth of the nitrogen laser beam inside the TLC plate. The feasibility of direct IR-MALDI mass spectrometry for the analysis of gangliosides from TLC plates has already been demonstrated [2]. Suitable IR matrices, such as frozen water [3] and glycerol [4] would make TLC-IR MALDI MS very attractive for the growing area of proteomics. Glycerol is commonly used as a stabiliser for fluorescence zones on the TLC plate [5], and hence its use would address both issues: to act as a matrix and to store fluorescence. Lasers of other wavelength, such as VIS laser radiation would be also of interest for direct TLC-MALDI mass spectrometry. Only little work has been carried out using VIS-MALDI MS due to the lack of suitable matrices. Preliminary studies have been reported using dye molecules, such as rhodamine 6G [6] and indocyanine green [7] as matrices. In this situation, it would be challenging to investigate ninhydrine as a suitable matrix for TLC-VIS MALDI, since the dye molecule is commonly used as spray reagent for derivatisation purposes on TLC plates.

The TLC-MALDI experiments presented in this thesis were conducted on a liner TOF instrument with delayed extraction. Hence, the porosity of the silica gel layer caused peak broadening to a certain extent and with this correlated mass inaccuracies. These problems can mainly be overcome by employing a FTICR or q-TOF instrument, in which the mass analyser does not require the TOF time of the ions. Initial experiments, carried out on the latter instrument, were presented in this thesis, highlighting the potential of this approach. Thus, determinations of small organic molecules by TLC-MALDI MS and tandem MS with a q-TOF instrument may be pursued further.

The search for new UV matrices to suppress the matrix background is an area of ongoing research [8]. Recently, meso-tetrakis(pentafluorophenyl)porphyrin has been studied as a replacement for an organic matrix [9], and also the addition of the surfactant cetrimonium bromide to an organic matrix [10] has been investigated. Both approaches seemed to be interesting for the TLC-MALDI analysis of small molecules, such as pharmaceuticals. Alternatively, the use of porous silicon from silicon wafers, which have been investigated by Wei *et al.* [11] maybe another promising path. First attempts to use porous silicon as a microscale chromatographic platform have been presented recently [12]. Thus, the first steps in this direction have already succeeded, and it seems a promising area for further research.

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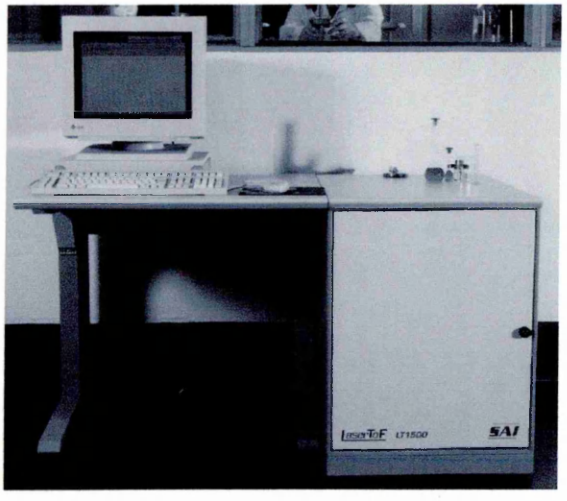
10.1 List of Abbreviation

AP	atmospheric pressure
ATP	adenosine triphosphate
CA	trans-cinnamic acid
CE	capillary electrophoresis
α-CHCA	α-cyano-4-hydroxy cinnamic acid
CI	chemical ionisation
CID	collision-induced dissociation
Co-UFP	cobalt ultra fine powder
CTC	chlortetracycline
Da	dalton
DE	delayed extraction
DHB	2,5-dihydroxy benzoic acid
DHBs	mixture of 2,5-dihydoxy benzoic acid and 2-hydroxy-5-methoxy benzoic acid in the volume ratio 9:1
DIOS	desorption/ionisation on porous silicon
EDTA	ethylenediaminetetraacetic acid
EI	electron ionisation
ESI	electrospray ionisation
FAB	fast atom bombardment
FEL	free-electron laser
FT	fourier transform
FTICR	fourier transform ion cyclotron resonance
FW	formula weight
FWHM	full width at half maximum
HABA	2-(4-hydroxyphenylazo) benzoic acid
HMB	2-hydroxy-5-methoxy benzoic acid
3-HPA	3-hydroxy picolinic acid
HPLC	high-performance liquid chromatography
HPTLC	high-performance thin-layer chromatography
IP	ionisation potential
IR	infrared
IT	ion trap
L2 MS	two step laser mass spectrometry
LC	liquid chromatography

LD	laser desorption
LDI	laser desorption/ionisation
LOD	limit of detection
LOQ	limit of quantification
LSI	liquid secondary ionisation
m/z	mass-to-charge ratio
MALDI	matrix-assisted laser desorption/ionisation
MC	minocycline
MS	mass spectrometry
MS-MS	tandem mass spectrometry
Nd:YAG	neodymium yttrium aluminum garnet
NSAID	non steroidal anti-inflammatory drug
OPO	optical parametric oscillator
OTC	oxytetracycline
PALDI	particle-assisted laser desorption/ionisation
PSD	post-source decay
PX	piroxicam
q-TOF	quadrupole time-of-flight instrument
R2PI	resonance-two-photon ionisation
REMPI	Resonance-enhanced multi-photon ionisation
R_f	retardation factor
RSD	relative standard deviation
SALDI	surface-assisted laser desorption/ionisation
SD	standard deviation
SEM	scanning electron microscopy
TC	tetracycline
TFA	trifluoroacetic acid
TiN	titanium nitride
TiO_2	titanium dioxide
TLC	thin-layer chromatography
TLC-MS	thin-layer chromatography mass spectrometry
TOF	time-of-flight
TX	tenoxicam
UV	ultraviolet
VIS	visible

10.2 MALDI TOF Mass Spectrometers

10.2.1 SAI LaserTOF 1500 (LT1500)



Performance

Resolution: ca. 540 (FWHM) on UK-124,912

Mass Accuracy: 0.03% RMS (internal calibration)

Mass Range: greater than 500,000 Da.

Sensitivity: sub picomol

Laser

Type: pulsed nitrogen laser 337 nm (LSC Inc.)

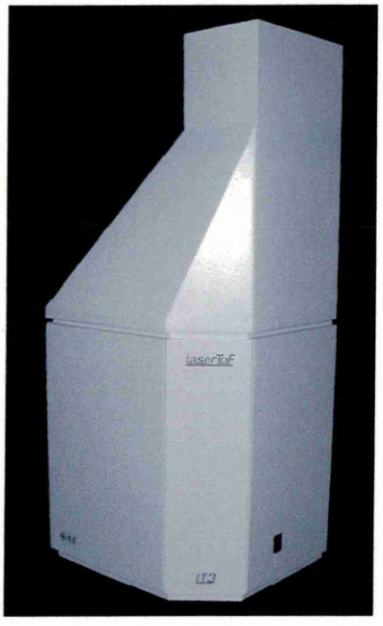
Spot size: elliptical 101 × 41 µm

(The laser spot size was determined by measuring the size of burnmarks on thermal paper using a high resolution microscope. The presented result is the average of three measurements.)

Stepper motor

Accuracy: 6 µm

10.2.2 SAI LaserTOF 3 (LT3)



Performance

Resolution:

(i) Reflectron mode: <12,000 (FWHM) ACTH (MH+2465)

(ii) Linear mode: 4,000 (FWHM) ACTH (MH+2465)

(iii) Precursor ion selection: 100 (FWHM)

Mass Accuracy:

(i) External calibration: <100ppm RMS

(ii) Internal calibration: <15ppm RMS

Mass Range: greater than 500,000 Da.

Sensitivity: less than 1fmol

Laser

Type: pulsed nitrogen laser 337 nm (LSC Inc.)

More information about both MALDI mass spectrometers can be found under http://www.saiman.demon.co.uk.

10.3 Calculation of Range Errors

In chapter 7 the precision of the constructed calibration curves is evaluated on the range errors obtained for the replicate TLC-MALDI analysis (method 1,3,4 n=3 and method 2 n=4) of each piroxicam quantity.

The range errors were calculated according to the following equation:

$$\frac{x_h - x_l}{\bar{x}} \cdot 100\%$$

x_h = highest piroxicam-to-tenoxicam ratio at given piroxicam quantity
x_l = lowest piroxicam-to-tenoxicam ratio at given piroxicam quantity
$\bar{x}$ = mean of piroxicam-to-tenoxicam ratios at given piroxicam quantity

Table 10.1 gives a summary of the calculated range errors for the corresponding quantities of piroxicam.

Table 10.1. Range errors of the five calibration points in the four different TLC-MALDI methods for the determination of piroxicam.

PX amount [ng]	Method 1	Method 2	Method 3	Method 4
400	25 %	63 %	86 %	130 %
500	21 %	50 %	70 %	125 %
600	16 %	22 %	74 %	69 %
700	13 %	22 %	41 %	59 %
800	2.0 %	15 %	32 %	55 %

10.4 Presentations

Parts of the presented work has been presented as posters or talks at various conferences.

10.4.1 Posters

A. Crecelius, M.R. Clench, D.S. Richards, G. Nichols, and V. Parr.
ANALYSIS OF PHARMACEUTICAL COMPOUNDS AND RELATED SUBSTANCES BY TLC-MALDI TOF MS.
15th IMS Conference, 27 August - 1 September 2000, Barcelona, Spain.

A. Crecelius, M.R. Clench, D.S. Richards, and V. Parr.
TLC-MALDI TOF MS USING PARTICLE SUSPENSION MATRICES.
49th ASMS Conference on Mass Spectrometry and Allied Topics, 27-31 May 2001, Chicago, USA.

A. Crecelius, M.R. Clench, D.S. Richards, and V. Parr.
TLC-MALDI TOF MS USING PARTICLE SUSPENSION MATRICES.
Analytical Research Forum of the Royal Society of Chemistry, 16-18 July 2001, Norwich, U.K.

A. Crecelius, M.R. Clench, D.S. Richards, and V. Parr.
TLC-MALDI TOF MS USING PARTICLE SUSPENSION MATRICES.
Annual BMSS Conference, 9-12 September 2001, Southampton, U.K.

10.4.2 Talks

A. Crecelius, M.R. Clench, D.S. Richards, and V. Parr.
ANALYSIS OF PHARMACEUTICAL COMPOUNDS AND RELATED SUBSTANCES BY TLC-MALDI TOF MS.
TLC forum of the Chromatographic Society, 22 November 2000, London, U.K.

A. Crecelius, M.R. Clench, D.S. Richards, and V. Parr.
ANALYSIS OF SMALL DRUG MOLECULES AND RELATED SUBSTANCES BY TLC-MALDI TOF MS.
Annual DGMS Conference, 3-6 March 2002, Heidelberg, Germany.

A. Crecelius, M.R. Clench, D.S. Richards, and V. Parr.
ANALYSIS OF SMALL DRUG MOLECULES AND RELATED SUBSTANCES BY TLC-MALDI TOF MS.
Analytical Research Forum of the Royal Society of Chemistry, 15-17 July 2002, London, U.K.

A. Crecelius, M.R. Clench, D.S. Richards, and V. Parr.
ANALYSIS OF SMALL DRUG MOLECULES AND RELATED SUBSTANCES BY TLC-MALDI TOF MS.
Annual BMSS Conference, 8-11 September 2002, Loughborough, U.K.

10.5 Publications

S. Mowthorpe, M.R. Clench, A. Crecelius, D.S. Richards, V. Parr, and L.W. Tetler.
MATRIX ASSISTED LASER DESORPTION IONISATION -TIME OF FLIGHT/THIN LAYER CHROMATOGRAPHY/MASS SPECTROMETRY - A RAPID METHOD OF IMPURITY TESTING.
Rapid Commun. Mass Spectrom. 13: 264-270 (1999).

A. Crecelius, M.R. Clench, D.S. Richards, J. Mather, and V. Parr.
ANALYSIS OF UK-224,671 AND RELATED SUBSTANCES BY THIN-LAYER CHROMATOGRAPHY/MATRIX ASSISTED LASER DESORPTION IONISATION/TIME OF FLIGHT MASS SPECTROMETRY.
J. Planar Chromatogr. 13: 76-81 (2000).

A. Crecelius, M.R. Clench, D.S. Richards, and V. Parr.
THIN-LAYER CHROMATOGRAPHY – MATRIX-ASSISTED LASER DESORPTION IONISATION - TIME-OF-FLIGHT MASS SPECTROMETRY USING PARTICLE SUSPENSION MATRICES.
J. Chromatogr. A. 958: 249-260 (2002).

A. Crecelius, M.R. Clench, D.S. Richards, D. Evason, and V. Parr.
THIN-LAYER CHROMATOGRAPHY - POSTSOURCE-DECAY MATRIX-ASSISTED LASER DESORPTION IONISATION TIME-OF-FLIGHT MASS SPECTROMETRY OF SMALL DRUG MOLECULES.
J. Chromatogr. Sci. (special issue: thin-layer chromatography) in press.

