Thin-Layer Chromatography with Ultraviolet and Mass Spectrometric Detection: From Preparative-Layer to Miniaturized Ultra-Thin-Layer Technique

by

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Academic Dissertation

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PREFACE

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Piia Salo

ABSTRACT

This thesis summarizes the principles of classical planar chromatographic separation combined with ultraviolet (UV) and mass spectrometric (MS) detection. In the experimental work, preparative-layer chromatography was used for purification and high-performance and ultra-thin-layer chromatography for analytical studies. UV or UV/VIS detection was carried out by visualization under a UV lamp or in daylight after derivatization, or by UV densitometry. In MS detection, flow-injection analysis–electrospray ionization (FIA–ESI), atmospheric pressure matrix-assisted laser desorption/ionization (AP–MALDI), vacuum MALDI, or desorption electrospray ionization (DESI) was applied. Small drug or "drug-like" molecules were utilized for the establishment, testing, and evaluation of these methods.

The purification of target compounds from crude synthesis products were carried out by preparative-layer chromatography (PLC). The usefulness of PLC for parallel purification of several samples on a singe plate was investigated, and six samples were purified in parallel. A new and simple scraping device was introduced for isolation of the PLC-separated target compounds from the plate. With this device, and PLC, synthesized products were successfully purified in sub-milligram amounts. To achieve the reliable purity for biological activity screening and early ADME testing (i.e. 80%), the resolution in PLC needed to be greater than 0.8.

High-performance thin-layer chromatography (HPTLC) was introduced and evaluated for fast semi-quantitative assessment of the purity of target compounds produced by solid- or liquid-phase synthesis. HPTLC-separated sample zones were detected and identified by measuring UV densitograms, in situ UV spectra, and, if necessary, MS spectra. The purities obtained by HPTLC–UV were equal (r^2 = 0.8053–0.8795) to those obtained by liquid chromatography, confirming the suitability of HPTLC for purity analysis of target compounds of synthesized products. ESI–MS and AP–MALDI–MS were used to identify and confirm the product zones on the plate after separation. For ESI–MS analysis, a new, simple, and fast scraping device was developed for removing the zone of the compound from the plate. AP–MALDI–MS was rapid, and easy to carry out directly on the plate without scraping. The new HPTLC methods enabled rapid, efficient, easy, and parallel analyses of several samples on one plate.

Ultra-thin-layer chromatography with atmospheric pressure matrix-assisted laser desorption ionization mass spectrometric detection (UTLC–AP–MALDI–MS) was introduced and studied for the first time. UTLC and HPTLC methods relying on UV and AP–MALDI–MS detection, and UTLC combined with AP–MALDI–MS and vacuum MALDI–MS were compared in the analysis of small drug and new synthesized molecules. Because of their thinner adsorbent layer, the monolithic UTLC plates provided 10–100 times better sensitivity in MALDI analysis than did conventional HPTLC plates. Limits of detection down to low picomole range were demonstrated for UTLC–AP–MALDI–MS. Other advantages of UTLC over HPTLC included faster separations and lower solvent consumption. In a comparison of AP– and vacuum MALDI–MS detection for UTLC plates, desorption from the irregular surface of the plates with the combination of an external AP–MALDI ion source and an ion

trap instrument provided clearly less variation in mass accuracy than the vacuum MALDI–time-of-flight (TOF) instrument. UTLC–AP–MALDI–MS was successfully applied to the purity analysis of synthesized products.

The combination of UTLC plates and desorption electrospray ionization mass spectrometry (DESI–MS) was also applied for the first time in the separation and identification of compounds. The limits of detection (LODs) for six different test compounds on the UTLC plate were determined to be in the picomole (ng) range, and the plates were successfully used as a rapid means of chromatographic separation before DESI–MS analysis.

An analysis of biological samples by two-dimensional (2D) UTLC separation with AP–MALDI–MS detection was presented for the first time. The performance of the method for bioanalysis was studied with benzodiazepines as model substances in human urine, and the influence of the urine matrix on the separation and repeatability was evaluated. 2D UTLC was shown to be an efficient technique for the separation of benzodiazepines. Separations occurred in 4–12 minutes. And AP–MALDI–MS was well suited for in situ detection and identification of the separated compounds. The limits of detection with both AP–MALDI–MS and AP–MALDI–MS/MS were in picomole range and thus low enough for bioanalysis. The applicability of 2D UTLC–AP–MALDI–MS was demonstrated in the detection of metabolites in an authentic urine sample.

In summary, HPTLC and UTLC combined with UV and MS detection, as presented here, were found to be rapid, easy, and low-cost techniques, and thus to provide powerful, alternative tools for qualitative and quantitative analysis of small molecules. And classical PLC was successfully utilized for the parallel purification of several samples on a single PLC plate.

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following five original publications, which will be referred to in the text by their Roman numerals (I–V):

I Piia K. Salo, Anniina M. Pertovaara, Vesa-Markku A. Salo, Hannele E. M. Salomies, and Risto K. Kostiainen; High-Performance Thin-Layer Chromatography Method for Assessment of the Quality of Combinatorial Libraries, and Comparison with Liquid Chromatography–Ultraviolet–Mass Spectrometry. *J. Comb. Chem.* 2003, 5, 223-232.

II Piia K. Salo, Annika Essén-Suuronen, Hannele Salomies, Raimo A. Ketola, and Risto Kostiainen; HPTLC, with UV and MS Detection, and Preparative-Layer Chromatography for Analysis and Purification of Synthesis Products. *J. Planar Chromatogr*. 2006, 19, 371-377.

III Piia K. Salo, Hannele Salomies, Kirsi Harju, Jari Yli-Kauhaluoma, Tapio Kotiaho, Raimo A. Ketola, and Risto Kostiainen; Analysis of Small Molecules by Ultra Thin-Layer Chromatography–Atmospheric Pressure Matrix–Assisted Laser Desorption/ Ionization Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* 2005, 16, 906-915.

IV Piia K. Salo, Suvi Vilmunen, Hannele Salomies, Raimo A. Ketola, and Risto Kostiainen; Two-Dimensional Ultra-Thin-Layer Chromatography and Atmospheric Pressure Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry in Bioanalysis. *Anal. Chem*. 2007, 79, 2101-2108.

V Tiina J. Kauppila, Nari Talaty, Piia K. Salo, Tapio Kotiaho, Risto Kostiainen, and R. Graham Cooks; New Surfaces for Desorption Electrospray Ionization Mass Spectrometry: Porous Silicon and Ultra-Thin Layer Chromatography Plates. *Rapid Commun. Mass Spectrom.* 2006, 20, 2143-2150.

ABBREVIATIONS

ACN	acetonitrile
ADME	pharmacokinetic parameters of drugs: absorption, distribution, metabolism, and excretion
AMD	automated multiple development
APCI	atmospheric pressure chemical ionization
AP–MALDI	atmospheric pressure matrix assisted laser desorption/ionization
α-CHCA	α-cyano-4-hydroxycinnamic acid
DAD	diode array detection
DESI	desorption electrospray ionization
DMSO	dimethylsulfoxide
1D/2D	one-dimensional/two-dimensional
ESI	electrospray ionization
F	fragment
FAB	fast atom bombardment
FIA	flow injection analysis
FTIR	Fourier-transform infrared spectroscopy
H	plate height
HPLC	high-performance liquid chromatography
HPTLC	high-performance thin-layer chromatography
LC	liquid chromatography
LOD	limit of detection
LSIMS	liquid secondary ion mass spectrometry
M	analyte
MALDI	matrix-assisted laser desorption/ionization
$[M+H]^+$	protonated molecule
$[M+Na]^+$	sodium adduct ion of the analyte
MeOH	methanol
MS	mass spectrometry / mass spectrometer
MS/MS	tandem mass spectrometry
m/z	mass-to-charge ratio
NP	normal phase
S/N	signal-to-noise ratio
SPE	solid-phase extraction
TLC	thin-layer chromatography
TOF	time-of-flight
OPLC	over-pressured layer chromatography
PEC	planar electrochromatography
PLC	preparative-layer chromatography
PRISMA	optimization system in TLC method development
R_F	retardation factor
RSD	relative standard deviation
RP	reversed phase
RPC	rotation planar chromatography

SALDI	surface-assisted desertion/ionization
SPE	solid-phase extraction
TIC	total ion chromatogram
UTLC	ultra-thin-layer chromatography
UV	ultraviolet
UV/VIS	ultraviolet/visible

1. INTRODUCTION

The present challenge in drug discovery is to synthesize new compounds efficiently in minimal time and identify drug candidates that can replace existing drugs whose patents are expiring. The trend is towards carefully designed and well-characterized compound libraries because fast and effective synthesis methods, combinatorial chemistry for example, easily produce thousands of new compounds. The need for rapid and reliable methods for quality control of the synthesized products is increased at the same time. Quality assessment is highly important since false (negative or positive) results, for instance in tests of biological activity or determination of early-ADME parameters in vitro (the pharmacokinetic study of drug absorption, distribution, metabolism, and excretion), must absolutely be avoided. These preliminary tests are essential for screening out compounds with unfavorable ADME properties as early as possible because every successive stage in the process of developing new drugs is more expensive than the previous one. Thus, the development of fast and reliable analytical methods for the quality control, including the identification of synthesis products and purity tests, is both important and challenging.

Liquid chromatography (LC) combined with ultraviolet (UV) or diode-array (DAD) and mass spectrometric (MS) detection is currently the most common technique for purity assessment. Quite often, to fulfill the purity requirements, the synthesis procedure must be developed further, or the product must be purified. With state-of-the art technology, bioactivity and ADME tests can be conducted on sub-milligram amounts of a compound, and it is often easier, therefore, to purify the sample than to develop the synthesis further. Current requirements for purification – automation and efficiency – have been satisfied by semi-preparative LC, especially with UV- and/or MS-triggered fraction collection. Although LC is the main technique in synthesis quality control and purification, thin-layer chromatography (TLC) and preparative-layer chromatography (PLC) continue to be important. Both enable simultaneous analysis of many samples on one plate, solvent consumption is low, plates are disposable so that there are no memory effects, and several detection methods can be applied in sequence. TLC has the further advantage of being an easy, low-cost method that can be used in any laboratory. The disadvantages compared with LC are lower separation efficiency and higher limits of detection (LODs). The separation efficiency of TLC can be improved by applying two-dimensional (2D) elution.

Interest in TLC has increased in the past few decades along with the improvements in TLC instrumentation and methods and further in the last few years with the development of new MS methods for detection. The combination of modern high-performance thin-layer chromatography (HPTLC) with automated sample application and densitometric scanning makes this sensitive and reliable technique highly suitable for qualitative and quantitative analysis in a broad area, in pharmaceutical, environmental, toxicological, and forensic research, for example. The recently introduced miniaturized ultra-thin-layer chromatography (UTLC) combined with UV detection provides faster elution times, lower solvent consumption, and lower detection limits than conventional TLC or HPTLC methods. One weakness of UTLC compared with HPTLC is the reduced resolution due to the shorter elution distances and smaller overall specific adsorption surface area.

The simplest TLC identifications are based on either color reactions of the separated sample zones or on a comparison of the R_F values (defined as the migration distance of the substance divided by migration distance of the mobile phase) of the analyte and a standard compound visualized under a UV lamp. Quantitative TLC measurements are performed by densitometric scanning. With densitometric measurements the analytes are identified by their (corrected) R_F values and by inspection of UV/VIS spectra of the analytes and standard compounds measured in situ. If standard compounds are not available (as in the screening of new natural agents or combinatorial chemistry samples), the identification of unknowns has to be done with a more specific technique, such as MS detection. TLC–MS can be performed off-line, on-line, or in situ. In off-line TLC–MS, the sample is scraped from the plate and extracted from the adsorbent material before separate MS analysis. In on-line TLC–MS, the sample is extracted from the plate with continuous solvent flow using for example a special sampling probe. The analyte is then ionized, for example by atmospheric pressure chemical ionization (APCI) or electrospray (ESI) ionization technique, and finally detected on-line with MS. In in situ TLC–MS, the sample is detected directly on the plate using, for example, matrix-assisted laser desorption/ionization (MALDI) or desorption electrospray ionization (DESI) technique.