A. Crecelius, M.R. Clench, D.S. Richards, and V. Parr.
QUANTITATIVE DETERMINATION OF PIROXICAM BY TLC-MALDI TOF MS.
J. Pharm. Biomed. Anal. submitted.

Rapid Commun. Mass Spectrom. **13**, 264–270 (1999)

Matrix-assisted Laser Desorption/Ionisation Time-of-flight/Thin Layer Chromatography/ Mass Spectrometry—A Rapid Method for Impurity Testing

Siew Mowthorpe[1], Malcolm R. Clench[1]*, Anna Cricelius[1], Don S. Richards[2], Vic Parr[3] and Lee W. Tetler[1]

[1]Division of Chemistry, School of Science and Mathematics, Sheffield Hallam University, Pond Street, Sheffield, S1 1WB, UK
[2]Pfizer Central Research, Ramsgate Road, Sandwich, Kent CT13 9NJ, UK
[3]SAI Ltd, New Elm Lane, Manchester M3 4JW, UK

Thin layer chromatography/matrix-assisted laser desorption/ionisation mass spectrometry (TLC/MALDI-MS) has been previously used to obtain mass spectra from a variety of compounds, principally peptides. For pharmaceutical compounds, which are often of relatively low molecular weight, it is important that any matrix materials employed do not interfere with the mass spectra obtained. The key step to successful TLC/MALDI-MS is hence the preparation of the TLC plate prior to mass spectrometry. Crucial to this is the deposition of matrix material into the plate to promote co-crystallisation with the analyte. In this work we have examined the literature methods for plate preparation and developed two new approaches. The first involves brushing the TLC plate with a supersaturated solution of matrix and the second involves electrospraying the TLC plate with a matrix solution. Data are presented from the direct analysis of tetracycline and its impurities. Using the electrospray method the limit of detection for tetracycline is 1 ng from a TLC plate. A commercial MALDI-TOF mass spectrometer has been modified to allow the acquisition of chromatographic data from TLC plates. Chromatograms from replicate spots of 100 and 1 μg of tetracycline are shown.

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Thin layer chromatography (TLC) is a widely used, relatively low technology method for the examination of compound purity. However it provides little information about compound identity and is not quantitative. In this situation, after initial examination by TLC, high performance liquid chromatography (HPLC) methods must then be developed in order to identify and quantify unknown species observed on TLC plates. Hence the ability to examine a TLC plate and obtain mass spectral information directly would be advantageous to a variety of industries (particularly the pharmaceutical industry) who utilise TLC to examine product purity.[1,2]

Several methods have been developed for the coupling of TLC to mass spectrometry (MS). These have included the extraction of spots from TLC plates with subsequent analysis of the extract by fast atom bombardment,[3,4] ^{252}Cf plasma desorption,[5] and laser desorption followed by laser ionisation mass spectrometry directly from TLC plates.[6] Each of these techniques suffers from either sensitivity limitations or requires complex and expensive MS instrumentation. Recently, work by Gusev *et al.*[7] has demonstrated the use of matrix-assisted laser desorption/ionisation (MALDI) for the direct analysis of TLC plates. The method of plate preparation developed by Gusev *et al.* involves pressing a previously prepared layer of matrix crystals into the TLC plate. Using this method, imaging of TLC spots of guinea green B, rhodamine and bradykinin have been reported.

In this work we are interested in developing methods of TLC/MALDI-TOFMS that would allow the scanning of a full TLC plate in order to obtain chromatographic as well as mass spectral data.

It is also an objective of this project that the method developed for matrix deposition should be capable of being automated to allow routine use of the technique. Hence alternative methods to the matrix pressing method have been investigated. Bristow *et al.*[8] reported TLC/MALDI-MS of dyestuffs using plates prepared by spraying the matrix solution onto the plate with a TLC reagent sprayer. Some preliminary work carried out in our laboratory showed that this approach led to the TLC spots spreading to an unacceptable degree on the TLC plate, and would therefore not be suitable for the acquisition of chromatographic data. Electrospraying MALDI matrices has been reported for the direct analysis of biological samples,[9] and, since it was felt that the fine spray droplets produced by electrospray would lead to minimal spreading, this technique was chosen for extensive investigation.

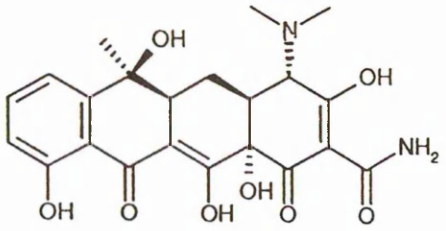
EXPERIMENTAL

Materials and instrumentation. The analyte tetracycline (compound (i), free base, FW 444.4) was purchased from Sigma-Aldrich (Dorset, UK), samples of known

*Correspondence to: M. R. Clench, Division of Chemistry, School of Science and Mathematics, Sheffield Hallam University, Pond Street, Sheffield S1 1WB, UK.
Contract/grant sponsor: Pfizer Ltd., UK.

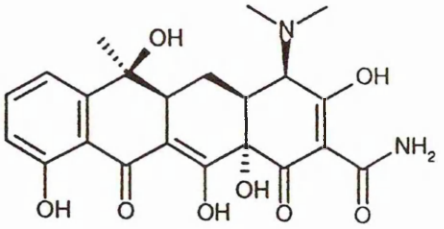
CCC 0951–4198/99/040264–07 $17.50

(i)



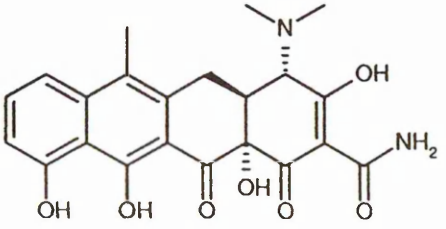
Tetracycline (FW 444 4)

(ii)



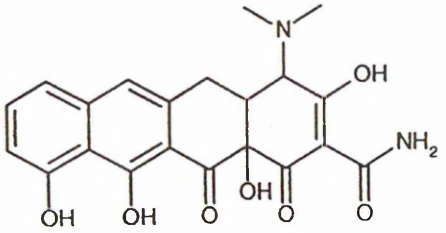
Epitetracycline (FW 444.4)

(iii)



Anhydrotetracycline (FW 426.4)

(iv)



Compound (iv) (FW 412.4)

Figure 1. Structures of tetracycline and some of its known impurities.

manufacturing impurities, epitetracycline (compound (ii), FW 444.4), anhydrotetracycline (compound (iii), FW 426.4) and compound (iv) (FW 412.4) were supplied by Pfizer Central Research, Sandwich, Kent, UK. The structures of these compounds are as shown in Fig. 1. In all cases the formula weight (FW) quoted is the molecular mass calculated using the isotope-averaged atomic masses.

The following MALDI matrices were used for MALDI/TLC coupling: α-cyano-4-hydroxycinnamic acid (αCHCA), 2-(4-hydroxyphenylazo)benzoic acid (HABA) and 2,5-dihydroxybenzoic acid (DHB). αCHCA, HABA and DHB were purchased from Sigma-Aldrich (Dorset, UK). The solvents acetone, methanol, ethanol and dichloromethane used were all HPLC grade. Trifluoroacetic acid (TFA) (AR grade) was typically added at 0.1% to the matrix solutions.

The TLC plates employed were silica gel $60F_{254}$, 0.2 mm thickness and aluminium-backed (Riedel-de-Haen).

The time-of-flight (TOF) laser mass spectrometer employed in these investigations was a modified LaserTOF 1500 (Scientific Analytical Instruments, Manchester, UK). This is a linear TOF instrument with a nitrogen laser (337 nm). The sample holder of this instrument was modified to allow TLC strips of approximately 65 × 2 mm to be fitted into a recessed slot. The sample probe and software were also modified to allow free movement along the z (vertical) axis, and software was developed that allowed the construction of TIC and mass chromatograms from the acquired data sets. Only the positive ion mode was used in this study.

Sample preparation

(i) Deposition of matrix material into the TLC plate by pressing. On an aluminium plate (25 × 25 mm) saturated solutions of α-cyano-4-hydroxycinnamic acid in acetone were deposited in 50 μL aliquots in order to create a homogenous crystal layer.

Sample spots cut from the TLC plate were wetted with an extraction solution (ethanol or methanol/water, 1:1). The previously prepared matrix layer on the aluminium plate was then pressed into the TLC plate for at least 2 minutes using a small press. After transfer the aluminium plate was removed and the thus coated TLC spots were stuck to a conventional MALDI target using double-sided tape.

(ii) Deposition of matrix material into the TLC plate by brushing with supersaturated solutions. A 65 × 2 mm strip of a developed (or uneluted) aluminium-backed TLC plate was mounted onto a conventional MALDI target with double-sided tape. An extraction solvent (ethanol) was applied to the TLC spots. A hot supersaturated slurry of the matrix (αCHCA) in acetone was then dabbed onto the TLC plate with a stiff brush. The prepared target was dried at room temperature for at least 10 minutes before analysis.

(iii) Deposition of matrix material into the TLC plate by electrospraying matrix solutions. For sample preparation via electrospray, a home-made device was constructed. This consisted of a syringe pump (Harvard 11) fitted with a 25 or 100 μL syringe which was connected via PTFE/PEEK tubing to a HPLC zero dead volume fitting to a capillary steel needle, to which a high voltage (2–4 kV) was applied. The capillary needle was further threaded through a HPLC T-piece zero dead volume fitting through which dry nitrogen could be applied coaxial to the capillary tube. The TLC plate was earthed and held horizontally during spraying and set at a distance of ca. 3–5 mm from the spray needle. The voltage and distance between the capillary needle tip and the TLC plate were adjusted such that a stable Taylor cone was visible and remained stable.

A 65 × 2 mm strip of a developed (or uneluted) aluminium-backed TLC plate was stuck onto a conventional MALDI target with double-sided tape. An extraction

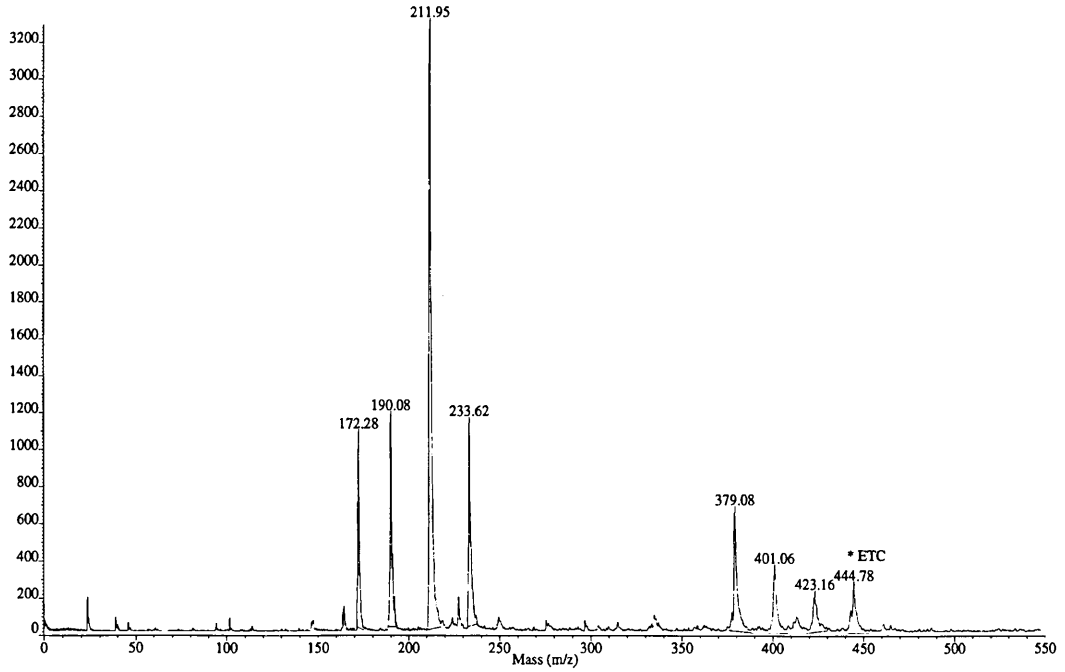


Figure 2. Mass spectrum of epitetracycline, TLC R_f value of 0.11.

solvent (ethanol) was applied to the TLC spots, when required. Matrix concentrations used were between 10–100 mg/mL depending on the matrix solubility. Two solvent systems were tested; Solvent system A, methanol/0.1% TFA and Solvent system B, methanol/water (1:1, v/v)/0.1% TFA. Typically, a 25 μL syringe was filled with a matrix solution and electrosprayed at a rate of 2.5 μL/min while the TLC plate was moved at ca. 0.6 cm/min. The voltage applied to the capillary needle of the electrospray device was typically 2.5–3.0 kV.

TLC separation

Tetracycline and its impurities were separated according to the procedure described by Weng *et al.*[9] Before use the silica gel plates were sprayed with an aqueous solution of disodium ethylenediaminetetraacetic acid (10% w/w, adjusted to pH 9.0), dried horizontally at room temperature for half an hour and then at 120 °C for ca. half an hour. The TLC development tank was lined with filter paper and saturated with development solvent dichloromethane/methanol/water (59:35:6, v/v) for an least an hour prior to use. Typically, an aliquot (10 μL) of a sample solution (20 mg/mL, methanol) was applied to a plate with a micropipette. The plate was developed to a distance of 7 cm, air dried at room temperature and visualisation of the TLC spots was by ultraviolet (UV) light at 254 nm.

RESULTS AND DISCUSSION

Selection of matrix

The selection of matrix was initially based on an examination of their behaviour during MALDI-MS on stainless steel targets.[10,11] A premixed solution of a matrix solution and sample solution (1–2 μL) in a series of matrix to analyte ratios was pipetted onto a stainless steel target. The matrix was selected on the following criteria: absence of interfering peaks in the spectral regions of interest, and reproducible analyte signal intensity from laser spot to spot over the target (i.e. good crystal homogeneity). For tetracycline, all three matrices, (αCHCA, HABA and DHB) appeared suitable based on the normal MALDI sample preparation procedure. However the lower solubility of HABA (10 mg/mL in methanol) excludes its use for electrospray deposition. αCHCA showed possible interfering peaks at *m/z* 423 $[2M - H + 2Na]^+$ and 439 $[2M - H + Na + K]^+$ which made its use as a matrix difficult since mass spectral resolution appears to slightly deteriorate on TLC/MALDI coupling. This effect may be due to the uneven nature of the TLC plate surface although it is not at present completely understood.

Optimum matrix to analyte (tetracycline) molar ratios varied from 5:1 (HABA), 100:1 (αCHCA) to 700:1 (DHB). This small excess of matrix to analyte is a reflection of the low molecular mass of the analyte species in contrast with the situation for large molecular weight analytes such as biopolymers where very large matrix excess is used.[12] The mass spectrum obtained with the use of αCHCA at a molar ratio to tetracycline of 100:1 showed considerable matrix suppression effects as observed by other workers.[13]

Comparison of different methods of deposition of matrix material

(a) Pressing. The indirect means of matrix deposition by pressing a predeposited matrix layer into a wetted TLC plate has been reported to work successfully by Gusev *et al.*[14] In our hands, the procedure was found to be extremely difficult, only small sections of silica gel plate could be handled. Therefore a developed TLC plate was cut up into small sections of 1.5 × 1.5 cm. The silica gel plate was quite fragile and frequently cracked during the procedure. Mass spectrometric analysis of successfully prepared TLC spots showed areas of localised analyte-doped matrix crystals, but the number of points from which analyte signals were obtained was insufficient to allow chromatographic information to be obtained from a spot.

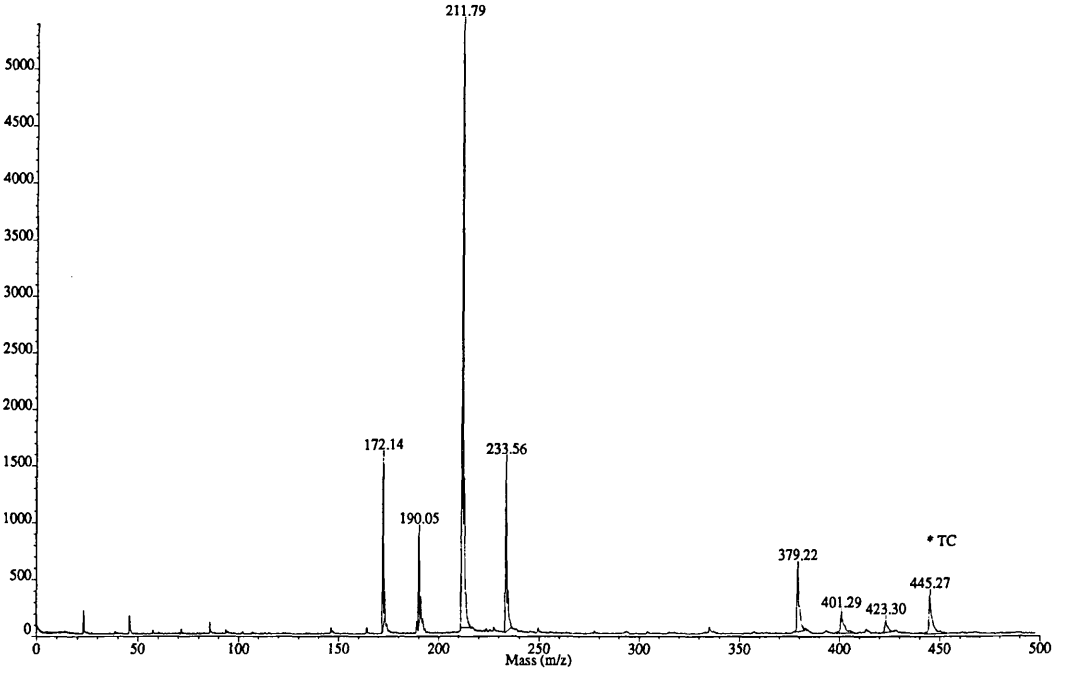


Figure 3. Mass spectrum of tetracycline, TLC R_f value of 0.25.

(b) Brushing. Deposition of matrix material by brushing a supersaturated solution of matrix into a TLC plate showed some incorporation of analyte-doped crystals and did yield analyte mass spectra. Figures 2–5 show mass spectra from TLC spots obtained from the analysis of tetracycline and some of its known manufacturing impurities. The TLC procedure was as described in the experimental section. Figure 2 shows the mass spectrum obtained from epitetracycline retardation factor (R_f) = 0.11. The (MH^+) ion can be clearly observed at *m/z* 444.8. Figure 3 shows the mass spectrum of tetracycline, itself (R_f) value = 0.25, MH^+ at *m/z* 445.3), and two unknown impurities at R_f) values of 0.34 and 0.60 are shown in Figs 4 and 5 respectively. The pre-treatment of the TLC plate with disodium ethylenediaminetetraacetic acid solution was necessary for the separation of tetracycline from the impurities. Without this pre-treatment, streaking was observed on the plate, due to the interaction of the basic moieties of tetracycline with metallic impurities in the silica. However, this procedure frequently contributed to the enhancement of signals from sodium and potassium adducts of tetracycline (*m/z* 467 and 483, respectively) and its impurities which complicates the interpretation of the mass spectra. Because of cation adduction, the close range of molecular weights of potential

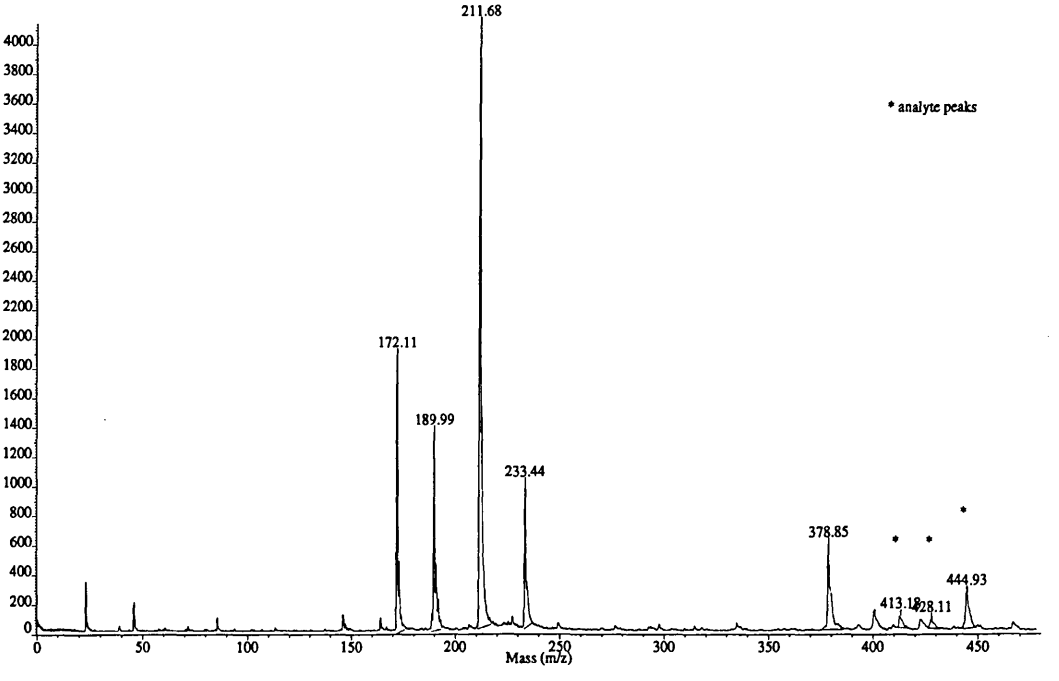


Figure 4. Mass spectrum of unknown impurity in tetracycline, TLC R_f value of 0.34.

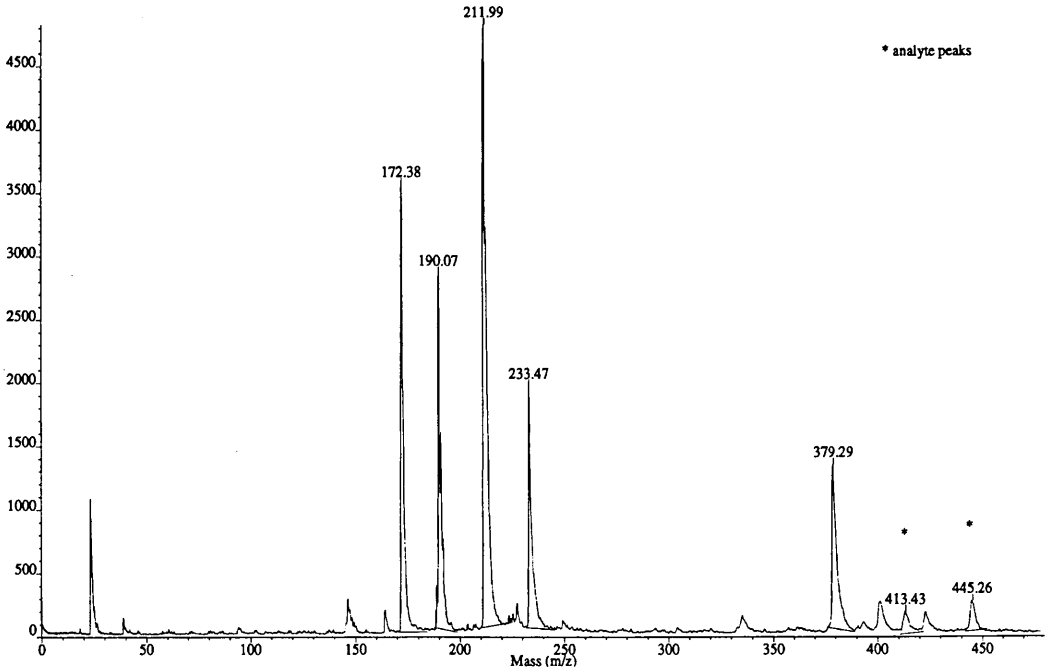


Figure 5. Mass spectrum of unknown impurity in tetracycline, TLC R_f value of 0.60.

impurities, and deterioration of resolution on TLC MALDI coupling, unambiguous identification of the impurities on the basis of assignment of MH^+ mass peaks proved challenging. In such a situation, only the use of good quality impurity reference standards would enable conclusive identification.

The procedure worked well only with very volatile solvents such as acetone, and in such cases lateral spreading of analyte species was minimal. MALDI-MS analysis targets prepared in this way showed analyte signals from localised areas on the TLC plate. However, it was difficult to produce consistent results in terms of signal intensity since the procedure did not yield a consistent loading of matrix onto the plate. Since the analyte signals from TLC plates prepared by the brushing method are localised they are not obtained from a sufficient number of points on the plate to generate chromatographic information from TLC spots. A degree of skill is needed to prepare a good plate, and the method does not seem to lend itself to automation. Therefore it was not investigated further.