This thesis comprises several parts. The following review of the literature gives a brief description of the TLC technique and an introduction to TLC–MS. The actual experimental work, reported in the five appended publications, is summarized in chapter five. In study I, HPTLC method was introduced and its suitability was evaluated in an assessment of the quality of a small and focused combinatorial library obtained by solid-phase synthesis. Study II investigated the suitability of the PLC method to isolate target compounds from crude synthesized products and purify them for bioactivity and preliminary ADME tests. In both study I and study II, the HPTLC method was compared with the LC method. In study III, HPTLC and UTLC methods were compared for the separation of synthesized products and commercial drug substances. Also, two MALDI ionization techniques (in atmospheric pressure and in vacuum) were used with UTLC plates for the first time, and the results were compared with those obtained with HPTLC plates. In study IV, a 2D UTLC method for pharmaceuticals in urine matrix was developed, and the influence of the urine matrix on separation and repeatability was evaluated. Finally in study V, the performance of DESI in the analysis of the UTLC-separated compounds was evaluated. Overall, the results of these five publications confirm the potential of planar chromatography combined with UV and MS detection to perform as a fast, efficient, and alternative tool in drug analysis.

2. REVIEW OF THE LITERATURE

2.1 Thin-layer chromatography (TLC)

After a look at the principles of thin-layer chromatography, a description is given of the techniques used in this work: classical preparative-layer chromatography, high-performance thin-layer chromatography, and the newly introduced ultra-thin-layer chromatography.

2.1.1 Principles of thin-layer chromatography

Thin-layer chromatography (TLC), also known as planar chromatography (PC), is one of the oldest methods in analytical chemistry still in use. The history of TLC as we know it today goes back to the early 1950s [1]. In TLC, the different components of the sample are separated by their interaction with the stationary phase (bonded to the glass, aluminum, or plastic support) and the liquid mobile phase that moves along the stationary phase [1, 2]. TLC is a fast, simple, and low-cost method suitable for any laboratory. A particular advantage is that it allows the analysis of many samples simultaneously. In contrast to liquid chromatography (LC), TLC offers separation without or at least with minimal sample preparation. Also, the plates are disposable, and there is no memory effect, such as may occur in LC. TLC is also an off-line method: sample application, separation, and detection take place in different processes. Because of its off-line character, TLC allows the use of a number of detection methods and appropriate derivatization reagents in sequence, which improves the reliability of the detection. The improvements in TLC instrumentation and methods over the past few decades, as well as the introduction of new, in situ MS methods that can be combined with TLC, have increased interest in the technique.

With over 20 different parameters listed as affecting the separation in TLC [3], method development is essential, and includes several steps. Perhaps the most important of these are the selection of the plate material (i.e. the stationary phase and its special characteristics), the optimization of the mobile phase composition, and the choice of elution technique (vertical, horizontal, capillary flow, forced-flow) [1, 2, 4]. The most common stationary phase in TLC continues to be unmodified (normal-phase; NP) silica, although several other phases are utilized as well [5], and are also commercially available. These other phases include for instance alumina and diol phases, C_8 and C_{18} reversed phases (RP), amino-, cyano-, and phenyl-modified phases, and chiral phase. The selection of mobile phases for RP separation is mainly limited to methanol, isopropanol, and acetonitrile, but almost all organic solvents can be used with unmodified silica. The optimization of the mobile phase is highly important for a successful separation. This can be done by methods such as trial and error, PRISMA, and mathematical, computer-aided models [6–10]. PRISMA [10], the most widely applied model, is based on the solvent classification system of Snyder [11], and the choice of the solvents consists of three steps. First, individual solvents in different classes are tested and normally the three best in order of separation efficiency are selected for further study. In the second step, the optimization is continued by combining the previously selected solvents in various ratios. And in the third step, the final fine tuning of the solvent composition for achieving adequate resolution is carried out.

Thin-layer chromatography can be divided into the capillary-flow (i.e. classical) and forced-flow techniques according to the flow type of the mobile phase [1, 2, 12, 13]. In classical TLC, the mobile phase flow is achieved with the weak capillary forces arising from the decrease in free energy of the solvent as it enters the porous structure of the layer [2, 12–14]. Thus, the separation efficiency of capillary-flow TLC is limited to the velocity at which the capillary forces drive the mobile phase through the stationary phase [2, 12–14]. The velocity of the mobile phase varies with time and elution distance, i.e. the velocity declines as the mobile phase migrates further along the plate. Moreover, as the capillary forces are unable to generate optimal and constant mobile phase velocity along the plate, zone broadening due to diffusion takes place, increasing with the migration distance [2, 12–14]. Thus, when capillary-flow technique is used, the variable and non-optimal mobile phase velocity results in limited separation efficiency.

In forced-flow technique, the velocity of the mobile phase is adjusted (i.e. increased) to give a constant and optimal velocity. This is done by applying external force, such as pressure, centrifugal force, or an electric field [12, 15, 16]. Examples of these methods are over-pressured layer chromatography (OPLC) [15, 17–20], rotation planar chromatography (RPC) [15, 21–24], and planar electrochromatography (PEC) [15, 25–28]. The sample capacity and resolution that can be achieved with OPLC and RPC are significantly better than those obtained with capillary-flow technique [2] although the separation efficiency of capillary-flow TLC can be increased, too, by using the two-dimensional (2D) elution technique [12, 13, 29]. In the following, the focus is on the capillary-flow techniques that were used in this work.

2.1.2 Preparative-layer chromatography (PLC)

Preparative-layer chromatography (PLC) can be used for the fractionation and/or isolation of compounds in amount up to 1000 mg. The amounts depend on the sample, layer thickness, and the elution mode to be used [30, 31]. According to the elution mode, PLC can be classified into classical PLC (i.e. conventional capillary-flow) and forced-flow PLC (e.g. OPLC and RPC) [32]. Classical PLC is a simple technique but the analysis time, which is about 2–4 hours, is rather long, and partly due to the time needed to achieve saturated conditions in the chamber before the separation.

The parameters most affecting the separation and resolution with classical PLC are the thickness, particle size, and particle size distribution of the stationary phase. [30]. The thickness of the adsorbent layer in the PLC plate is normally 0.5–2 mm, the particle size is about 25 μm, and the particle size distribution usually ranges between 5 and 40 μm. The large mean particle size and wide particle size distribution of the adsorbent layer cause a limited resolution with classical PLC, meaning that the optimization of the mobile phase is a highly important part of the method development. The isolation of a completely pure component from a complex sample with classical PLC is difficult due to the weak resolution. Classical PLC has typically been applied for purification and isolation of individual compounds from natural extracts or from other biomaterials [31, 32]. PLC is also a well-known and common tool, used, for example, in the isolation of impurities from the drugs [33] for identification studies or in the purification of target compounds from a crude synthesized

products [34] for further studies (e.g. screening of biological activity or the determination of early ADME parameters in vitro). Classical PLC can generally be applied for separation if two to five compounds or about 10–150 mg of pure sample needs to be isolated [30]. Larger amounts, even up to 1000 mg, can be fractionated by the forced-flow techniques such as OPLC and RPC [30, 31].

2.1.3 TLC and high-performance thin-layer chromatography (HPTLC)

TLC and high-performance thin-layer chromatography (HPTLC) plates are the most widely applied plates in analytical planar chromatography. The main differences between analytical TLC/HPTLC plates and preparative PLC plates are the layer thickness, mean particle size, and particle size distribution [1, 30]. In TLC and HPTLC, layer thickness is typically 0.2 or 0.25 mm. Mean particle size is about 12 μm in TLC and 5 μm in HPTLC, and the particle size distribution is up to 20 μm for TLC and about 10 μm for HPTLC. The smaller particle size and narrower distribution in HPTLC allows higher packing density of the particles and thus a more homogenous stationary phase. As a consequence, HPTLC offers better resolution and lower LODs than conventional TLC. The migration distance in HPTLC is up to 5 cm, and is about one half that in TLC (9 cm). When the normal vertical elution technique is used, both types of plates allow the analysis of about 20 samples on one 10 x 20 cm plate. However, the number of samples can be doubled to about 40 in HPTLC by carrying out the elution from the two sides simultaneously, i.e. by horizontal elution mode instead of vertical mode. The combination of modern HPTLC with automated sample application and densitometric scanning makes this sensitive and reliable technique highly suitable for qualitative and quantitative analysis in pharmaceutical, environmental, toxicological, and forensic applications [2, 12, 35–42].

2.1.4 Ultra-thin-layer chromatography (UTLC)

Miniaturized analytical methods [43] are under intense development in analytical chemistry – also in planar chromatography – and a new planar chromatographic method, ultra-thin-layer chromatography (UTLC), was recently introduced by Hauck et al. [44] The layer thickness in UTLC is only about 10 μm, and the adsorbent consists of monolithic material (not particles as in PLC, TLC, and HPTLC plates). Thus the surface of the UTLC plate is more even and thinner providing lower LODs than with the other plates [45]. Relative to TLC or HPTLC methods, UTLC also provides faster elution times (1–6 min) and lower solvent consumption (1–4 ml) [45]. One weakness of UTLC compared with HPTLC is the reduced resolution caused by the shorter elution distances and smaller overall specific adsorption surface area [45].

As UTLC is a new technique, only a few studies with UTLC plates have been published before ours. In these other studies, UTLC plates were employed with UV or DAD detection, and the method was applied for only a few, mainly preliminary, separations of steroids, pesticides, and dyeing compounds, amino-acids, phenols, surfactants, and some pharmaceuticals [44–46]. MS was first combined with UTLC in our laboratory [47]. Just recently, time-of-flight–secondary-ion-mass spectrometry (TOF–SIMS) has been used with the UTLC plates by Oriňák et al [48]. Also, tentative results with RP-UTLC and vacuum MALDI–TOF–MS [49] have been introduced.

2.1.5 Two-dimensional (2D) TLC systems

A primary disadvantage of TLC over LC is lower separation efficiency. However, the separation efficiency of TLC can be improved by using two-dimensional (2D) elution. In a 2D TLC system, the first-dimension elution (1D) is performed normally and, after drying, the plate is turned 90 degrees and eluted again in the second dimension [1, 2, 12, 13, 29]. Separation efficiency can be increased by applying different solvent compositions in the two dimensions. Likewise, the selectivity can be improved by using a plate covered with two different phases. The dual-plate of Whatman [50] combines two phases (3 cm C-18 strip on silica gel layer) and can be used for the separation of mixed polarity samples. Alternatively, a plate with diol- or cyano-modified stationary phase, for example, enables normal and reversed phase separation on the same plate [29, 51] merely by changing the solvent composition between the dimensions; i.e. RP separation takes place with an aqueous solvent and NP separation with an organic solvent. 2D TLC technique has been successfully applied to the analysis of drugs, biological samples, and plant extracts [29, 35, 40, 51–55]. It is worth adding that the nature of the planar chromatography technique means that 2D TLC is simpler to carry out than 2D LC.

2.2 "Cleave and analyze" detection

Separation and detection in TLC are normally performed in separate, off-line processes. The possibility to use several detection methods in sequence provides flexibility and improves the reliability of detection. Detection in TLC generally is achieved using on-line, "cleave and analyze" (i.e. off-line), or in situ detection directly on the plate [1, 56]. TLC on-line detection is a new and emerging technique. In situ UV detection is the presently dominant technique, though it provides only limited structural information. These two techniques (on-line and in situ) are discussed in section 2.3.