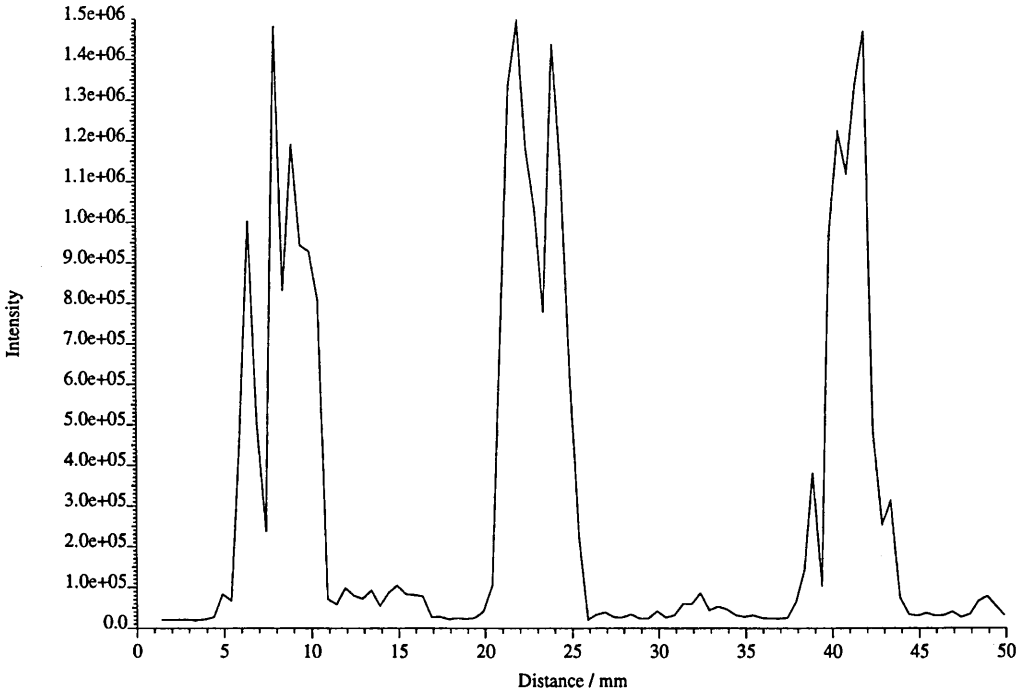


Figure 6. Summed ion chromatogram from *m/z* 440 to 460 for 3 replicate tetracycline spots of 100 µg on silica gel substrate.

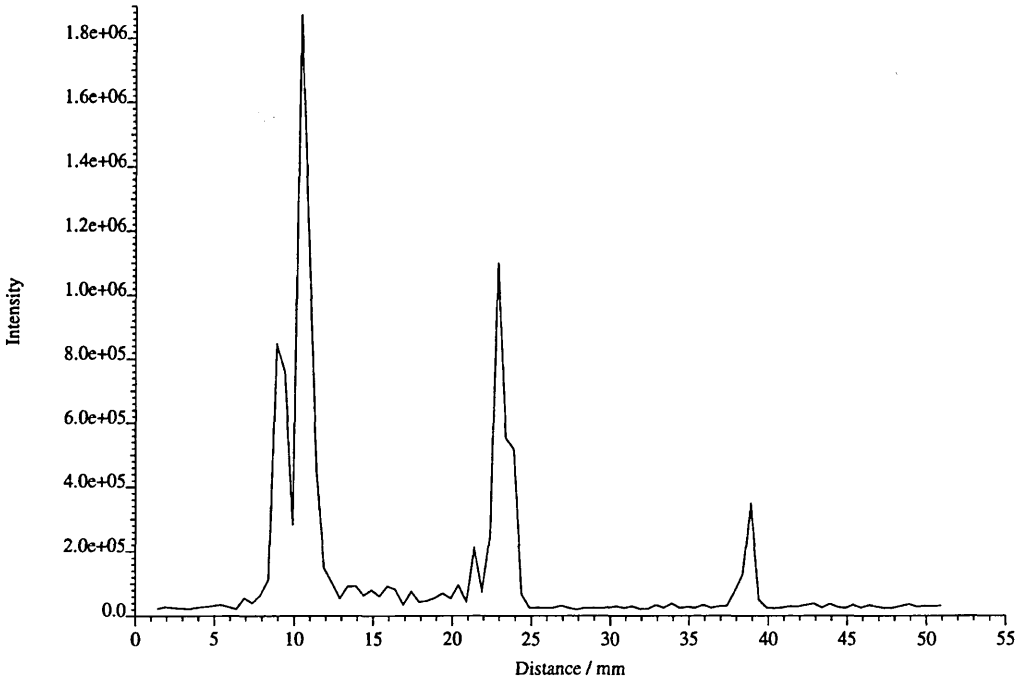


Figure 7. Summed ion chromatogram from *m/z* 440 to 460 for 3 replicate tetracycline spots of 1 μg on silica gel substrate.

(c) Electrospraying. For tetracycline the best data from plates prepared by electrospray were obtained using DHB as the matrix (100mg/mL in Solvent system B). It was found that a single spray pass was sufficient. A second pass showed either no improvement in signal intensity or a deterioration in the signal obtained, possibly because the initial layer of crystals which is co-crystallised with analyte would be redissolved by the second pass of matrix solution.

Using the modified instrument, chromatographic data were successfully obtained from TLC plates directly spotted with three replicate tetracycline spots each of 100 and 1 μg, prepared using the electrospray method. Figures 6 and 7 show summed ion chromatograms over the range *m/z* 440–460 for 3 tetracycline spots of 100 μg (Fig. 6) and 1 μg (Fig. 7). Typical mass spectra obtained from 100 and 1 μg tetracycline spots are shown in Figs 8 and 9, respectively. The ultimate limit of detection for tetracycline (localised signals) is, however, much lower at 1 ng, but so far we have not attempted scanning at these levels. These data further illustrate the poor mass resolution, and hence mass measurement accuracy, obtained in the TLC/MALDI-MS experiments to date. These appear to be quite poor in the chromatographic acquisition experiments, possibly due to the movement of the TLC plate, which is in essence now the MALDI target, over several millimetres during the acquisition of each spectrum. The small variations in flight time

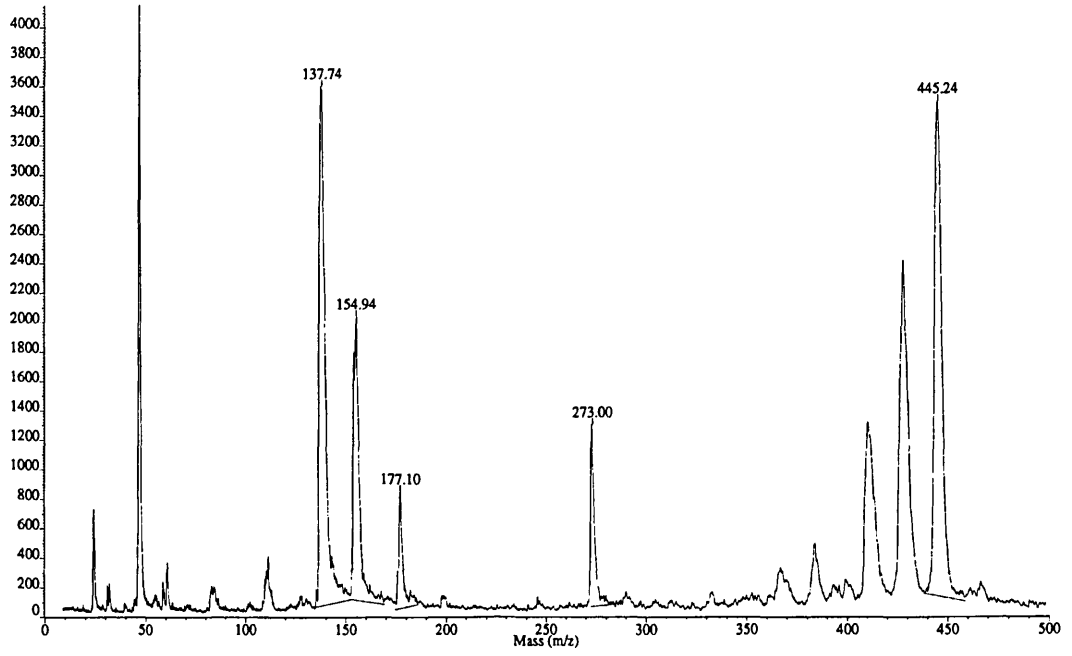


Figure 8. A typical mass spectrum obtained from scanning a 100 μg tetracycline spot.

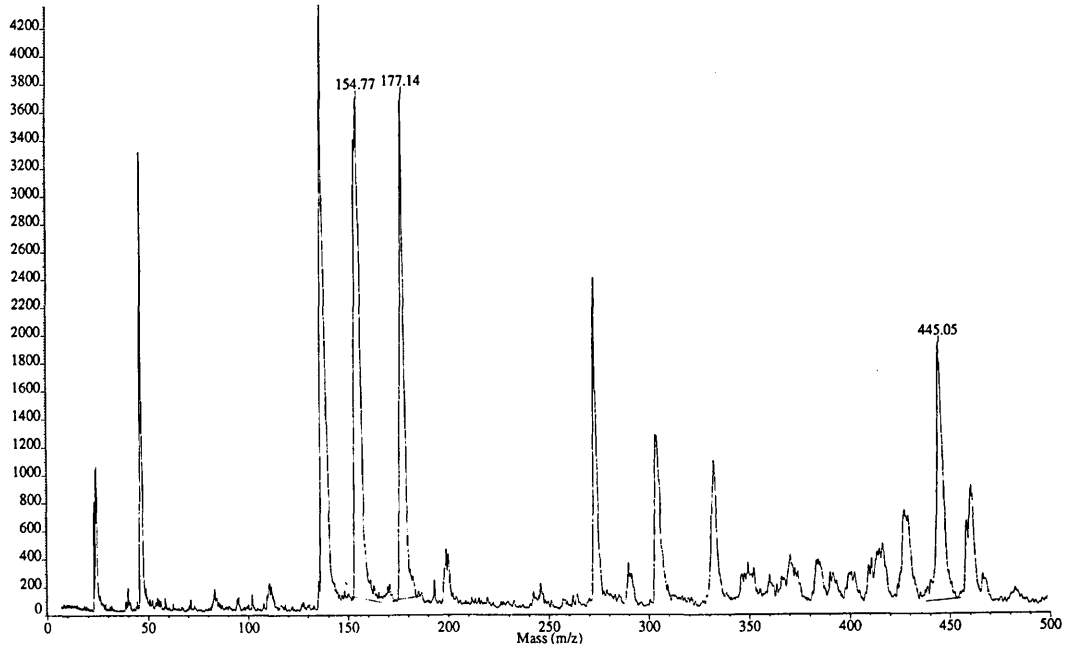


Figure 9. A typical mass spectrum obtained from scanning a 1 μg tetracycline spot.

that would cause these phenomena arising from the uneven nature of the TLC plate surface, would obviously be greater the larger the area of TLC plate surface that data has been acquired from in order to produce one spectrum. However, as previously stated, this effect is not at present fully understood.

CONCLUSIONS

We have examined the two literature methods for TLC plate preparation for coupling to MALDI-MS, i.e. pressing a previously prepared matrix layer into the TLC plate surface and the use of a reagent spray. We have also developed two new approaches; brushing a supersaturated matrix solution and deposition of matrix material by electrospraying. All procedures gave prepared TLC/MALDI targets which on analysis produced mass spectra from analyte-doped matrix crystals. However, of the three methods of matrix deposition into a TLC plate only electrospray deposition has been shown to produce analyte signals from enough points on a sample spot to allow chromatographic information to be obtained from a TLC plate. By modification of a commercial MALDI-TOF mass spectrometer, and its control and acquisition software, we have succeeded in obtaining chromatographic data from replicate spots of tetracycline at the 1 ug level. This is, to the best of our knowledge, the first time that the acquisition of chromatographic data from a TLC plate by MALDI-MS has been successfully demonstrated. The limit of detection is lower, about 1 ng (for localised signals). However it is likely that a modified environment which promotes more homogeneous crystallisation is still needed in order to improve the ultimate limit of detection further and hence allow acquisition of chromatographic data at these lower levels.

Acknowledgements

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JPC Original Research Paper

Analysis of UK-224,671 and Related Substances by Thin-Layer Chromatography–Matrix-Assisted Laser-Desorption Ionization–Time of Flight Mass Spectrometry

Anna Crecelius, Malcolm R. Clench*, Don S. Richards, Joanne Mather, and Vic Parr

Key Words:

TLC
Matrix-assisted laser-desorption ionization
Mass spectrometry

Summary

We have previously reported some preliminary data on the development of methods for the direct analysis of TLC plates by matrix-assisted laser-desorption ionization–time of flight mass spectrometry (TLC–MALDI–TOFMS). In that paper we described the electrospraying of matrix solutions on to the plate a new method of preparing TLC plates for MALDI–MS. We demonstrated that mass spectra could be obtained directly from plates prepared in such a manner and showed that modification of a commercial MALDI–TOF mass spectrometer enabled chromatographic data to be obtained from such plates.