"Cleave and analyze" represents the off-line method, in which the separated sample zone is scraped from the plate, extracted from the adsorbent material, and then analyzed by some appropriate technique. The analyte zone can be isolated from the plate not only by one of the methods normally used, i.e. extraction after scraping with a razor blade, scissors, or spatula, but also with a suction apparatus [57, 58] or a microcapillary extraction device [59]. "Cleave and analyze" can be used in both qualitative and quantitative evaluation [60]. Formerly, in quantitative TLC, the zone was wholly scraped and then analyzed, for example by titrimetry, gravimetry, or HPLC. At present, the modern and fast spectroscopic techniques available for in situ TLC measurements make this indirect quantitation unnecessary. The "cleave and analyze" method is still very much in use in qualitative analysis, for the identification and characterization of unknown compounds after scraping and extraction. The technique applied for further study could be infrared (IR) or nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS) [56, 59, 61–64].

2.3 In situ and on-line detection

In situ detection can be done visually or by a variety of spectroscopic methods and mass spectrometric techniques. The MS techniques also include some on-line methods.

2.3.1 Visual detection

TLC detection is simplest with compounds that absorb light in visible (VIS) wavelength range; the analytes are colored and visible as such. The detection of TLC-separated compounds under a UV lamp relies on the addition of a fluorescent indicator to the adsorbent [1]. For example, F_{254} fluorescent indicator is excited with UV wavelength at 254 nm and emits green fluorescence [1]. Compounds that absorb radiation at 254 nm reduce this emission on the layer, and a dark violet spot on a green background is observed where the compound zones are located. Derivatization is another way to make compounds visible [4]. In classical post-chromatographic derivatization, which normally is achieved through spraying or dipping, an analyte on the plate is dyed with an appropriate universal or group specific reagent [4, 65], after which the compounds are visualized as derivatives under a visible light or UV lamp. After visualization, the compounds can be identified by comparing the R_F values of the analytes with those of standards. Thus, visual detection alone cannot be used for quantitative analysis.

2.3.2 Spectroscopic methods

Visual detection is suitable for qualitative analysis, but a more specific detection method is needed for quantitative analysis and for obtaining structural information on separated compounds. UV, diode-array (DAD) and fluorescence spectroscopies, mass spectrometry (MS), and Fourier-transform infrared (FTIR) and Raman spectroscopies have all been applied for the in situ detection of analyte zones on a TLC plate [12, 56, 66–68]. The most common of these is UV densitometric measurements, though in recent years MS has also often been used.

Slit-scanning densitometry is the most usual method in TLC to perform the UV detection. In slit-scanning, the analyte zone or spot on the plate is scanned with a light beam, the length and width of which can be varied by changing the slit size of the monochromator [1, 4, 12, 67]. Densitometric measurement is usually performed in absorbance and reflectance mode using one or several wavelengths, although measurement in a fluorescence mode is also available. In UV reflectance mode, the photosensor of the densitometer measures diffusely reflected light (the difference in the optical signals of the blank plate and the analyte zone), which is converted to an electrical signal. After densitometric scanning, the analytes can be identified by comparing the recorded R_F values and in situ UV/VIS spectra of the analytes and standard compounds. Ojanperä and co-workers [69, 70] have established special libraries and software based on corrected R_F values, in situ UV spectral correlation, and spectrum maximum site comparison, to assist the identification of drugs in biological samples.

The slit size [67, 71, 72], the roughness of the plate surface and the thickness of the adsorbent layer [71, 73], and also the shape and size of the analyte zone [67, 73, 74] affect the signal intensity and the repeatability in slit-scanning UV densitometry. Thus it is advisable to optimize the slit size for each analysis separately, to apply the sample as a band rather than a spot, and to use an HPTLC plate with its smoother surface. Because of light scattering at the particles of the layer, the calibration curve is linear only at low concentrations and in narrow calibration range [1, 4, 71]. At higher concentration and wider calibration range, the calibration curve is non-linear and often follows a polynomial regression or the Michaelis-

Menten equation [71, 75]. In the detection of the zones by fluorescence scanning, a cutoff filter interposed between sample and photosensor of the densitometer eliminates diffusely reflected light [1, 4, 67], and therefore, the intensity of the measured light is linear with the analyte concentration. In fluorescence mode, the signal is also nearly independent of the size and shape of the analyte zone. Also, the sensitivity is better in fluorescence than in UV absorbance mode: The LODs are typically in nanogram range with UV and in picogram range with fluorescence [4].

Additionally to slit-scanning UV/VIS densitometry, image analysis, known also as video densitometry, is increasingly being employed in TLC detection [2, 12, 13, 76, 77]. The equipment includes an imaging detector (i.e charged-coupled device, CCD, video camera), a computer with a video digitizer, a light source, and an optical system. With this imaging method, data can be acquired simultaneously for all analytes in the plate, which is an advantage especially in two-dimensional separation. However, the sensitivity and the number of wavelengths available for measuring are limited in imaging.

The identification of TLC-separated compounds in UV and fluorescence is mostly based on reference standards. If standard compounds are not available, as in the screening of new natural agents or combinatorial chemistry samples, identification has to be done with a more specific technique, such as FTIR or MS detection. FTIR is an efficient technique for the characterization of separated zones directly from the plate [78, 79]. FTIR has also been used to characterize the modified surfaces of TLC plates with the purpose of evaluating the possibility of using commercial UTLC plates in reversed-phase separation mode [49]. We modified a commercially available normal-phase (NP) UTLC plate with octadecyl-trichlorosilane (ODS) and the success of the modification was verified by in situ FTIR spectroscopy (Figure 1). Although FTIR gives specific structural information about analytes directly on the plate, similarly to MS, the sensitivity of MS is much better, and the combination of TLC and MS is at present more popular.

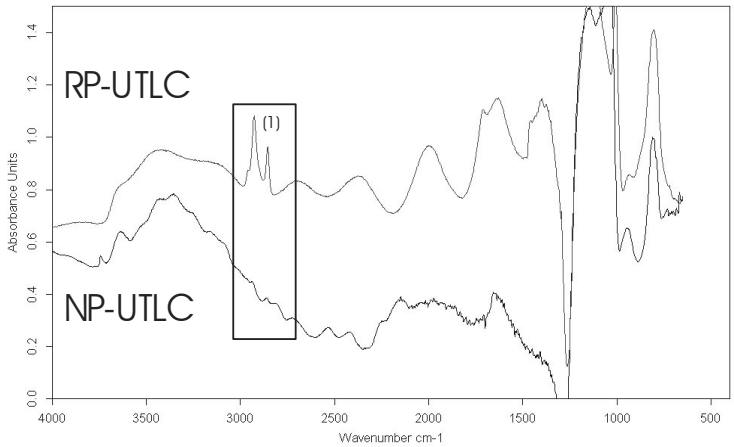


Figure 1. The reflection FTIR spectrum measured from the surface of RP–UTLC and NP–UTLC plates; The C-H stretching vibrations (1) observed with RP–UTLC are not observed with NP–UTLC.

2.3.3 Mass spectrometry (MS)

TLC–MS has been actively studied during the last few years. Nevertheless, MS is not as generally and widely used a detector for TLC as for liquid chromatography. Probably this is because of the off-line character of TLC; transfer of the separated sample from plate to MS is not so straightforward as transfer of the LC effluent and difficulties have been encountered. However, TLC has been successfully combined with various in situ MS techniques [66], including secondary ion mass spectrometry (SIMS), fast atom bombardment (FAB), and matrix-assisted laser desorption/ionization (MALDI). TLC–FAB [80–82] is a rather old-fashioned technique that is decreasing in popularity. TLC–SIMS, though also an old technique, is re-emerging [83–85]. TLC–MALDI, in turn, as a simple, fast, and soft technique, is becoming increasingly popular for direct TLC–MS analysis [86–99, II–IV]. In addition, several interesting couplings of TLC with ESI [100–107] and with APCI ionization [108–109] techniques have been introduced. In these techniques, the analyte is transferred on-line from the plate to the ionization source using special surface sampling probes.

TLC–MALDI–MS

MALDI–MS was introduced in the late 1980s [110–112]. The method has been widely used in analysis of low and high molecular weight compounds and has been interfaced with column and planar separations [113, 114]. The coupling of MALDI and TLC was mainly established in the mid-1990s by Hercules and co-workers [86–90]. Crecelius et al. too, have worked on the combination of TLC and MALDI [91–96]. Their studies include qualitative and quantitative measurements using MALDI–time-of-flight (TOF)–MS. In addition, Costello and co-workers have achieved important results with TLC–MALDI–TOF–MS [97, 99] and also with TLC– MALDI–FTMS [98].

In MALDI–MS, the analyte is ionized and analyzed with the help of a matrix compound [115–119]. After matrix addition, the TLC plate is attached to the MALDI target plate, and the laser beam is focused to the desired spot on the plate (Figure 2A). The UV laser (e.g. a nitrogen laser) is most commonly used in MALDI applications, but IR laser has been used as well [120]. The energy of the laser causes the analyte to be vaporized and ionized, and the ions formed are transferred to the mass analyzer. The ion formation mechanism in UV–MALDI is not known exactly but it is proposed to take place in two main steps [115–117]. Schematic drawing of MALDI desorption process is presented in Figure 2B. In a primary ionization reaction, the matrix compound absorbs the UV radiation of the laser, and the matrix is ionized. In a secondary ionization reaction, the charges of the matrix are transferred to analyte molecules either by charge exchange or by proton transfer reaction. This means that choosing the right matrix is essential for successful MALDI–MS analysis. Also, using the right analyte to matrix ratio and adding the matrix to the plate in the right manner are highly important as they affect the quality of the MALDI–MS spectra. It has been observed, for example, that the analyte to matrix ratio should be lower for low mass molecules than for high mass molecules [64, 91, 121, 122]. Different ways of adding the matrix, for example as dried droplets, spin-coating, thin films in a layered manner, airspray, and electrospray have also been investigated [87, 91, 119, 123, 124]. Airspraying and electrospraying of the matrix

on top of the separated analyte zones are the most promising techniques for TLC [91, 93, 94, 96, II–IV]. Solid, liquid ion, and suspension matrixes have been used and compared [86, 91, 93, 97, 99, 113, 118, 119, 125–128]. Also, different variations of the MALDI technique have been introduced. In SALDI (surface-assisted laser desorption ionization), the ionization takes place, for example, with the assistance of graphite or carbon powder and glycerol [129–132]. In PALDI (polymer-assisted laser desorption ionization), non-polar, small polymers may be used [133]. Many of the matrixes for TLC–MALDI–MS cause interfering mass peaks at low m/z range. These can be minimized through use of a suitable analyte-to-matrix molar ratio [64, 91, 134–136]. New potential matrixes, such as ion liquids and SALDI matrix, which produce a low background, are also being investigated [113, 127, 128, 132].