In the work presented here, we report for the first time, the generation and characterization of an impurity profile of a pharmaceutical compound by TLC–MALDI–TOFMS. UK-224,671 is a compound synthesized, by Pfizer Ltd as part of a drug-development program. A mixture of UK-224,671 and its known related impurities was separated by TLC on silica gel and the plate was prepared for TLC–MALDI–TOFMS by electrospraying the surface with a solution of α-cyano-4-hydroxycinnamic acid (α-CHCA) by means of an electrospray deposition device. A modified SAI Ltd Laser TOF 1500 instrument was then used to record mass chromatograms directly from the TLC plate; this enabled identification of the main component and three major related substances. Higher extraction efficiency was observed when each spot was wetted with methanol before electrospraying of the matrix. The mass spectra obtained contained mainly $[M + H]^+$ ions. The degradation in mass-measurement accuracy observed in our previous TLC–MALDI–MS experiments has been corrected by use of newly developed software that recalibrates each mass spectrum acquired during a run by using a matrix ion or other selected ion as a 'lock mass'. Data acquired with and without the use of the 'lock mass' are compared.

A. Crecelius and M.R. Clench, Division of Chemistry, School of Science and Mathematics, Sheffield Hallam University, Pond Street, Sheffield; D.S. Richards, Pfizer Central Research, Ramsgate Road, Sandwich, Kent; and J. Mather and V. Parr, SAI Ltd, New Elm Lane, Manchester, UK.

1 Introduction

Although thin-layer chromatography (TLC) is widely used for the rapid qualitative examination of compound purity [1,2], the amount of qualitative information is limited to the retention factor (R_F) of the components. Coupling of TLC with mass spectrometry (MS) provides confirmatory evidence of components which are subsequently identified by comparison of their R_F values with those of reference standards on the thin layer chromatogram.

Several methods have been developed for coupling TLC with MS [3,4]. These include both indirect and direct methods. In indirect methods, TLC is often used for purification and the spots are usually visualized under UV illumination. Subsequently the components of interest are excised from the TLC plate and analyzed by fast-atom bombardment (FAB) or liquid secondary-ion mass spectrometry (LSIMS) [5,6]. Further development in this direction led to systems whereby the sample introduction inlet was modified to enable direct insertion of the TLC plate. This approach has been used for 1- and 2-dimensional acquisition of thin layer chromatograms by TLC–FABMS [7–9]. One of the drawbacks of this technique is the unsuitability of liquid matrices such as glycerol and thioglycerol. If the chromatographic resolution of the TLC plate is to be maintained the MS experiment must be performed within ca 10 min of matrix application.

TLC–MS has also been accomplished by the use of laser desorption followed by laser ionization directly from the TLC plate [10]. More recent work by *Gusev* et al. [11] has demonstrated the use of matrix-assisted laser-desorption ionization (MALDI) for the direct analysis of TLC plates. The method of plate preparation developed by *Gusev* et al. involves pressing a previously prepared layer of matrix crys-

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tals into the TLC plate. This method has been used for imaging of TLC spots of guinea green B, rhodamine, and bradykinin.

We are interested in developing TLC–MALDI–TOFMS methods that enable the scanning of a complete TLC plate to obtain both chromatographic and mass spectral data. It is also an objective of this work that the method developed for matrix deposition should be capable of automation to enable routine use of the technique. Hence, alternative methods to matrix pressing have been investigated. Electrospraying of MALDI matrices has been reported for the direct analysis of biological samples [12] and for imaging experiments conducted by MALDI [13].

We have recently reported some preliminary data from the preparation of TLC plates for MALDI by electrospraying of matrix solution [14]. In this work we were able to show that chromatographic information could be acquired from a plate prepared in this manner and were able to acquire data from replicate spots of 1 µg tetracycline. In this work we commented on errors in mass measurement observed in mass spectra acquired by TLC–MALDI–MS. In the work presented here, we report for the first time the generation of an impurity profile of a pharmaceutical compound and some of its related substances by TLC–MALDI–TOFMS entailing direct acquisition of data from a developed TLC plate. We also describe some results from the use of a software 'lock mass' to correct for mass-measurement inaccuracies observed in TLC–MALDI–MS.

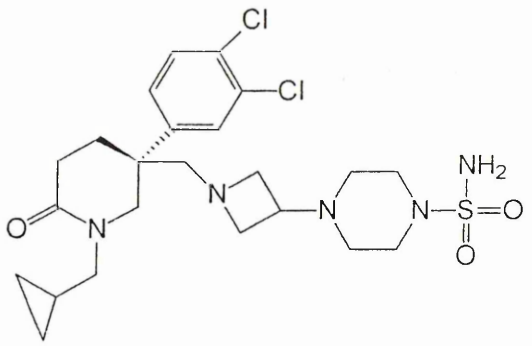
2 Experimental

2.1 Chemicals

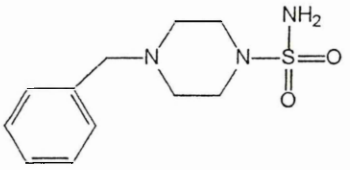
UK-224,671 ($C_{24}H_{35}Cl_2N_5O_3S$), a pharmaceutically active compound, and known related substances UK-256,327 ($C_{11}H_{17}N_3O_2S$), UK-253,501 ($C_{20}H_{26}N_4O_2S$) and UK-260,489 ($C_{19}H_{23}Cl_2NO_3$), were supplied by Pfizer Central Research, Sandwich, Kent, UK The structures of the compounds are shown in **Figure 1**. In all cases the relative molecular mass (*RMM*) quoted is calculated by using the mono-isotopic atomic masses for the most abundant isotopes.

The suitability of the organic MALDI matrices α-cyano-4-hydroxycinnamic acid (α-CHCA), 2-(4-hydroxyphenylazo)benzoic acid (HABA), 2,5-dihydroxybenzoic acid (DHB), *trans*-cinnamic acid (CA), and 3-hydroxypicolinic acid (3-HPA) (Sigma–Aldrich, Dorset, UK) for MALDI–TLC coupling was examined.

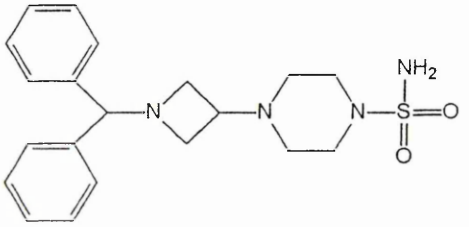
Methanol, ethanol, tetrahydrofuran, and dichloromethane were all HPLC grade. Ammonia (35%) was prepared by dilution of a concentrated ammonia SG 0.88. Water was deionized MilliQ water generated in-house (>10 MΩ). TLC was performed on aluminum-backed plates coated with 0.2 mm layers of silica gel $60F_{254}$ (Merck, Germany).



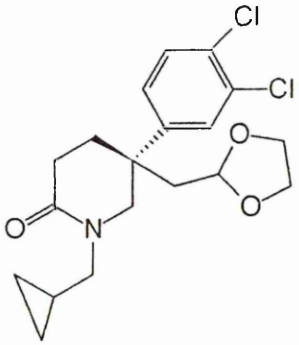
UK-224,671 RMM: 544.55 Da.



UK-256,327 RMM: 255.34 Da.



UK-253,501 RMM: 386.52 Da.



UK-260,489 RMM: 384.30 Da.

Figure 1

The structures of UK-224,671 ($C_{24}H_{35}Cl_2N_5O_3S$), a pharmaceutically active compound, and some of its known impurities UK-256,327 ($C_{11}H_{17}N_3O_2S$), UK-253,501 ($C_{20}H_{26}N_4O_2S$) and UK-260,489 ($C_{19}H_{23}Cl_2NO_3$).

2.2 TLC Separation

TLC separation was conducted by a procedure adapted from an in-house method developed at Pfizer Ltd. Silica gel plates were used as supplied without pretreatment. The TLC development tank was lined with filter paper and saturated overnight with the mobile phase, dichloromethane–methanol–ammonia (35%), 60 + 10 + 1 before use. Sample solutions (10 µL) were applied to the plates with a syringe. Plates were developed to a distance of 7.0 cm, dried in air at room temperature, and visualization of the TLC spots was by UV light at λ = 254 nm.

2.3 Matrix Selection

For organic matrices, the analytes were dissolved in methanol (10 mg mL^{-1}). The organic matrices investigated (α-CHCA, HABA, DHB, CA and 3-HPA) were dissolved in a polar solvent, typically methanol, at 10–100 mg mL^{-1} depending on solubility. Trifluoroacetic acid (TFA; AR grade; 0.1%, *v/v*) was added to the prepared matrix solution. The analyte and matrix solutions were mixed in suitable ratios (typically 1:1 and 1:10 *v/v*, respectively). Approximately 1–2 µL of the mixture was spotted on to a stainless steel MALDI target which was then analyzed.

2.4 MALDI Sample Preparation

The method used to prepare the TLC plates for MALDI–MS was to electrospray the matrix on to the plate. A 'home made' electrospray deposition device was constructed from a syringe pump (Harvard 11) fitted with a 25-µL syringe which was connected via PTFE/PEEK tubing and an HPLC 'zero dead volume' fitting to a capillary steel needle, to which a high voltage (1–4 kV) was applied. The capillary needle is further threaded through a 'zero dead volume' HPLC T-piece through which dry nitrogen could be applied coaxial to the capillary tube. The TLC plate was earthed and held horizontally during spraying, at a distance of ca 2–4 mm from the spray needle. The voltage and distance between the capillary needle tip and TLC plate were adjusted such that a stable Taylor cone was visible and remained stable.

In all the experiments a 65 mm × 2 mm strip of the developed aluminum-backed TLC plate was attached to the modified MALDI target with double-sided tape. Typically, a 25 µL syringe was filled with matrix solution which was electrosprayed at a rate of 150 µL h^{-1} while the TLC plate was moved at ca 0.6 cm min^{-1}. The voltage applied to the capillary needle of the electrospray device was usually ca 1.5 kV.

2.5 Mass Spectrometry

The time-of-flight (TOF) laser mass spectrometer employed in these investigations was a modified Laser TOF 1500 (Scientific Analytical Instruments, Manchester, UK), a linear TOF instrument with a nitrogen laser (λ = 337 nm). The sample holder of this instrument has been modified to enable TLC strips of approximately 65 mm × 2 mm to be fitted into a recessed slot. The sample probe and software have been modified to enable free movement along the *z* (vertical) axis, and software has been developed that enables the construction of total ion (TIC) and mass chromatograms from the acquired data sets. 'Lock mass' software has been developed to enable recalibration of each mass spectrum during the scanning of a TLC plate on the basis of measured *m/z* values of one or more peaks in the recorded mass spectra. Its use in these experiments is described more fully in Section 3.5. The positive ion mode only was used in this study.

3 Results and Discussion

3.1 Matrix Selection

Selection of the organic matrix was based on examination of MALDI–MS behavior on stainless steel targets. A premixed solution of a matrix solution and sample solution (1–2 µL) in a range of matrix-to-analyte ratios was pipetted on to a stainless steel target. The matrix was selected on the basis of the solubility of the matrix and the analyte in similar solvents, the absence of interfering peaks in the spectral regions of interest, and reproducible analyte signal intensity from laser spot to spot over the target (i.e. good crystal homogeneity). By use of these criteria for UK-224,671 and related substances, α-CHCA was found to give the best results. Optimum matrix-to-analyte molar ratios were between 16:1 and 40:1. This small excess of matrix to analyte reflects the low molecular mass of the analyte species, in contrast to large molecular weight analytes such as biopolymers, for which a very large matrix excess is used [15].

3.2 Electrospraying MALDI Matrices on to TLC plates

For UK-224,671 the best data from plates prepared by electrospraying were obtained by use of α-CHCA as the matrix (20 mg mL^{-1} in methanol containing 0.1% TFA). Use of this matrix, however, gives a mass spectral peak at *m/z* 379 $[2M + H]^+$ which can interfere with signals from UK-253,501 (*RMM* 386.52 Da.) and UK-260,489 (*RMM* 384.3 Da.) if adequate mass measurement stability and resolution are not achieved. Instrumental resolution was, however, adequate for separation of analyte- and matrix-related signals, as shown in Figures 3c, d and 5c, d, respectively.