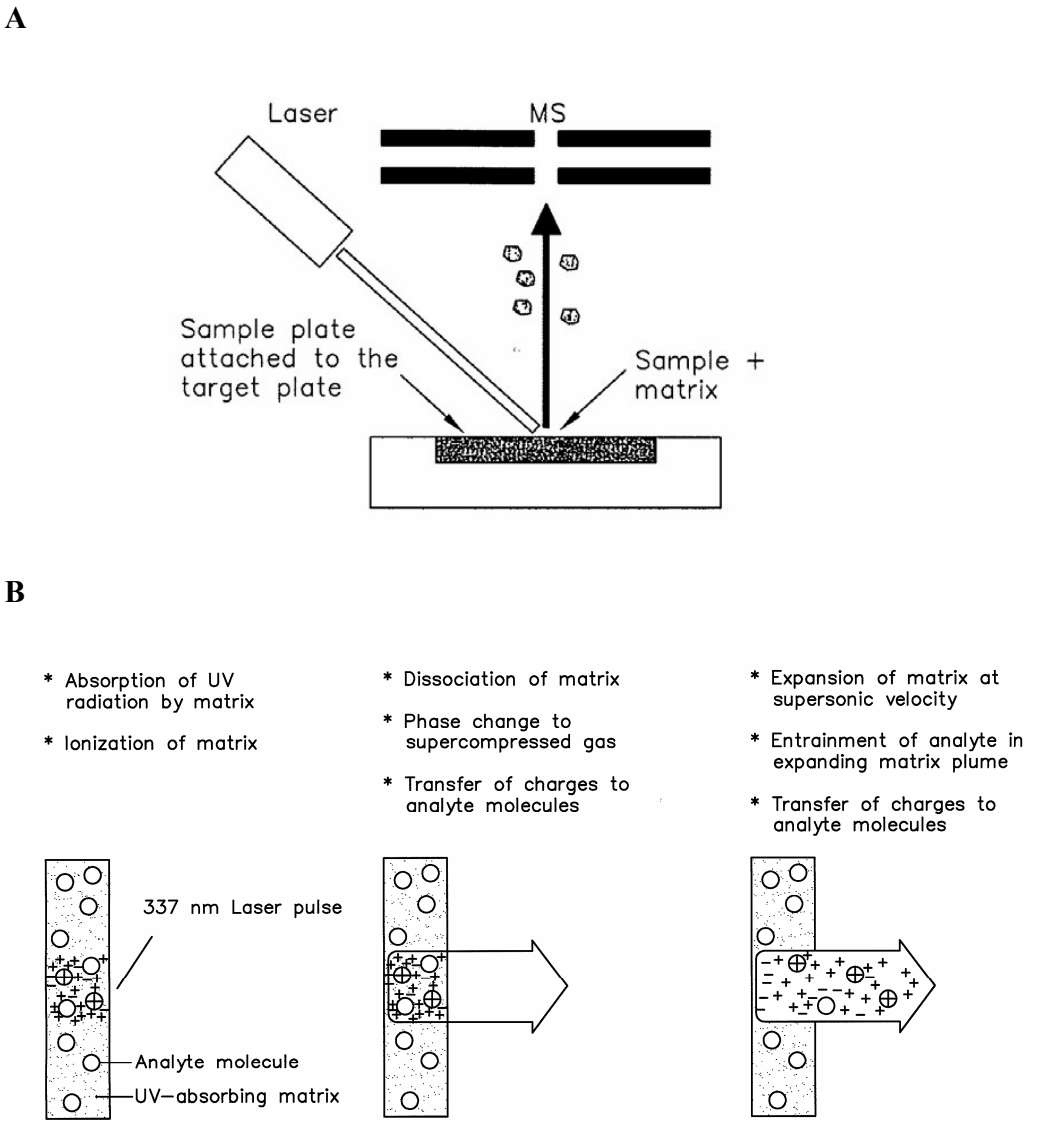


Figure 2. Schematic drawing of A) TLC–MALDI–MS and B) MALDI desorption process [118].

A disadvantage of the MALDI method has been the relatively poor repeatability in quantitative analysis. However, research on quantitative MALDI is expanding [96, 137, 138] and Crecelius and co-workers, among others, have demonstrated good precision with an internal standard method [96] in which the internal standard is pre-developed over the plate. Furthermore, working with TLC and vacuum MALDI sources makes the method somewhat risky since large amounts of chromatographic material are directly introduced to the vacuum chamber of the mass spectrometer. This problem can be avoided by working with the recently introduced atmospheric pressure MALDI (AP–MALDI) [139–145] source, which can be combined with any kind of mass analyzer. Changing the sample plates is faster with AP–MALDI instruments than with vacuum MALDI instruments since pump down is not required. Additionally, the ionization process is softer in AP–MALDI than in vacuum MALDI and AP–MALDI produces more abundant protonated molecule and less fragmentation than vacuum MALDI [139, 141, 145]. Though desorption from the irregular surface of TLC plates with a vacuum MALDI time-of-flight (TOF) instrument (Figure 3A) has a detrimental effect on the repeatability of the measured m/z values [97, 146], the problem can be overcome, for example, by using AP–MALDI–ion trap–MS (Figure 3B) (study III) or MALDI–FTMS [98] because in these techniques the ions are formed externally, collected inside the analyzer, and subsequently mass analyzed.

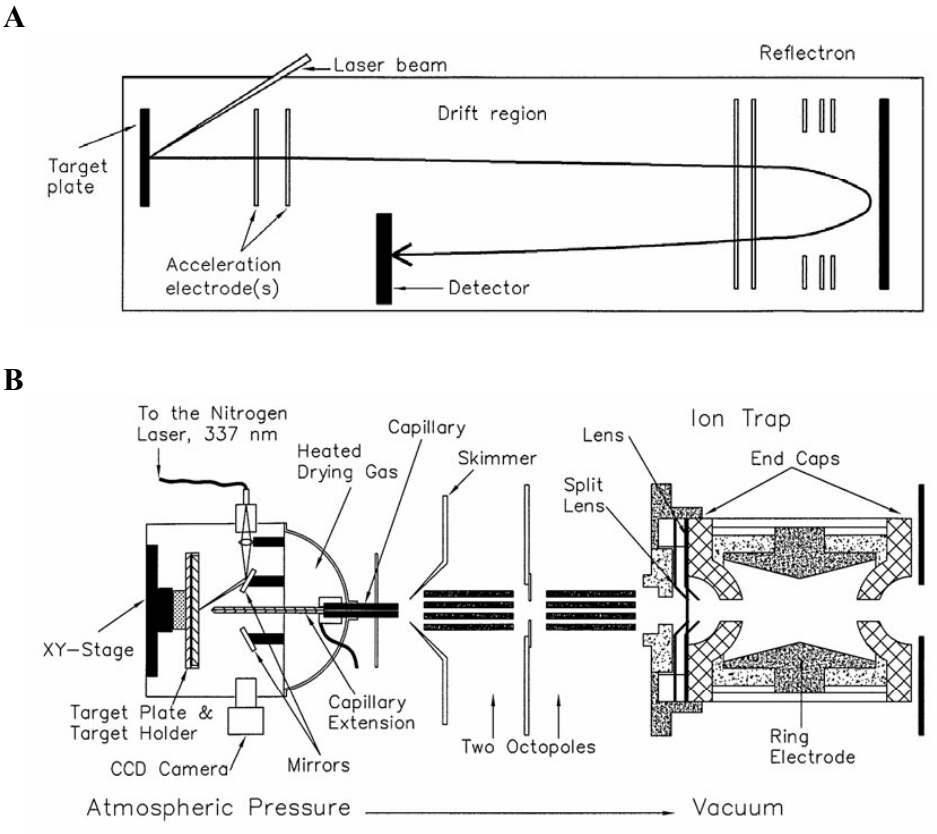


Figure 3. Schematic drawing of A) vacuum MALDI-TOF-MS and B) AP-MALDI-Ion Trap-MS.

TLC–APCI–MS and TLC–ESI–MS

Van Berkel and co-workers have recently described couplings of TLC to atmospheric pressure chemical ionization (APCI) [108] and electrospray ionization (ESI) [100–104]. In both couplings, a special surface sampling probe (Figure 4) is used for extracting the analyte on-line from the TLC plate to MS analysis. The sampling probe is pressured against the TLC-separated analyte zone, and the eluting solvent, delivered with a syringe driver, is pumped through the analyte zone on the plate. During the pumping the analyte is extracted into the eluent, which is then transferred to the APCI or ESI source, and finally the ions are directed to the mass analyzer.

These techniques [100–104, 108] are designed to reduce the post-treatment of the eluted plates before MS analysis, and the chemical noise in the low m/z range usually obtained with MALDI technique. Furthermore, in both techniques continuous mass spectrometric data acquisition can be achieved by scanning the probe along the separated track or line of the TLC plate. This feature makes these couplings highly interesting for the analysis of unknowns since MS and MS/MS spectra can then be measured from the separated compounds. Analysis with a normal-phase plate may be problematic, however, as the eluting solvent may spread along the plate and not be able to flow back to the probe (i.e. phase wettability). Further, since the performance of these couplings is dependent on the successful sampling of the analyte(s) from the plate, a compromise in the solvent system is needed for efficient extraction and the phase wettability.

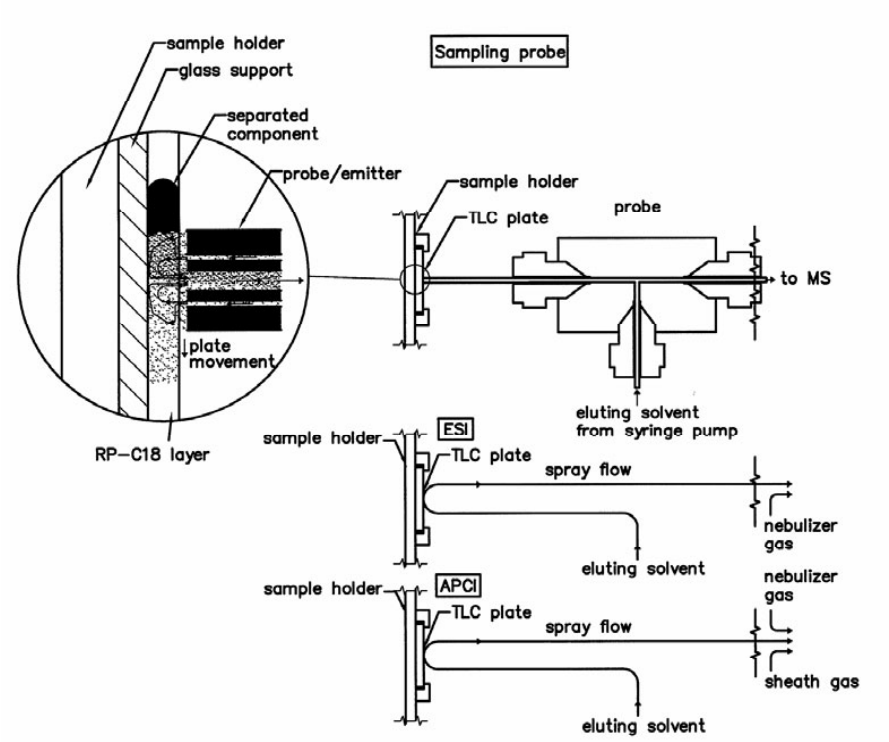


Figure 4. Schematic drawing of the TLC/ESI/APCI–MS surface sampling probe [100, 108].

TLC–DESI–MS

Desorption electrospray ionization (DESI) is a relatively new atmospheric pressure ionization mass spectrometry technique, introduced by Cooks and co-workers [147] for the analysis of analytes on surfaces. The mechanisms of DESI are an active area of investigation [147–150]. In brief, the charged droplets formed in an electrospray are directed toward a surface where their impact causes the release of analytes from the surface and their ionization (Figure 5). The desorbed ions are transferred to the MS and mass-analyzed. DESI analyses are routinely performed directly from the surface with no sample pretreatment, the typical analysis time for one sample being a few seconds. These features make DESI of interest as a method for in situ analyses. For example, rapid analysis of natural substances and pharmaceuticals without sample pretreatment has been demonstrated from such biological matrixes as plants, animal tissue, urine, and blood [147, 150–154].

Like DESI alone, the coupling of TLC and DESI–MS is a novel technique; only three papers have been published so far [155, 156, V]. Two of these are from the group of Van Berkel and one is ours (V). In the work of the Van Berkel group, the fundamentals of TLC–DESI–MS, such as basic experimental setup and the optimization of measuring conditions, were demonstrated with various dyes and pharmaceuticals [155]. They also reported that DESI–MS overcomes the wettability problem encountered when their surface sampling probe is interfaced to ESI–MS [155]. At the same time they noted that aqueous solvents and mechanical forces of the pneumatic DESI gas jet damage the surface of the TLC plate and hinder the generation of the analyte ions. More frequent cleaning of the instrument is needed therefore, to remove sputtered stationary phase particles. In their second paper [156], the group introduced the first automated DESI–MS technique including spot sampling, scanning of multiple eluted lanes on the plate, and imaging of bands in the lane. We, in turn, were the first to apply monolithic UTLC plates and DESI–MS to the analysis of separated compounds (V).

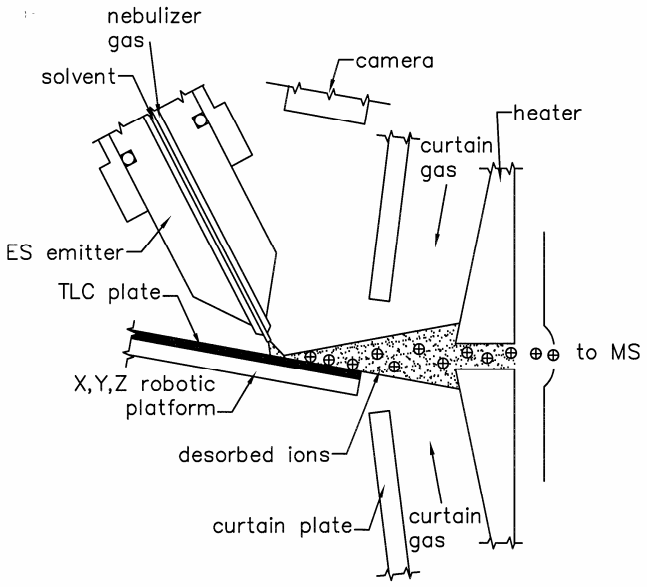


Figure 5. Schematic drawing of the TLC/DESI–MS experimental set-up [155].

3. AIMS OF THE STUDY

The aims of the study were to evaluate the feasibility of different thin-layer chromatography techniques with visual and UV densitometric detection, and electrospray, desorption electrospray, and matrix-assisted laser desorption ionization mass spectrometric detection in qualitative and semi-quantitative analyses of drugs and "drug-like" molecules.

The more specific aims of the research (I–V) were

- to evaluate the suitability of modern HPTLC with UV densitometric detection for assessing the quality of a small and focused combinatorial library and to compare the method with LC–UV–MS (I, II).

- to investigate the suitability and usefulness of PLC in purification of synthesis products for bioactivity and early ADME tests (II).

- to establish a novel UTLC with AP–MALDI–MS technique, and compare it with UTLC and HPTLC techniques relying on UV, vacuum MALDI–MS, and AP–MALDI–MS detection (III).

- to analyze biological samples by 2D UTLC with AP–MALDI–MS detection, and to evaluate the influence of urine matrix on the separation and repeatability, and to demonstrate the feasibility of 2D UTLC and AP–MALDI–MS for the analysis of an authentic urine sample (IV).

- to test the feasibility of DESI–MS for the analysis of drugs directly from the UTLC plate (V).

4. MATERIALS AND METHODS

The materials and methods used in the study are briefly presented in this section. The chemicals and instruments are listed in tables, while the methods of each publication are shortly described. Detailed descriptions can be found in the original publications I–V.

4.1. Chemicals, TLC plates, and HPLC columns

The chemicals used in the study are listed in Table 1. Synthesized products (named also as synthesis products) were produced in our synthesis laboratory, and were purified target compounds or unpurified crude product. All the other chemicals were of analytical or chromatographic grade. Structures of the studied compounds are shown in Figure 6. The TLC plates and HPLC columns are listed in Table 2.

4.2. Methods and instrumentation

The methods and instrumentation are presented in Tables 3 and 4 and Figure 7.

4.2.1 HPTLC for assessment of the quality of combinatorial libraries, and comparison with LC–UV–MS (I)

Nineteen compounds from a combinatorial library produced by solid-phase method and aimed at protein kinase C inhibitors were studied to evaluate the suitability of HPTLC in quality assessment. The HPTLC method was also compared with the LC–UV–MS method currently in use. For HPTLC studies, sample solutions (20 mM in MeOH) were sprayed on the plate in amounts of 3–10 µl. After elution with 2-propanol/hexane (1:8) the plates were observed visually under UV lamp and then measured by UV densitometry. In situ UV spectra of the compounds were measured at wavelength range of 190–450 nm. For the final confirmation by FIA–ESI–MS, the separated compounds were isolated from the plate with the device developed in this work (Figure 7A). Sample solutions were introduced to the ESI–MS by direct injection (10 µl). The eluent of water/methanol 1:1 (pH 4.5) was delivered with a microsyringe pump. Once the peak of the target compound was verified by in situ densitometric measurements and FIA–ESI–MS, the final purity of the target compound in the crude synthesized product, as a percentage, was calculated from the ratio of the peak area of the target compound to the total area of all peaks observed in densitogram measured by HPTLC–UV. For LC–UV–MS studies, sample solutions were prepared by diluting the standard solution (20 mM in MeOH) to concentration 1:10. The diluted sample (2 µl) was injected to the column. The flow rate of the mobile phase was 0.1 ml/min, and the eluent flow was directed to the electrospray ion source of the mass spectrometer without splitting. The purities were determined from total ion chromatograms and UV chromatograms.

4.2.2 HPTLC method with UV and MS detection, and PLC for analysis and purification of synthesized products (II)

Five crude isoflavone products synthesized in our laboratory and 2-phenyl-cromone reference standard were used to study the suitability of the HPTLC method for the analysis of purity and the suitability of the PLC for the purification of synthesis products for in vitro bioactivity and ADME tests. For the analytical HPTLC experiments, sample solutions (1 mg/ml in chloroform) were sprayed (10 µl) onto the adsorbent and eluted twice with chloroform or

dichloromethane. Elution time was 10 min per run. After elution the HPTLC plates were investigated visually under a UV lamp and then with a UV densitometer. After the separation, the zone of the target compounds was confirmed by AP–MALDI–MS. α-Cyano-4-hydroxycinnamic acid (α-CHCA) was used as a matrix compound for the AP–MALDI–MS studies. The matrix solution was sprayed over the eluted sample zone with Linomat IV, and the total amount of α-CHCA on the HPTLC plate was 100 nmol. After matrix addition, the HPTLC plate was cut to match the size of an in-house-modified AP–MALDI target plate and attached to it with double-sided conductive tape. The target compound was identified with MS and the purity, as a percentage, was calculated on the basis of the UV measurements (i.e. from the ratio of the peak area of the target compound to the total area of all peaks). For preparative layer chromatography, sample solutions were applied manually to the PLC adsorbent. Total amounts of the crude products applied to the plate varied between 1 and 5 mg. The mobile phase was chloroform or dichloromethane. The plates were eluted twice, with an elution time of 60 min per run. The separated zones were detected visually under a UV lamp, and the zone of the target compound was isolated with the device described in Figure 7B. Final purity of the target compounds isolated from the PLC plate was confirmed by HPLC–UV. The HPLC–UV method was also used to evaluate the suitability of the analytical HPTLC method. For HPLC–UV studies the samples were in DMSO, and water and methanol were the solvents in gradient program. The purity, as a percentage, was calculated as the ratio of the peak area of the target compound to the total area of all peaks.

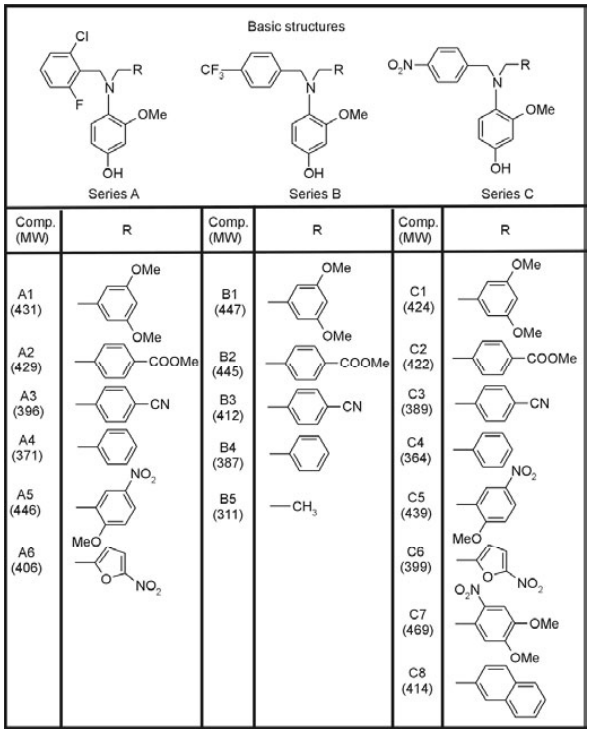
4.2.3 Analysis of small molecules by UTLC–AP–MALDI–MS (III)

Three commercial pharmaceutically interesting drug compounds (reference standards) and six heterocyclic 1,2,3-triazoles produced by solid-phase combinatorial chemistry were used in the comparison of UTLC and HPTLC methods with UV and AP–MALDI–MS detection, and also in comparison of UTLC with AP– and vacuum MALDI–MS. The stock solutions of the analytes (1 mg/ml) were prepared in dichloromethane/methanol (50:50 v/v), methanol, or acetonitrile and diluted with the same solvent to the final working solution. For UTLC and HPTLC separations, 1 µl of the working solution was applied to the adsorbent and, depending on the experiment, the total amount of the analytes on the plate was between 1 pmol and 10 nmol. Ethyl acetate/*n*-hexane (1:2 v/v) containing 2% acetic acid was the final mobile phase for the triazoles, and ethyl acetate containing 0.5% ammonium hydroxide the final mobile phase for the drugs. UTLC plates were eluted in a saturated chamber to the distance of 2 cm and HPTLC plates to the distance of 5 cm. The elution time was 2–4 min for UTLC and 5–8 min for HPTLC. After elution, HPTLC plates were investigated visually under UV lamp and with a UV densitometer. UTLC plates were measured with a UV densitometer. For MS studies, the plates were attached to an in-house-modified MALDI target plate after addition of α-CHCA matrix solution to the plate, 10 nmol for UTLC and 100 nmol for HPTLC. The AP–MALDI mass spectrometry system consisted of an AP–MALDI ion source combined with an ion trap instrument, and the vacuum MALDI measurements were performed using a MALDI–time-of-flight (TOF) instrument. The ion trap instrument was calibrated with an external calibration method and calibration mixtures provided by the instrument manufacturer. The calibration of MALDI-time-of-flight (TOF) instrument was done by using $[M + H]^+$ (m/z 190) and $[M + Na]^+$ (m/z 212) ions of the matrix as internal calibration points.

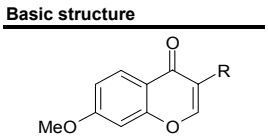
Table 1. Chemicals.