3.3 Acquisition of Mass Chromatograms and Mass Spectra

By use of the modified instrument chromatographic data were successfully obtained from a thin-layer chromatogram of a mixture containing 25 µg of each of UK-224,671, UK-256,327, UK-253,501, and UK-260,489. The overlaid ion mass chromatograms are shown in **Figure 2.** In these data, the peaks arising from the four TLC spots are clearly visible. The software labels the *x*-axis with the distance traveled from the origin by the spot, enabling ready calculation of R_F

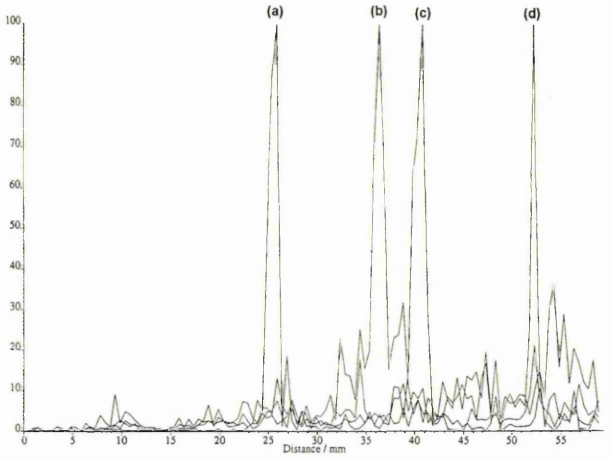


Figure 2

Overlaid ion chromatograms from TLC–MALDI–MS analysis of a mixture of: (a) UK-224,671, (b) UK-256,327, (c) UK-253,501, and (d) UK-260,489 (25 µg per component).

values. From these data the R_F values of UK-224,671, UK-256,327, UK-253,501, and UK-260,489 were calculated as 0.36, 0.51, 0.57, and 0.75, respectively, in accord with the R_F values obtained by UV detection. The ion mass chromatogram of the parent compound UK-224,671 comes from a different run. The corresponding mass spectra obtained for each spot are shown in **Figure 3**. Although these non-background-subtracted spectra show intense ions arising from the matrix, the protonated molecular species from each analyte is clearly visible.

3.4 Extraction of Analytes from TLC plates

Analytes separated by TLC are presumably located up to 100–500 µm inside the plate. To produce a MALDI–MS signal, the analyte must be transferred from inside the silica gel to the surface, and this must be followed by electrospraying of the matrix, and crystallization. The time available for extraction of an analyte from inside the TLC plate on to the surface is limited by the time of matrix crystallization, which is less than ca 10 min [16]. In this work extraction of the analytes from the interior of the TLC plate was improved by addition of an organic solvent, for example ethanol, tetrahydrofuran, methanol, and methanol–water, 1:1, (*v*/*v*). The best results were obtained when methanol was applied to each TLC spot. **Figure 4** shows the overlaid-ion mass chromatograms from TLC–MALDI–MS analysis of a mixture containing 25 µg of each of UK-224,671, UK-256,327, UK-253,501, and UK-260,489, using the approach described. The jagged nature of the chromatographic peaks is a typical characteristic of the use of methanol, which causes analyte spreading in a manner such that the concentration of the analyte at the spot center is reduced. The quantity of extraction solvent

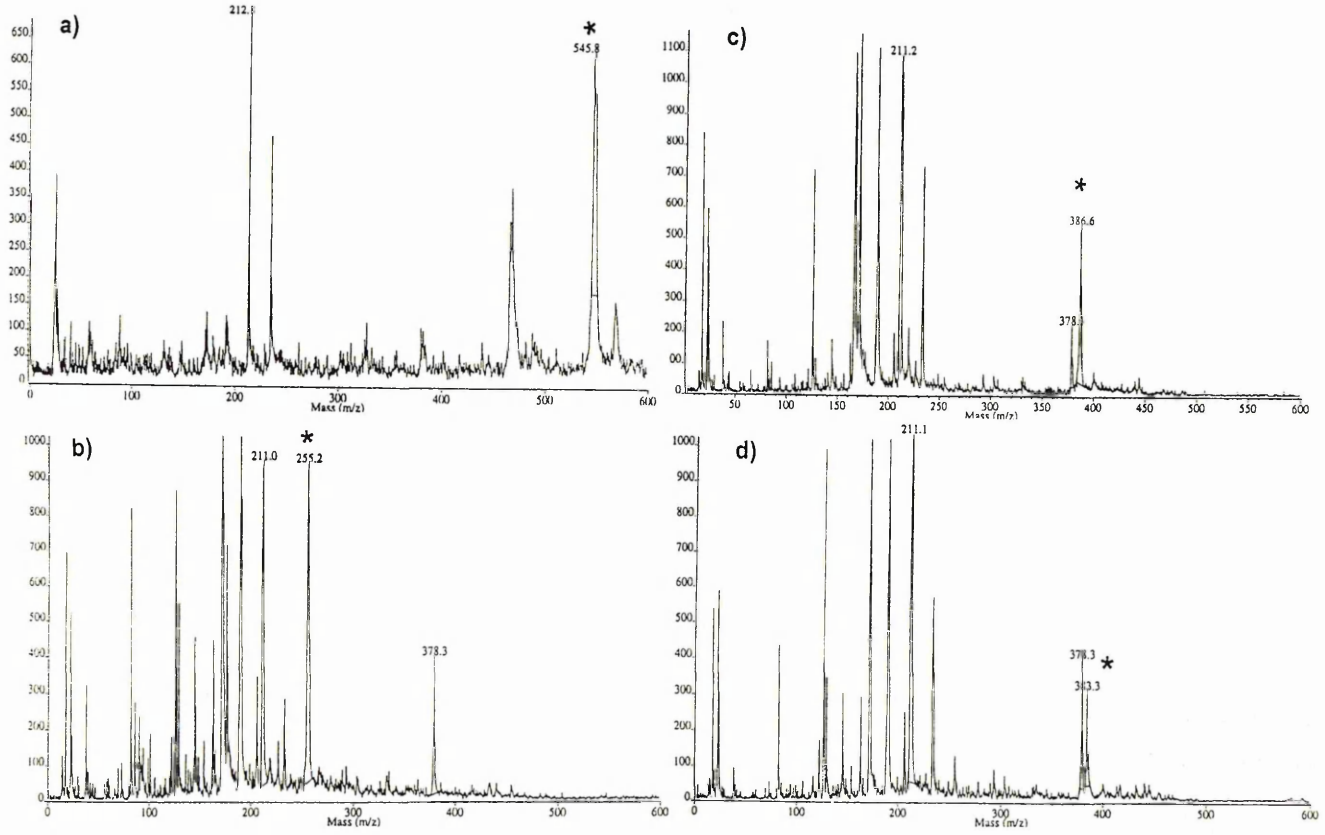


Figure 3

TLC–MALDI mass spectra of the peaks indicated in Figure 2: (a) UK-224,671, (b) UK-256,327, (c) UK-253,501, and (d) UK-260,489 (25 µg per component).

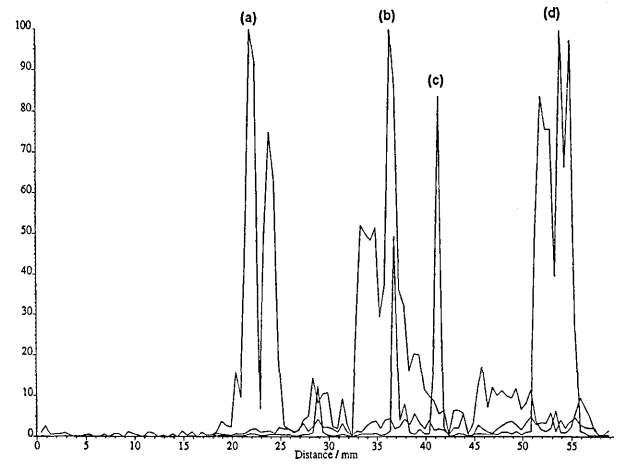


Figure 4

Overlaid ion chromatograms from TLC–MALDI–MS analysis of a mixture of: (a) UK-224,671, (b) UK-256,327, (c) UK-253,501, and (d) UK-260,489 (25 µg per component). The data were acquired after use of methanol as extraction solvent.

deposited on each TLC spot is critical – the amount should be as high as possible to increase the absolute quantity of analyte close to the surface but lateral spreading should be minimized as far as possible. It has been found that ca 1 µL methanol is appropriate. Lateral spreading was typically in the range 1–5 mm, depending on the analyte. Planar diffusion of UK-256,327 along the plate occurred even without application of an extraction solvent (Figure 2). Figure also illustrates that UK-256,327 and UK-253,501 could be found at the same position (37 mm from the starting point) because of the extraction procedure. The improvement of the sensitivity of TLC–MALDI–MS experiments performed on UK-224,671 and related compounds by use of an extraction solvent is demonstrated by the increase in the ion intensity of the protonated molecular species for each substance, observable in **Figure 5**, and also by the detection of all four analytes in a single experiment.

3.5 Recalibration of each Mass Spectrum

As shown in Figure 3d, in which the protonated molecular species of UK-260,489 (*RMM* 384.30 Da.) was observed at *m/z* 383.3 rather than at 385 Da., degradation of mass spectral resolution and mass measurement accuracy is observed when mass spectra are recorded directly from TLC plates. We have previously reported this effect [14], and others have observed such degradation even when reflectron based MALDI–MS (capable of much higher resolution than the instrument used in this work) was employed [17]. Because we attribute this effect to the uneven nature of the TLC plate surface, the use of a software 'lock mass' was investigated. This software recalibrates each mass spectrum acquired during the scanning of a TLC plate, on the basis of the error in mass measurement observed for one or more user-defined *m/z* values. Initially two matrix ions were cho-

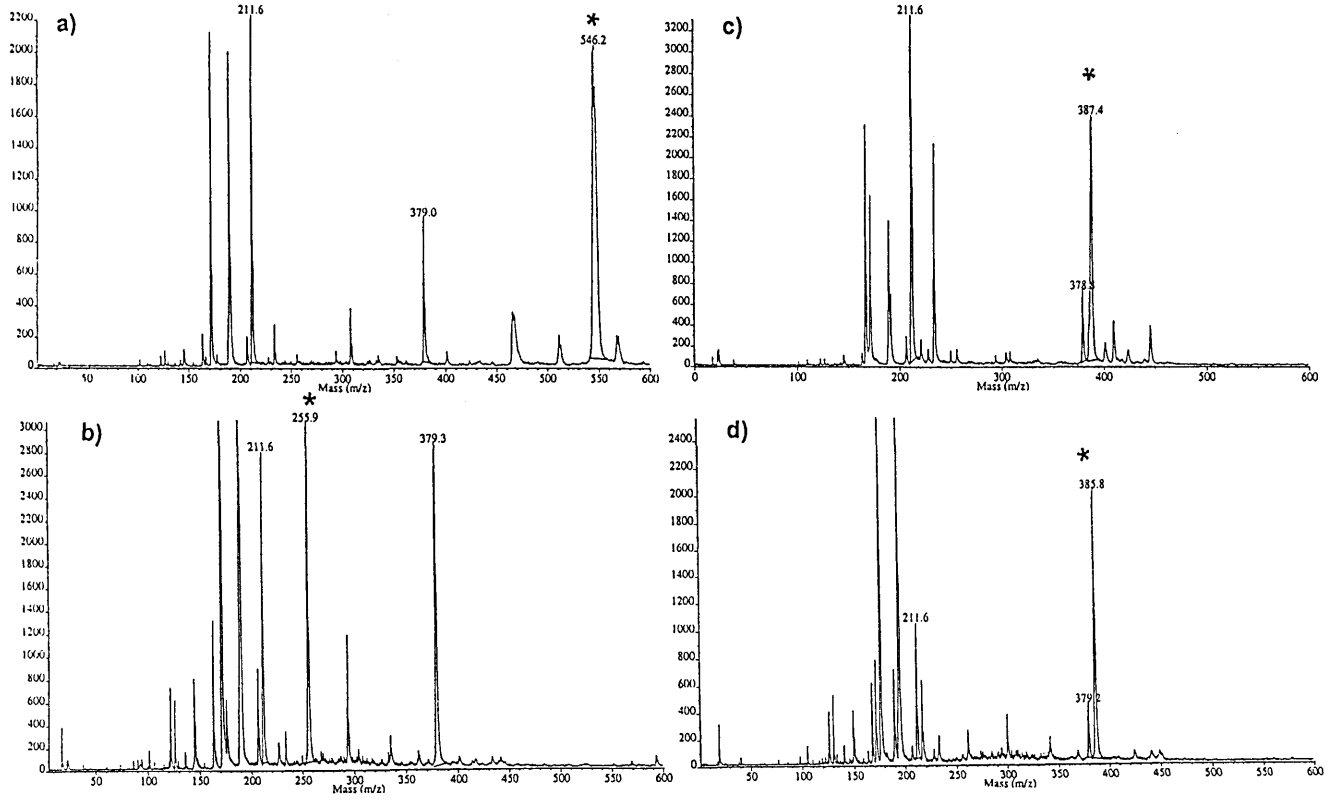


Figure 5

TLC–MALDI mass spectra of the peaks indicated in Figure 4: (a) UK-224,671, (b) UK-256,327, (c) UK-253,501, and (d) UK-260,489 (25 µg per component)

sen for the recalibration, but we have found that that one is sufficient. Thus, the $[M + Na]^+$ ion of α-CHCA was typically chosen. The mass spectra shown in Figure 5 were acquired by use of the 'lock-mass' software and illustrate that this approach is a possible solution of mass measurement inaccuracy, although the mass spectral resolution achieved in the chromatographic acquisition experiments is still relatively poor compared with conventional MALDI applications. This is possibly because of the movement of the TLC plate over a distance of 0.125 mm during the acquisition of each spectrum.