Chemical	Producer/ Supplier	Note	Publication
Acetic acid	Rathburn, Walkerburn, Scotland	For pH adjustment,	I, III, IV
	Bang&Bonsomer, Helsinki, Finland	In Dragendorff reagent	IV
Acetone	Merck, Darmstadt, Germany	Solvent	IV
Acetonitrile (ACN)	Rathburn, Walkerburn, Scotland	Solvent	II-IV
	Mallinckrodt, Phillipsburg, NJ, USA	Solvent	V
Acetylcholine	Sigma-Aldrich, Steinheim, Germany	Standard	V
α-Cyano-4-hydroxy-cinnamic acid (α-CHCA)	Fluka Chemie, Buchs, Switzerland	MALDI matrix	II-IV
Ammonia solution, 25%	Riedel-de Haën, Seelze, Germany	Solvent	III
	J.T. Baker, Deventer, Holland	Solvent	IV
	Fisher Scientific, Fair Lawn, NJ, USA	Solvent	V
Ammonium acetate	Merck, Darmstadt, Germany	For buffer solution	I
	Riedel-de Haën, Seelze, Germany	For buffer solution	IV
Ammonium carbonate	University Pharmacy, Helsinki, Finland	For buffer solution	IV
Authentic urine sample	From a healthy volunteer		IV
Bismuth subnitrate	Merck, Darmstadt, Germany	In Dragendorff reagent	IV
Blank urine	From a healthy volunteer		IV
Chloroform	J.T. Baker, Deventer, Holland	Solvent	II
Diazepam	Sigma-Aldrich, St. Louis, MO, USA	Standard	IV, V
Dichloromethane (DKM)	Rathburn, Walkerburn, Scotland	Solvent	II, III
	Mallinckrodt, Deventer, Holland	Solvent	IV
Dimethylsulphoxide (DMSO)	Riedel-de Haën, Seelze, Germany	Solvent	II
Dobutamine	Eli Lilly Laboratories, Indianapolis, IN, USA	Standard	V
Ethanol	Altia, Rajamäki, Finland	Solvent	IV
Ethyl acetate	Merck, Darmstadt, Germany	Solvent	III
	Mallinckrodt, Phillipsburg, NJ, USA	Solvent	V
Formic acid	Fisher Scientific, Fair Lawn, NJ, USA	Solvent	V
Helix pomatia (112 400 IU β-Glucuronidase / ml)	Sigma-Aldrich, St. Louis, MO, USA	Enzyme hydrolysis	IV
Isoflavones	Synthesis laboratory, Faculty of Pharmacy, Helsinki, Finland	Synthesis crude products	II
Lorazepam	Sigma-Aldrich, St. Louis, MO, USA	Standard	IV
Methanol (MeOH)	J.T. Baker, Deventer, Holland	Solvent	I-IV
	Mallinckrodt, Phillipsburg, NJ, USA	Solvent	V
3-Methoxy-4-*N,N*-substituted phenols	Synthesis laboratory, Faculty of Pharmacy, Helsinki, Finland	Synthesis crude products	I
Metoprolol tartrate	ICN Biomedicals, Aurora, OH, USA	Standard	III
Midazolam	Roche, Basel, Switzerland	Standard	III-V
N-Desalkylflurazepam	Sigma-Aldrich, St. Louis, MO, USA	Standard	IV
n -Hexane	J.T. Baker, Deventer, Holland	Solvent	I, III
Nitrazepam	Sigma-Aldrich, St. Louis, MO, USA	Standard	IV
Oxazepam	Sigma-Aldrich, St. Louis, MO, USA	Standard	IV
2-Phenyl-chromone	Fluka AG, Buchs, Switzerland	Standard	II
Potassium iodide	Riedel-de Haën, Seelze, Germany	In Dragendorff reagent	IV
2-Propanol	Riedel-de Haën, Seelze, Germany	Solvent	I
Sodium acetate	Merck, Darmstadt, Germany	For buffer solution	IV
Testosterone	Fluka Chemie, Buchs, Switzerland	Standard	V
Toluene	J.T. Baker, Deventer, Holland	Solvent	IV
Triazolam	Sigma-Aldrich, St. Louis, MO, USA	Standard	IV
1,2,3 -Triazoles	Synthesis laboratory, Faculty of Pharmacy, Helsinki, Finland	Crude and purified synthesis products	III
Trifluoroacetic acid (TFA)	Acros Organics, Geel, Belgium	For MALDI matrix	II-IV
Verapamil hydrochloride	Sigma-Aldrich, Steinheim, Germany	Standard	III, V
Water (Milli-Q purified)	Millipore, Molsheim, France	Solvent	I, II, IV
	Millipore, Billerica, MA, USA	Solvent	V

Publication I



Publication II



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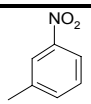
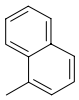
1	297	
2	302	
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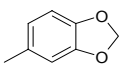
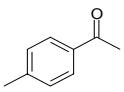
4	296	
5	294	

Publication III

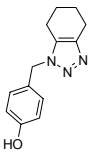
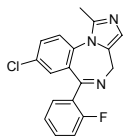
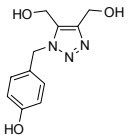
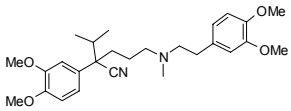
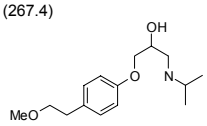
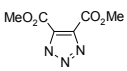
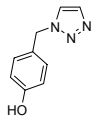
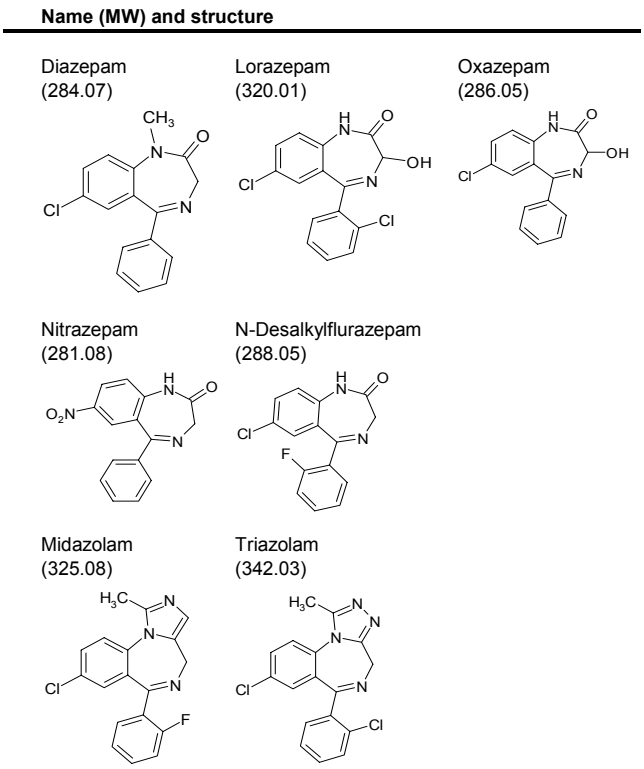
Comp.	Name (MW) and structure		Comp.	Name (MW) and structure	
1	Triazole 1 (229.3)		6	Midazolam (325.8)	
2	Triazole 2 (235.2)		7	Verapamil (454.6)	
3	Triazole 3 (145.2)		8	Metoprolol (267.4)	
4	Triazole 4 (185.1)				
5	Triazole 5 (175.2)				

Figure 6. Structures and molecular weights of the compounds investigated (continues).

Publication IV



Publication V

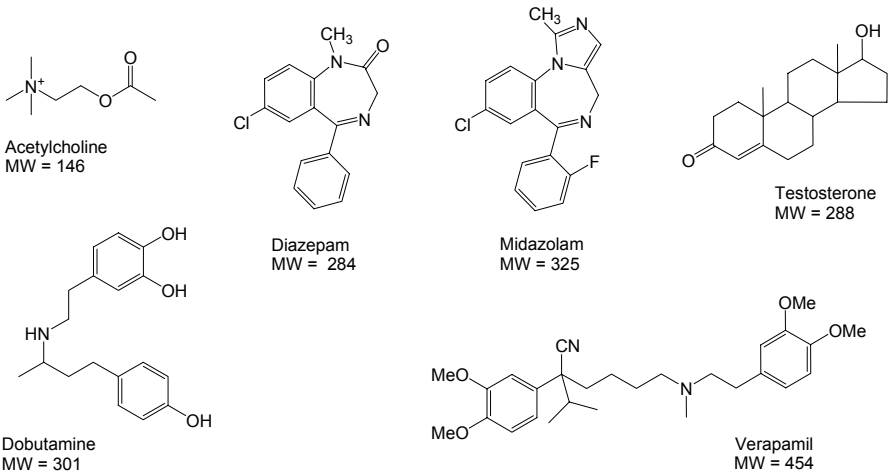


Figure 6. Structures and molecular weights of the compounds investigated (continued).

Table 2. TLC plates and HPLC columns.

Plates	Dimensions (cm)	Thickness (mm)	Producer/Supplier	Publication
HPTLC, silica gel 60 F_{254}	10 x 20	0.25	Merck, Darmstadt, Germany	I
HPTLC, silica gel 60 F_{254}	10 x 10	0.25	Merck, Darmstadt, Germany	I-IV
PLC, silica gel 60 F_{254}	20 x 20	2	Merck, Darmstadt, Germany	II
UTLC, monolithic	3.6 x 6	0.01	Merck, Darmstadt, Germany	III-V

Columns	Dimensions (mm)	Particle size (μm)	Producer/Supplier	Publication
Genesis C18	50 x 1	4	Jones Chromatography Ltd., Hengoed, U.K.	I
LichroCART Purospher RP-18e	125 x 3	5	Merck, Darmstadt, Germany	II, IV
Guard column, LiChroCART 4-4, Purospher RP-18e, 5μm			Merck, Darmstadt, Germany	II, IV

Table 3. Methods employed (continues).

Publication	Methods	Notes
I	HPTLC	UV detection (UV lamp and densitometer)
	FIA-MS	ESI-MS and API 3000 instrument
	HPLC	UV and ESI-MS; ion trap-MS instrument
II	HPTLC	UV detection (UV lamp and densitometer)
	MALDI-MS	Atmospheric pressure (AP) ionization; Q-TOF instrument
	PLC	UV detection (UV lamp)
	HPLC	UV detection
III	HPTLC	UV detection (UV lamp and densitometer)
	UTLC	Densitometric UV detection
	MALDI-MS	Atmospheric pressure (AP) ionization; ion trap instrument
	MALDI-MS	Vacuum ionization; time-of-flight (TOF) instrument
IV	HPTLC	UV detection (UV lamp)
	2D UTLC	Densitometric UV detection, Dragendorff dyeing
	MALDI-MS	Atmospheric pressure (AP) ionization; ion trap instrument
	HPLC	UV detection
V	UTLC	Desorption electrospray ionization detection;
	DESI-MS	LTQ instrument

Table 3. Methods employed (continued).

Publication	Purpose and materials and instrumentation used
I	To transfer HPTLC-separated analyte from HPTLC plate for MS *Injection syringe device (see Figure 7A);
II	To isolate separated analyte from PLC plate *Isolation device (see Figure 7B) *Centrifuge, Hettisch Universal
IV	Solid-phase extraction to purify urine samples *Oasis HLB 1 cc SPE columns, Waters Oasis, MA, USA *GeneVac Technologies -vacuum apparatus, England
IV	Enzymatic hydrolysis to hydrolyze authentic urine sample *Techne-incubator, Cambridge, UK *Centrifuge, Eppendorff, Germany

Table 4. Instrumentation.

Method	Instruments	Manufacturer	Publications
HPTLC, PLC, UTLC	Linomat IV TLC applicator	Camag, Switzerland	I-IV
	UV lamp	Desaga, Germany	I-IV
	Camag TLC Scanner II	Camag, Switzerland	I-IV
HPLC	HP 1100 liquid chromatograph	Hewlett-Packard/Agilent, Germany	I, II, IV
	Esquire-LC Ion Trap LC/MS^n with an electrospray ion source	Bruker, Germany	I
FIA-MS	Sciex API3000 triple quadrupole with an electrospray ion source	Sciex, Concord, Canada	I
	Rheodyne injector	Cotati, CA, USA	I
	Microsyringe pump	Harvard Apparatus, USA	I
Vacuum MALDI-MS	Bruker Autoflex MALDI-TOF	Bruker Daltonics, Germany	III
AP-MALDI-MS	Q-TOF microTM with an	Waters & Micromass, UK	II
	AP-MALDI ion source	MassTech Inc., Columbia, USA	II
	Esquire 3000+ ion trap with an	Bruker Daltonics, Germany	III
	AP-MALDI ion source	Agilent Technologies, Germany	III
	LC-MSD-Trap-XCT-plus ion trap	Agilent Technologies, Germany	IV
	with an AP-MALDI ion source	Agilent Technologies, Germany	IV
DESI-MS	LTQ mass spectrometer	Thermo Finnigan, CA, USA	V
	prototype of the OmniSpray Source	Prosolia Inc., IN, USA	V

A **B**

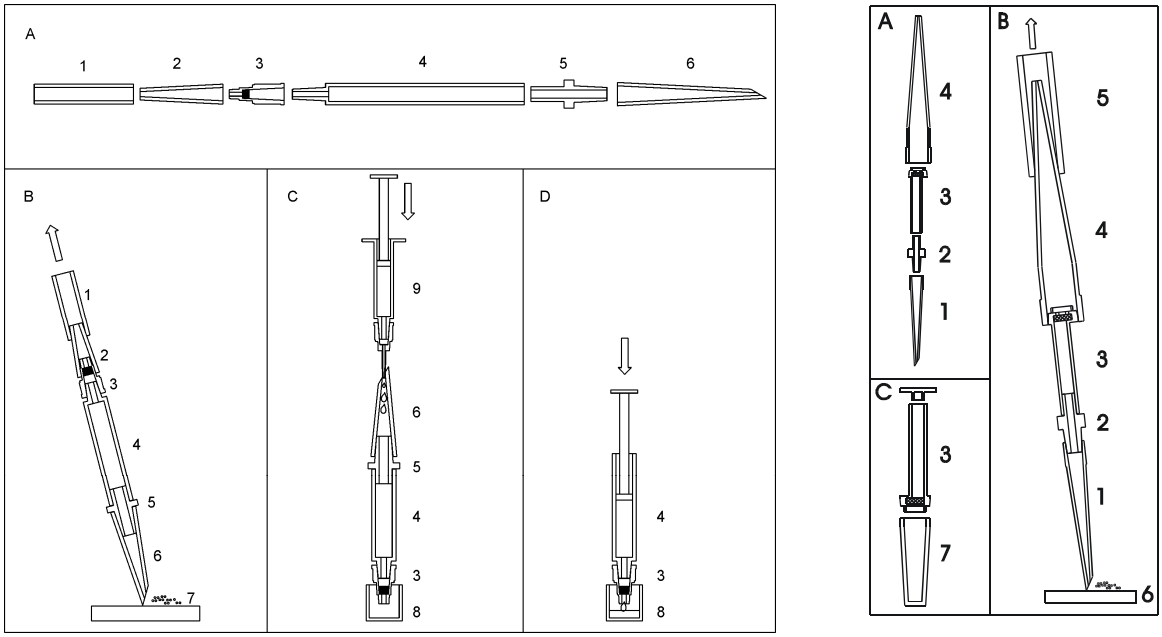


Figure 7.

A) Injection syringe device for transferring HPTLC sample from the plate to MS analysis:
(1) tube to vacuum; (2) Finntip® FT 300; (3) filter unit; (4) injection syringe; (5) hollow binding piece; (6) Finntip® FT 250 Universal; (7) HPTLC plate; (8) sample vial; (9) second injection syringe and needle. A. Unassembled pieces of the injection syringe device; B. With the help of the vacuum, the scraped adsorbent is sucked through the Finntip® (6), the binding piece (5), and the injection syringe (4) to the filter unit, on which the sample is retained according to particle size. C. Apparatus is turned vertically, and the injection syringe is removed from the vacuum. About 0.5 ml of appropriate organic solvent is introduced to the Finntip® (6). D. The compound of interest is eluted by pushing the solvent with the piston through the filter unit to the sample vial. The sample solution is now ready for MS analysis.

B) Device developed for isolation of analytes from a PLC plate.
A. Unassembled pieces of the isolation device. B. With help of a vacuum, the scraped adsorbent is sucked through the Finntip® 1000 (1) and attachment unit (2) to the sample reservoir unit with Bio-Inert membrane® (3), where the sample is retained according to its particle size. After the isolation, the apparatus is turned upside down, the vacuum is closed, and the Finntip® 10 ml (4) and the attachment unit (2) are removed. C. A filtrate reservoir unit (Microsep Centrifugal Devices) (7) is attached to the sample reservoir unit (3) and 1 ml of appropriate organic solvent is added. The isolated compound is eluted by centrifugation and the concentrate in the filtrate reservoir (7) is now ready for further procedure.

4.2.4 Two-dimensional UTLC–UV/VIS and UTLC–AP–MALDI–MS in bioanalysis (IV)

Seven reference standards of benzodiazepine were used in the development of a two-dimensional UTLC–AP–MALDI–MS method to screen and analyze for benzodiazepines and their possible metabolites in human urine. All stock solutions of the benzodiazepine reference compounds (1 mg/ml in acetone/ethanol 50:50 v/v) were diluted to appropriate concentrations. Spiked urine samples were prepared by adding 10 µl of benzodiazepine solutions (10 µg/µl) to a mixture of 1 ml of blank urine and 2 ml of ammonium carbonate buffer solution (0.01 M; pH 9.3). Authentic urine sample was collected from a healthy volunteer 53 hours after intake of 10 mg of diazepam. Urine samples were purified by solid-phase extraction (SPE) prior to UTLC separation, and the recovery tests of the SPE procedure were carried out using the HPLC–UV method. Enzymatic hydrolysis of the authentic biological sample was performed with *Helix pomatia* β-glucuronidase. Sample solutions were sprayed onto the UTLC plate in amounts of 1–10 µl (depending on the sample concentration) allowing the total amount of analytes on the plate to range between 0.1 pmol and 1 nmol. Dichloromethane/acetone (93+7 v/v) was used as the final mobile phase for the elution of the first dimension (1D) and toluene/acetone/ethanol/25% ammonia solution (70+20+3+1 v/v) for elution of the second dimension (2D). The plates were eluted to a distance of 2 cm (in both dimensions), and the elution time in one dimension was 2–6 min; i.e. the total elution time was 4–12 min. Benzodiazepines were detected visually as derivatives after spraying of the plate with Dragendorff reagent, with UV densitometric scanning, and with AP–MALDI–MS or MS/MS.

4.2.5 UTLC–DESI–MS (V)

Six common pharmaceuticals or otherwise biologically important compounds were used to test the performance of an ultra-thin layer chromatography (UTLC) plate as a new surface not previously used with DESI. Stock solutions (10 mM) of the analytes were prepared in water, water/methanol (50/50), or methanol, and diluted in water/methanol (50/50) to final concentrations of 100 nM–1 mM. For the separations, 1 µL of the sample solution containing 100 µM (100 pmol) of each analyte was applied to the adsorbent. After elution (with mobile phase of ethyl acetate containing 0.5 % ammonium hydroxide), the plate was scanned using the ion trap mass spectrometer equipped with a desorption electrospray ion source consisting of a solvent delivery line, a coaxial nozzle for delivering the nebulizing gas (N_2), a high voltage power supply, and two x,y,z moving stages for independent control of the positions of the sample and the source in relation to the inlet of the mass spectrometer. A manual 360° rotational stage that housed the DESI source was used to control the impact angle. The UTLC plate was scanned by moving the surface stage and the DESI sprayer manually. Water/methanol/formic acid (50/50/0.1%) was used as the sprayer solvent.

5. RESULTS AND DISCUSSION

The results obtained in this work are summarized in this section in three parts. More details can be found in the original publications (I–V). The results of the one-dimensional separations with PLC, HPTLC, and UTLC plates are presented and discussed first. Then, the results obtained by "cleave and analyze" and in situ spectroscopic and MS detections are reported and discussed. Finally, the procedure for the two-dimensional separation with UTLC plates is described and evaluated.

5.1. Separations with PLC, HPTLC, and UTLC

The success of TLC separation critically depends on the application technique, stationary phase, mobile phase, and elution mode. PLC (II), HPTLC (I–III), and UTLC plates (III–V) were used in this work. The main features of the plates are presented in Table 5. Of the three plates, PLC has the thickest adsorbent layer (2 mm), largest particle size (about 25 μm), and broadest particle size distribution (5–40 μm) making it suitable only for preparative use. The adsorbent layer in the HPTLC plates is 10 times thinner (0.2–0.25 mm), and that in the UTLC plates is 20 times thinner still (10 μm). Also, the mean particle size in the HPTLC plates is smaller (about 5–7 μm) than that in the PLC plates, and the particle size distribution is narrower (~2–10 μm). UTLC plates do not contain particles but monolithic material. Thus, HPTLC and UTLC plates are more suitable for analytical use. The dimensions of the plates vary as well: the UTLC plate (3.6 x 6 cm) is about one-twentieth the size of the PLC plate (20 x 20 cm). All of these physical properties of the plates have a significant effect on the capacity, elution time, resolution, and sensitivity achieved with UV and MALDI–MS.

Table 5. Description of the UTLC, HPTLC, and PLC plates (I–V).

	UTLC	(HP)TLC	PLC
Layer thickness (mm)	~ 0.01	0.20 / 0.25	0.5 - 2
Mean particle size (μm)	-	~ 5 - 7	~ 25
Particle size distribution (μm)	-	up to ~ 10	5 - 40
Fluorescent indicator	no	yes	yes
Plate size (cm)	3.6 x 6	10 x 10 / 10 x 20	20 x 20
Elution distance (cm)	2 - 2.5	5	18
Elution time	4 - 6 min	15 min	~ 1 - 2 h
Solvent consumption (ml)	3	10	100

Application of the samples to the UTLC and HPTLC plates using the spray-on technique with a Linomat application device (I–IV) provided considerably narrower bands than the spot application technique, and better resolution was obtained in separation. In the spray-on technique the solvent is fast vaporized, and the sample zone broadening was minimal. Narrow sample zones were especially important with UTLC plates, where the migration distance is short and separation efficiency limited. The solvent composition also had a significant effect on the sample zone broadening. With silica stationary phases, less volatile aqueous solvents caused significantly broader sample zones than did more volatile organic solvents. This was particularly noticeable in the analysis of benzodiazepines in urine sample (IV). Direct application of the urine sample to the UTLC plate led to decreased resolution relative to the benzodiazepine standard solution prepared in acetone/ethanol (50:50 v/v). To resolve this problem, the benzodiazepines were extracted with SPE, the extraction solvent was evaporated to dryness, and residues were reconstituted to acetone/ethanol (50:50 v/v) before application to the plate.

The plate thickness determines the maximum amount of compound that can be applied to the plate without decrease in resolution. While capacity was not explicitly examined with UTLC or HPTLC plates, studies I–IV indicated that HPTLC can be used at least up to microgram level and UTLC up to nanogram level. The capacity of PLC was examined in detail to determine the maximum amount of compound that can be isolated with acceptable resolution (study II). More than 2 mg could be applied to the 2-mm-thick PLC plate, but resolution with PLC plate thickness of 0.5 and 1.0 mm began to decrease at sample amounts of 1–2 mg. The plate size and the width of the sample zone determine how many samples can be analyzed in parallel. With the sample application technique used in this work, about eight samples can be analyzed in parallel with UTLC plates, and about 18–20 samples with HPTLC plates (10 x 20 cm). However, the number of samples can be doubled by using the horizontal elution technique, carrying out the elution from two sides simultaneously instead of in the conventional vertical mode. In PLC the number of parallel samples is case dependent. Although a wider application zone enables the isolation of a larger amount from one plate, narrower zones enable more samples to be purified in parallel. In our work (study II), using a zone width of 1.5 cm and a distance of 1.5 cm between the samples, parallel purification of six samples on one 20 x 20 cm PLC plate was achieved without decrease in the performance of the system.

Optimization of the mobile phase in order to achieve the required resolution is highly important in TLC since the gradient elution technique is relatively difficult to employ. The optimization can be performed with several systems [6–10], but with the PRISMA model [10], which is widely used in TLC, the procedure is easy and relatively fast to perform: the overall time required for the optimization is a few hours (I, II). Good or at least adequate resolution was achieved for the compounds (I–III) with of help of the PRISMA model. Although the optimization of the mobile phase took a few hours, the analysis time itself was relatively short: with UTLC about 2–6 min and with HPTLC 5–20 min. The elution time in PLC was about 1–2 hours, however.

The separation efficiency is greatly dependent on the physical properties of the stationary phases on the plates (Table 5). The performances of UTLC and HPTLC plates were compared in the separation of triazoles and drug compounds (midazolam, verapamil, metoprolol) using the same eluent composition and UV densitometry for detection (study III). The R_F values of the compounds ranged between 0.1 and 0.97 with UTLC and between 0.03 and 0.54 with HPTLC plates (Table 6), providing good separation efficiency. The R_F values obtained with UTLC are higher because the total surface area is smaller, i.e. the adsorbent layer is thinner and the specific surface area is smaller (10 μm and about 350 m^2/g for UTLC and 0.2 mm and about 500 m^2/g for HPTLC) [45]. Furthermore, the plate heights (H) were in most cases higher with UTLC plates than with HPTLC plates (Table 6). The elution time with UTLC (2–4 min) was about half that with HPTLC (5–8 min), and the solvent consumption in the elution of one UTLC plate was 3 ml, which is about one third the amount needed in HPTLC. All these UTLC results are parallel with the results reported earlier by Hauck and Schulz [45]. The relative standard deviations (RSD) of the R_F values were between 1.7 and 3.1% (Table 2 in study III) indicating good repeatability of the separation. A comparison of the separation efficiencies of PLC and HPTLC was carried out in study II. The R_F values of the compounds were between 0.35 and 0.51 with HPTLC and between 0.18 and 0.35 with PLC plates (Table 7). Correspondingly, the resolutions were 0.7–3.28 with HPTLC and 0.54–1.1 with PLC. The lower separation efficiency with PLC than with HPTLC is due to the larger mean particle size of the adsorbent and the broader particle size distribution (Table 7). However, PLC proved suitable for small-scale purification if the analyte could be separated from the impurities with resolution of 0.8 or more.