4 Conclusions

We have succeeded in obtaining mass spectral and chromatographic data from a thin layer chromatogram of a mixture of 25 µg quantities of a pharmaceutical compound and some of its related substances. This is, to the best of our knowledge, the first time that the acquisition of genuine chromatographic data from a TLC plate by MALDI–MS has been successfully demonstrated.

The role of an extraction solvent has been investigated in detail. It has been demonstrated that its use results in a higher extraction efficiency and hence in an increase in the intensity of ions from the compounds studied. It also leads to planar spreading, however, which leads to broader chromatographic peaks in the chromatographic data obtained by TLC–MALDI–MS. The degradation in mass measurement accuracy that we previously reported in TLC–MALDI–MS experiments has been successfully overcome by the use of a software 'lock mass'.

We now intend to investigate further modification of the sample procedure to promote more homogeneous crystallization of matrix and analyte. This should enable acquisition of chromatographic data at lower levels. We will also examine the use of cobalt ultra fine powder (Co-UFP) as a matrix to reduce the background ions present in the mass spectrum which can interfere with the analyte ions.

Acknowledgments

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The Possible use of Filter Paper as a Concentrating Medium in TLC

Victor G. Berezkin, Irena Malinowska, Jan K. Różyło*, A.B. Makarov, and R.G. Mardanov

Key Words:

Adsorption TLC
Sample concentration

Summary

This paper describes the possibility of applying mixtures of substances to TLC plates by use of different types of filter (chromatographic) paper as concentrating zones. The resolution of chromatographic systems has been compared after use of different modes of solute application – applicator (point and linear application), micropipet, use of plates with concentrating phase, and use of different types of filter paper as the concentrating phase.

The effect of the method of solute application on the R_F values of the chromatographed substances was also tested. The proposed use of paper as a concentrating zone increases the resolution of a chromatographic system in comparison with systems in which a micropipet is used; resolution is comparable with that obtained on commercially available plates with concentrating phase. The use of the concentrating zone affects the migration of individual components of the solute. The effect depends on the kind of concentrating zone and on the adsorption of a given substance on the adsorbent surface.

1 Introduction

The basic problem in chromatography is the efficiency of the separation of mixtures. A measure of this efficiency is the resolution of chromatographic systems, R_S, which is defined by the relationship [1,2]:

$$RS = \frac{2(R_{F1} - R_{F2})}{(w_1 + w_2)} \quad (1)$$

where R_{F1} and R_{F2} are the R_F values of two adjacent substances and w_1 and w_2 are the widths of the respective spots, measured in the direction of migration of the mobile phase.

R_F values depend on substance identity and on the nature of the adsorbent and mobile phase. The broadening of a spot of a chromatographed substance depends not only on these factors but also on the size of the solute spot at the origin of a chromatogram, i.e. on the method of application of the substance. The smaller the size of a spot at the origin of a chromatogram, the smaller the spots of the chromatographed substances on the developed chromatogram, i.e. the method of application of substances to a TLC plate influences the resolution obtained when the mixture is separated. Correct application of a solute can thus be a means of improving the resolution and efficiency of a chromatographic system.

Solute application in planar chromatography can be performed by a variety of different methods. The method used is crucial for mixtures of components which are difficult to separate and the spots are situated close one to another. The oldest and most widely used method of sample application is the use of a micropipet – a calibrated capillary or automatic equipment with the possibility of changing the volume. The major advantages of the method are its simplicity and speed. Sample spots applied in this way are circular at the origin, the diameter of the spot depending on the volume of solute applied.

One method of improving the resolution of a chromatographic system is band (linear) application. The chromatographed substance or mixture is applied to the origin, either manually or automatically in the form of a band. Manual application is time-consuming and requires considerable skill – an uneven band is often obtained.

Bands can also be obtained by means of a special procedure entailing evaporation of solvent from the plate; the proce-

V.G. Berezkin, A.B. Makarov, and R.G. Mardanov, A.V. Topchiev Institute of Petrochemical Synthesis of the Russian Academy of Sciences, 29 Leninsky Pr., 11-79-12 Moscow, Russia; and I. Malinowska and J.K. Różyło, Faculty of Chemistry, Department of Adsorption and Planar Chromatography, M. Curie-Sklodowska University, M. Curie-Sklodowska Sq. 3, 20-031 Lublin, Poland.

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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

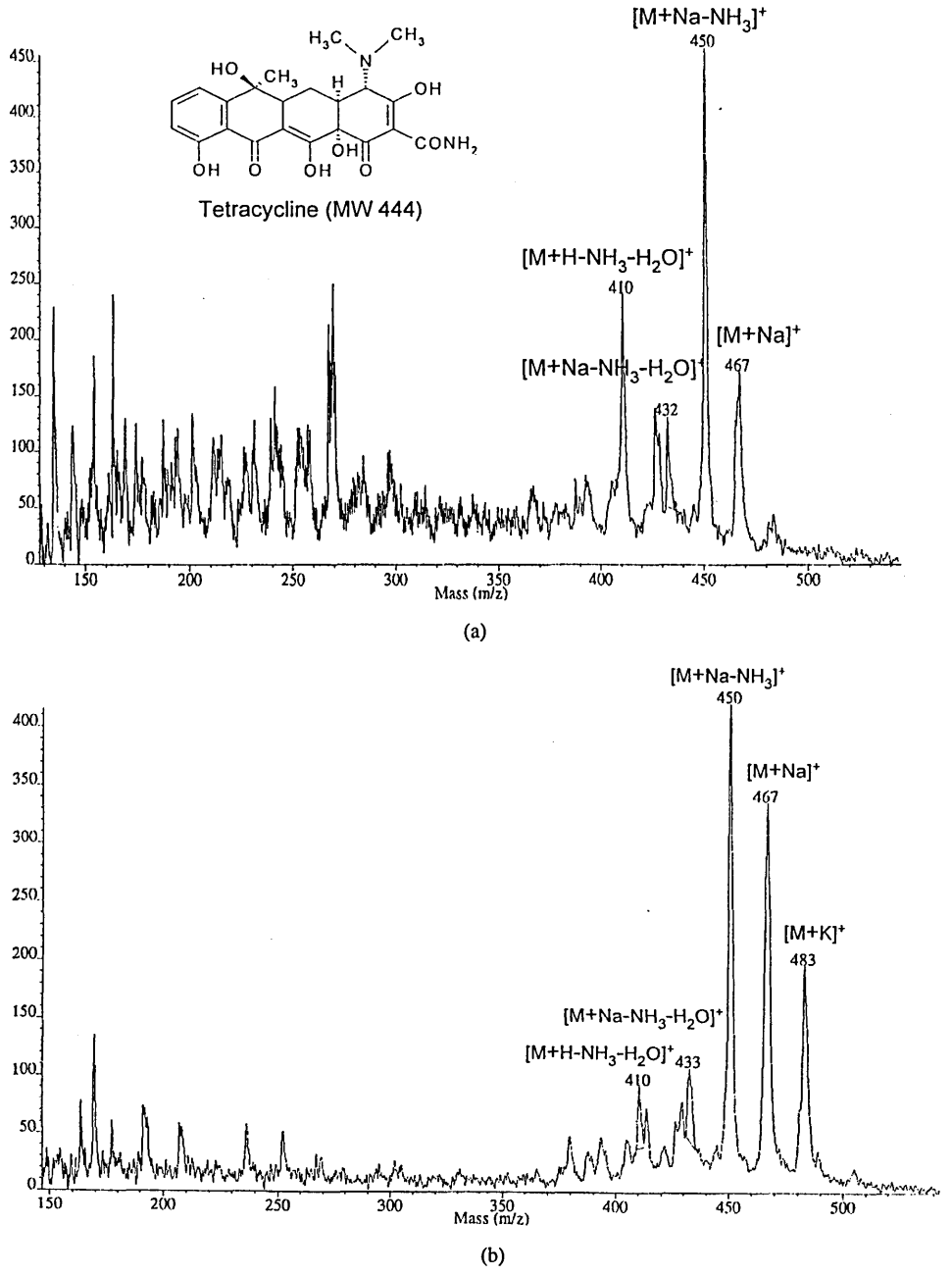
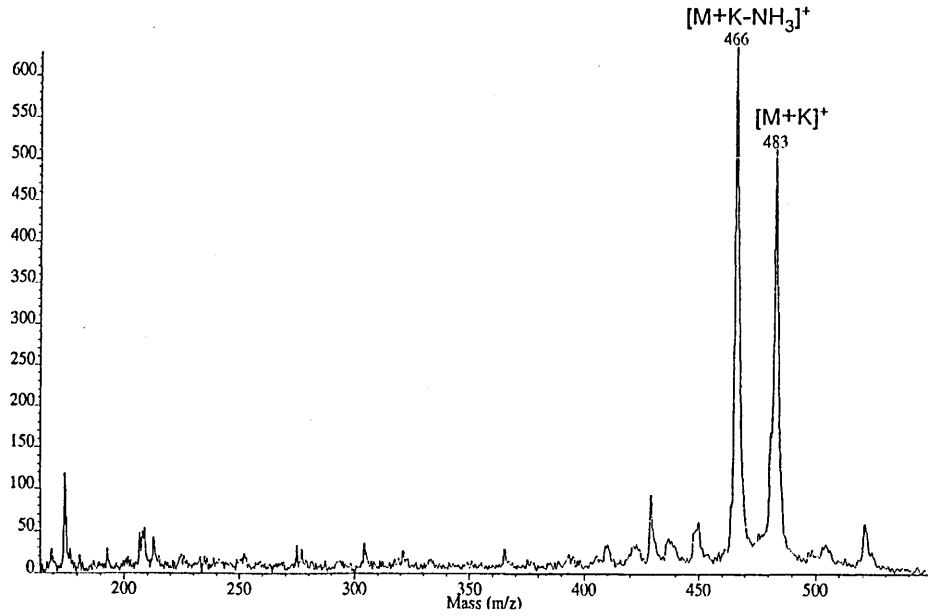
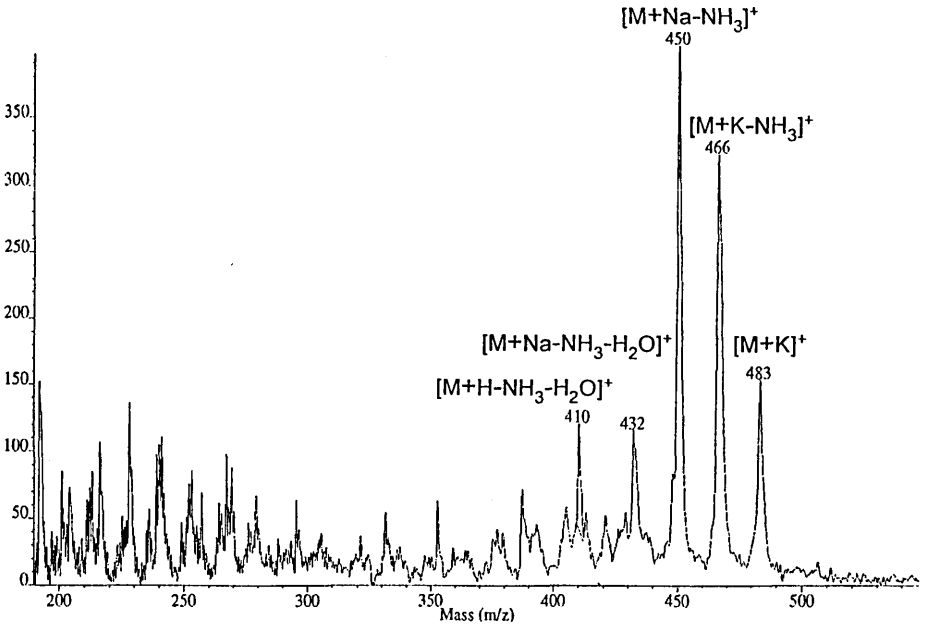


Fig. 1. MALDI mass spectra of tetracycline using nano- and micro-particles as suspension matrices, (a) Co-UFP, (b) TiN, (c) TiO_2, (d) Graphite and (e) Silicon.

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



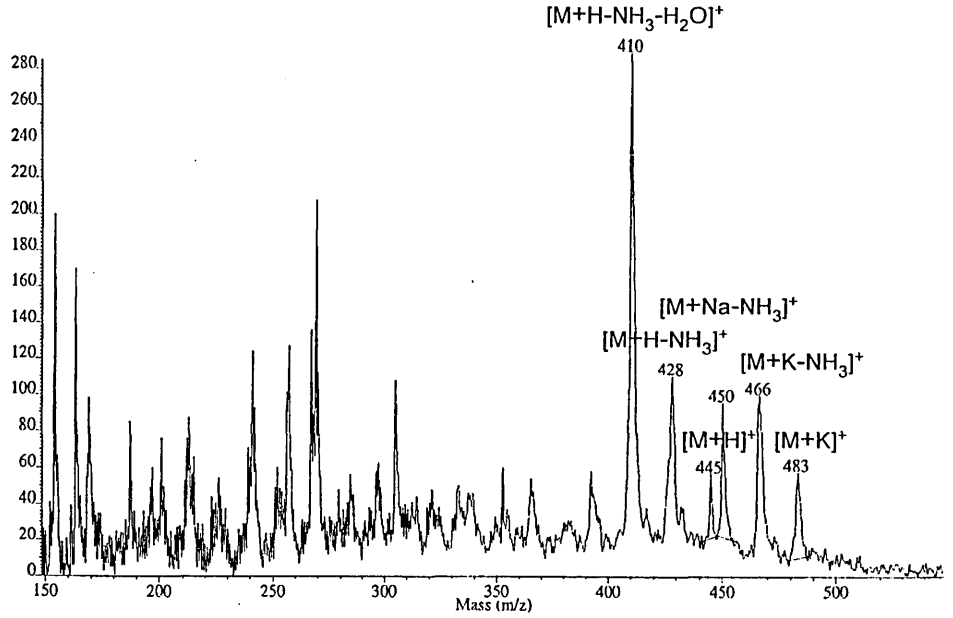
(c)



(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



(c)

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.

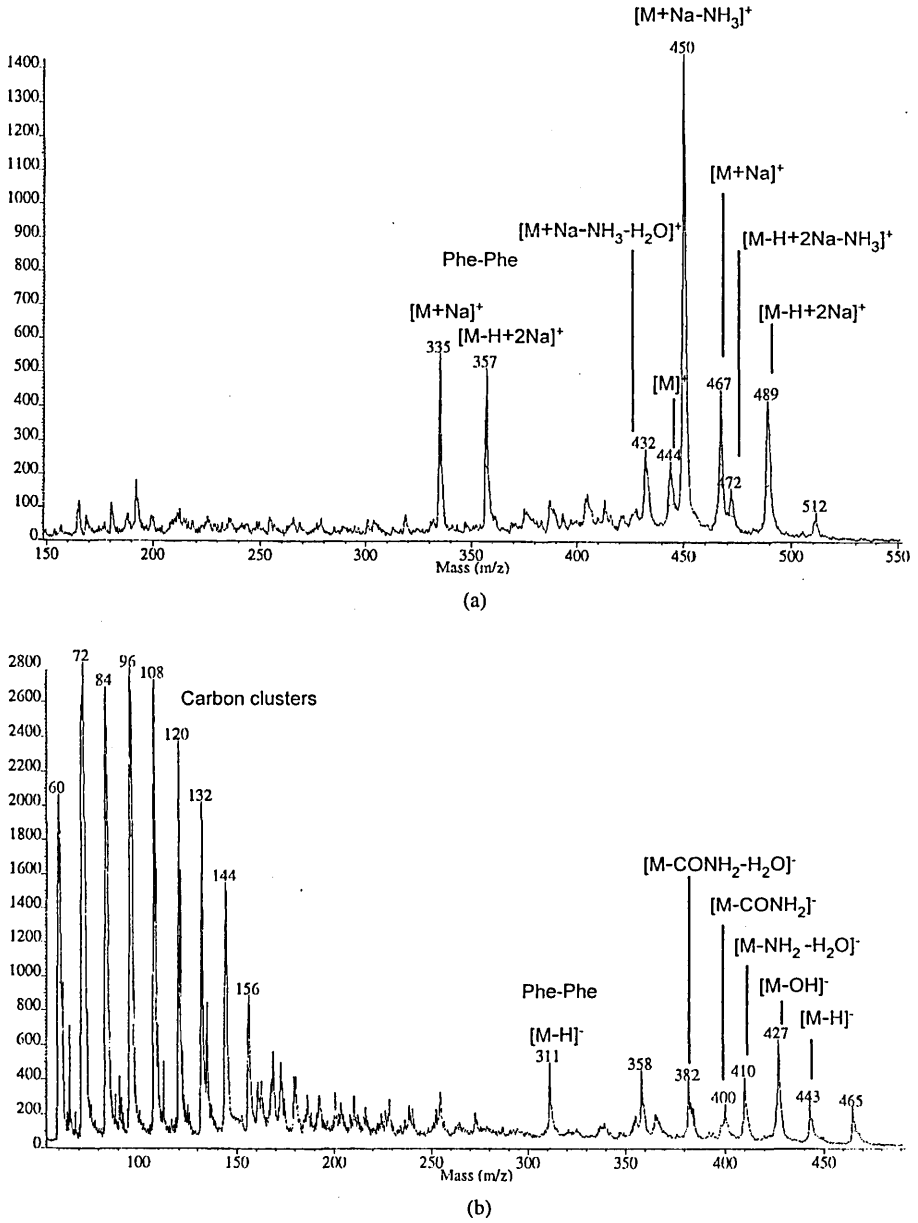


Fig. 2. TLC–MALDI mass spectra of tetracycline (200 μg) using a graphite matrix in: (a) positive; and (b) negative ion mode.

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

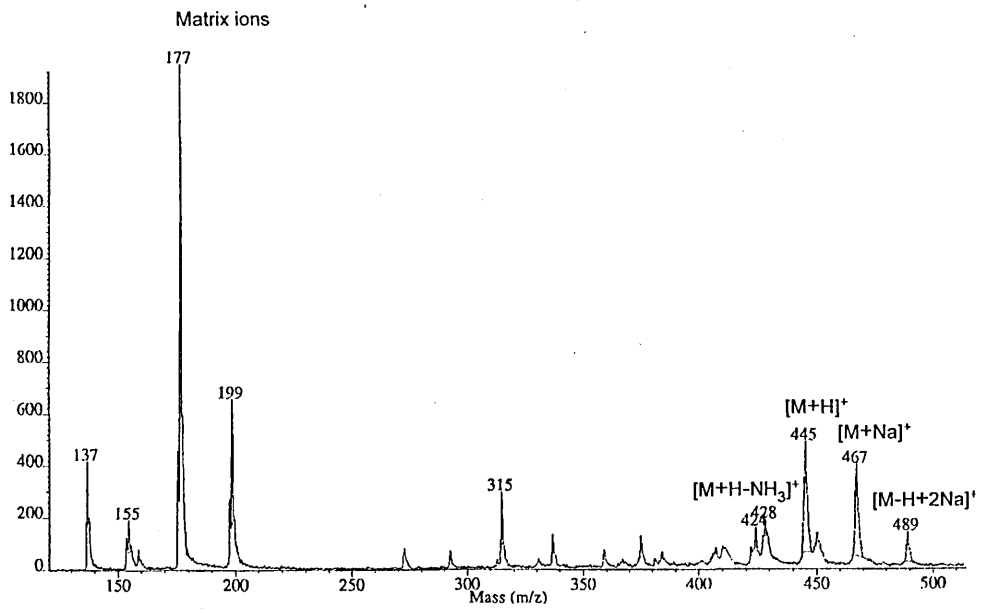


Fig. 3. TLC–MALDI mass spectrum of tetracycline (200 μg) using DHB.

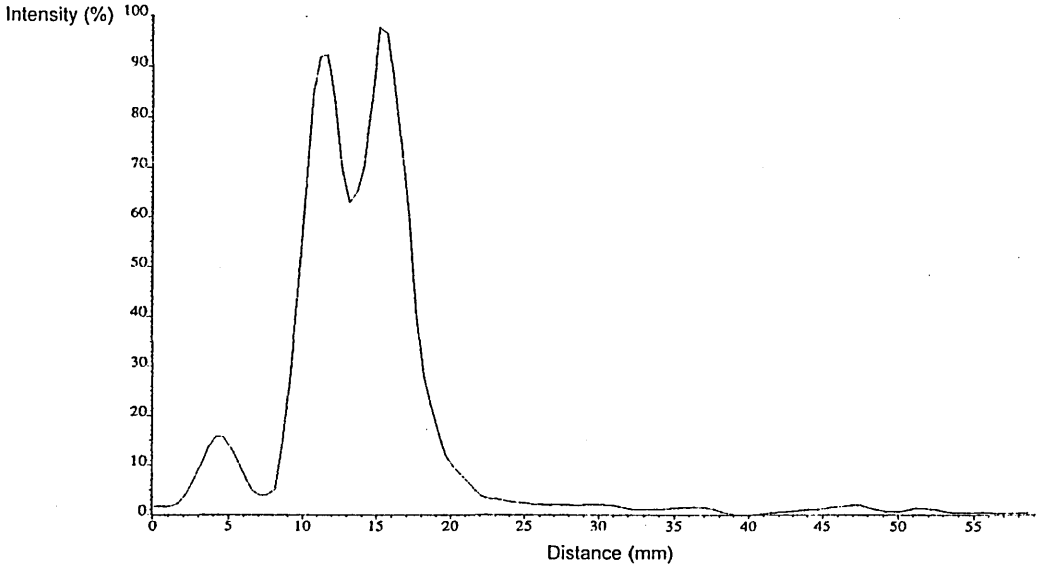


Fig. 4. The smoothed ion mass chromatogram of tetracycline (200 μg) from the experiment shown in Fig. 3. The $[M+H]^+$ ion (*m/z* 444–446) was used to construct the ion mass chromatogram.

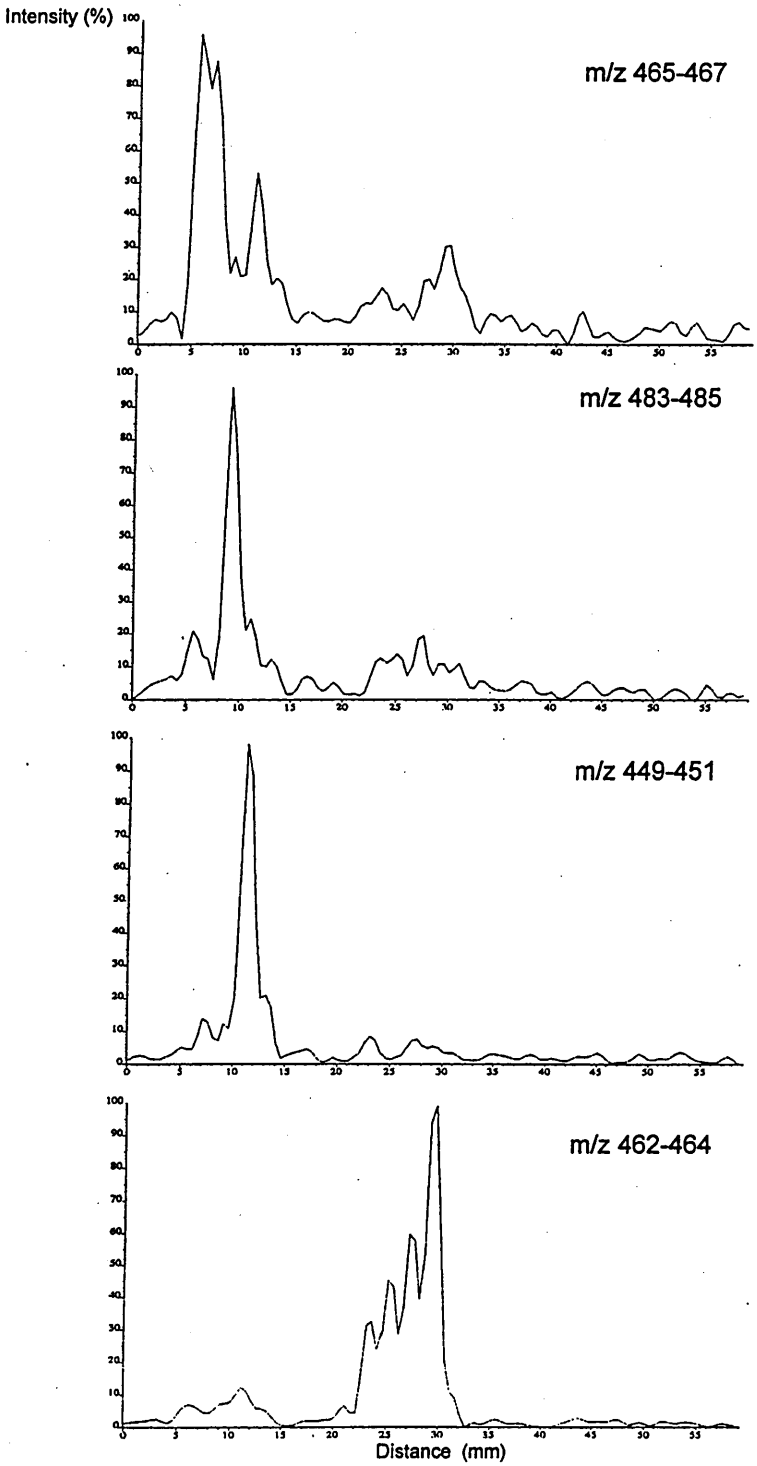


Fig. 5. Smoothed single ion mass chromatograms from the TLC–MALDI–TOF–MS analysis of OTC, CTC, TC and MC (10 μg per component).

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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