Table 6. Comparison of R_F values and plate heights (H) of the compounds used in study III measured by the UTLC and HPTLC methods. Compound 5 was a target compound in a crude synthesis sample, and identification of it is described at page 53.

Comp.	Name	R_F		H (μm)*	
		UTLC	HPTLC	UTLC	HPTLC
1	Triazole 1	0.19	0.11	328	125
2	Triazole 2	0.10	0.03	184	445
3	Triazole 3	0.80	0.54	87	35
4	Triazole 4	0.50	0.22	102	96
6	Midazolam	0.88	0.22	68	115
7	Verapamil	0.97	0.24	41	90
8	Metoprolol	0.37	0.04	314	296

*Calculated: $H = X/N$ and $N = 16Xz/W^2$; X indicates the distance of a mobile phase from the origin, z is the distance of a substance spot from the origin, and W is the diameter of the spot; i.e. the smaller plate height, the narrower peaks and better separation.

Table 7. R_F values and resolutions of the target compounds measured in study II with HPTLC and PLC.

Sample	R_F values		Resolution	
	HPTLC	PLC	HPTLC	PLC
1	0.44	0.34	1.41	0.77
2	0.51	0.28	0.85	0.84
3	0.47	0.35	0.70	0.54
4	0.35	0.29	3.28	1.05
5	0.35	0.18	1.36	1.10

5.2. Detection (visual, UV, and MS)

TLC detection can be accomplished with a UV lamp, by densitometric scanning at a selected wavelength, or by recording the whole UV spectrum. These methods were used in studies I–IV. MS detection is more specific, and it was applied in all studies (I–V). Flow injection analysis (FIA) with electrospray ionization (ESI–MS) was used in study I after scraping of the analyte from the plate. MS detection was carried out directly on plate by matrix-assisted laser desorption ionization mass spectrometry (MALDI–MS) in studies II–IV and by desorption electrospray ionization (DESI–MS) in study V.

5.2.1 Visual (UV lamp, dyeing) method and UV densitometry (I–IV)

Rapid TLC detection can be accomplished by visual observation of the separated zones under a UV lamp (I–III). This method is routinely used in method development of TLC, and also to monitor synthesis processes. Commercial UTLC plates lack a fluorescent indicator and the UV lamp cannot be used. Instead, the analyte(s) can be visualized as their derivatives after dyeing of the plate. The dyeing was tested in study IV to obtain and specify the exact location of the analyte(s) on the plate after a two-dimensional elution (IV). In the method development, the visualization of the analytes on the plate was rapid and easy to perform under a UV lamp, or with dyeing. However, visual detection offers only limited sensitivity and is best reserved for qualitative work.

UV densitometry provides a fast and reliable method for detection and also for identification of the TLC-separated analytes (I–IV). With densitometric measurements the analytes are identified by their R_F values and in situ UV spectra. The use of one wavelength is often sufficient for detection and recording of the R_F value of the analyte. If the analyte cannot be identified exactly on the basis of the R_F value, recording of the whole UV spectrum (for example between the wavelengths of 190 and 400 nm) of the analyte is required in addition. Combination of the R_F values and UV spectra information allowed nearly all of the synthesis target compounds examined in study I to be distinguished from the impurities (I). The limits of detection (LODs) achieved with the UV densitometer (S/N = 3) were about picomole range (Table 8), being lower than with visual detection (i.e. under a UV lamp or with dyeing) and thus providing better sensitivity for the detection of low concentrations of analytes. For

quantitative work, the UV densitometer was absolutely necessary, enabling a semi-quantitative purity determination from the ratio of the peak area of the analyte to the total area of all peaks recorded (I–III). The quantitative repeatability of the UTLC–UV method was tested in study III. RSDs, measured as peak heights or areas, were acceptable, ranging between 3.7 and 8.3%. These results are in accordance with the HPTLC data reported earlier [157–159] and show that the repeatability with UTLC–UV is comparable to that with HPTLC–UV. In conclusion, visual detection is preferred in method development, whereas UV densitometry provides more accurate and reliable results in quantitative and semi-quantitative work.

Table 8. Limits of detection (S/N =3) for the compounds in study III with UTLC/HPTLC–UV.

	UTLC		HPTLC	
Compound	non-eluted (pmol)	eluted (pmol)	non-eluted (pmol)	eluted (pmol)
Triazole 1	38	69	23	79
Triazole 2	88	154	25	75
Triazole 3	33	84	68	539
Triazole 4	42	79	266	819
Midazolam	1	25	4	326
Verapamil	7	66	9	622
Metoprolol	49	54	25	345

Comparison of HPTLC and LC–UV–MS (I, II)

HPLC–UV (I, II) and LC–UV–ESI–MS (I) were compared with HPTLC–UV in semi-quantitative purity analysis. Semi-quantitative purities, as a percentage, for the synthesis target compounds used in studies I and II were calculated from the ratio of the peak area of the synthesis target compound to the total area of all peaks in UV densitograms and chromatograms or total ion chromatograms (TIC). There was no significant difference between the results of calculations with HPTLC–UV and LC–UV–MS (Figure 8). The correlations between the LC–UV and HPTLC–UV methods were reasonable in publication I (r^2 = 0.8053) and good in publication II (r^2 = 0.8795), indicating that HPTLC–UV is as reliable a method as LC–UV for such semi-quantitative purity analysis. HPTLC nevertheless has some advantages over LC–MS. The most important advantage is that HPTLC is a cheaper and simpler method than LC–MS. The plates are disposable and the memory effect is not a problem as it may be in LC–MS. In addition, only volatile buffers and medium polar or polar solvents can be used in LC–ESI–MS, while a significantly wider selection of solvents and buffers can be used in HPTLC separation. Speed of analysis is also an important factor in method selection. HPTLC with densitometric detection allows the analysis of about

20–40 samples on one 10 x 20 cm HPTLC plate within one hour. Furthermore, the chromatographic step with several plates can be performed in parallel in different TLC chambers. In conventional LC–MS, only one sample can be analyzed per run, and since the analysis time is typically 5–20 min, only 3–10 samples can be analyzed in one hour. The sample throughput in LC–MS can be improved by using automated methods, special multiple probe autosamplers, and several LC-columns in parallel [160]. However, all this leads to an expensive, complicated, and less robust analysis. The time required for development of the method is normally shorter with LC–MS, since gradient elution with buffered water/methanol or water/acetonitrile often offers acceptable chromatographic behavior and ionization efficiency with ESI. In HPTLC, where isocratic conditions are usual, the development of a sufficient separation may take several hours, increasing the overall analysis time. The data processing can be automated with both methods. The specificity of LC–MS is superior to that of HPTLC with UV detection, and therefore the identification of a synthesis target compound with LC–UV–MS is fast and reliable even if the sample contains impurities. LC–MS also allows determination of the structures of impurities.

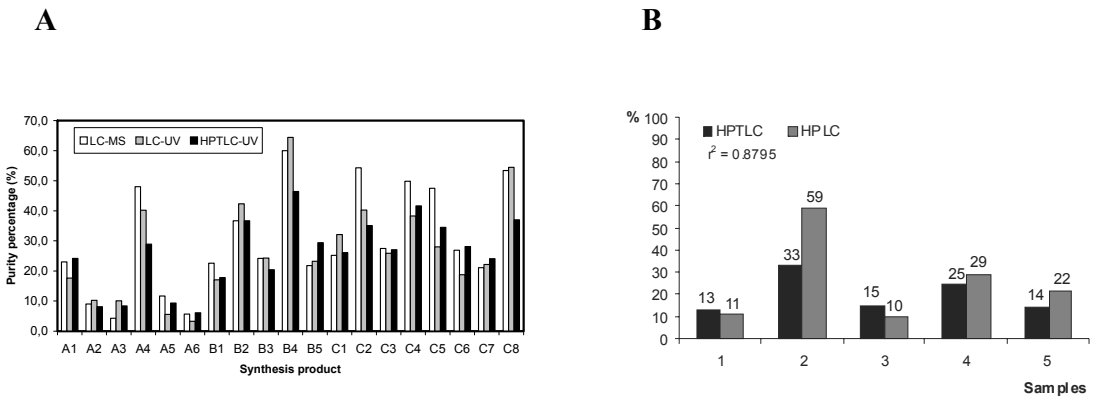


Figure 8.
A) The purities of the synthesis products A1–C8 (%) measured in study I by HPTLC and LC−UV−MS methods: LC–MS (TIC) and HPTLC–UV (r^2 = 0.8404), LC–UV and HPTLC–UV (r^2 = 0.8053), and LC–MS (TIC) and LC–UV (r^2 = 0.8310).

B) Purity (%) of the target synthesis product in crude synthesis samples (1–5) measured in study II by HPTLC–UV and HPLC–UV (r^2 = 0.8795).

5.2.2 Electrospray ionization mass spectrometry (I, V)

The measurements with electrospray ionization mass spectrometry (ESI–MS) were preformed using flow injection analysis (FIA) after scraping of the analyte from the plate (I) and using a direct in situ analysis with desorption electrospray ionization (DESI) mass spectrometry (V).

"Cleve and analyze" method for FIA–ESI–MS detection (I)

In the "cleave and analyze" method, the separated analyte zone needs to be isolated from the plate. When the usual methods – extraction after scraping with a razor blade and the suction method of Amorese *et al*. [58] – proved to be unsuitable for ESI–MS due to heavy background, we developed a new scraping method for isolation of the analyte from HPTLC (I) and PLC (II) plates. The method was combined directly with a sample clean-up procedure. In our device for HPTLC (Figure 7A), the cut end of a Finntip® allows the isolation of very narrow zones of the analyte without co-isolation of unwanted components. The adsorbent particles are sucked onto the filter, from which the particles are extracted with appropriate solvent for further analysis. The whole procedure, from assembling of the device to scraping, sample extraction, and filtration, is completed within 2–3 minutes. Memory effects are not a problem since all parts of the device in contact with the sample are disposable. In addition, the device is simple and easy to set up in any laboratory. The device for the PLC plate (Figure 7B) was much the same as that for the HPTLC plate, but it was scaled up for semi-preparative purpose (II).

The isolated compounds of a sample can be rapidly and simply identified by flow injection analysis (FIA) using ESI–MS. If more detailed characterization of the analyte is desired, additional structural information is easily obtained with tandem mass spectrometry. ESI offers very soft ionization. Since protonated or deprotonated molecules tend to be the main peaks in ESI spectra, the method is reliable, for example for the identification of TLC-separated synthesis target compounds. Figure 9 illustrates how FIA–ESI–MS was used in the identification of a synthesis product. Since normal HPTLC separation was carried out, the first tentative assessment of the quality of the synthesis was done by visual observation of the zones under a UV lamp. One zone may indicate successful synthesis while additional zones indicate impurities. With densitometric measurements the target compounds can be identified by their R_F values and by in situ UV spectra, because in small libraries with homologous series of compounds, the R_F values and UV spectra should not differ significantly, while those of the impurities can be expected to differ from those of the target compounds. The differences can be utilized in the identification of the target compounds (the method is described in detail in study I). If unambiguous identification of the target compounds is still not possible, the isolated compounds can be identified by FIA–ESI–MS. Figure 9C shows as an example the ESI–MS spectrum of synthesis target compound (sample C8), obtained by using the developed scraping and sample clean-up procedure. The spectrum shows only a very abundant protonated molecule (m/z 415) with minimal fragmentation. The lack of background ions indicates efficient clean-up of the sample.

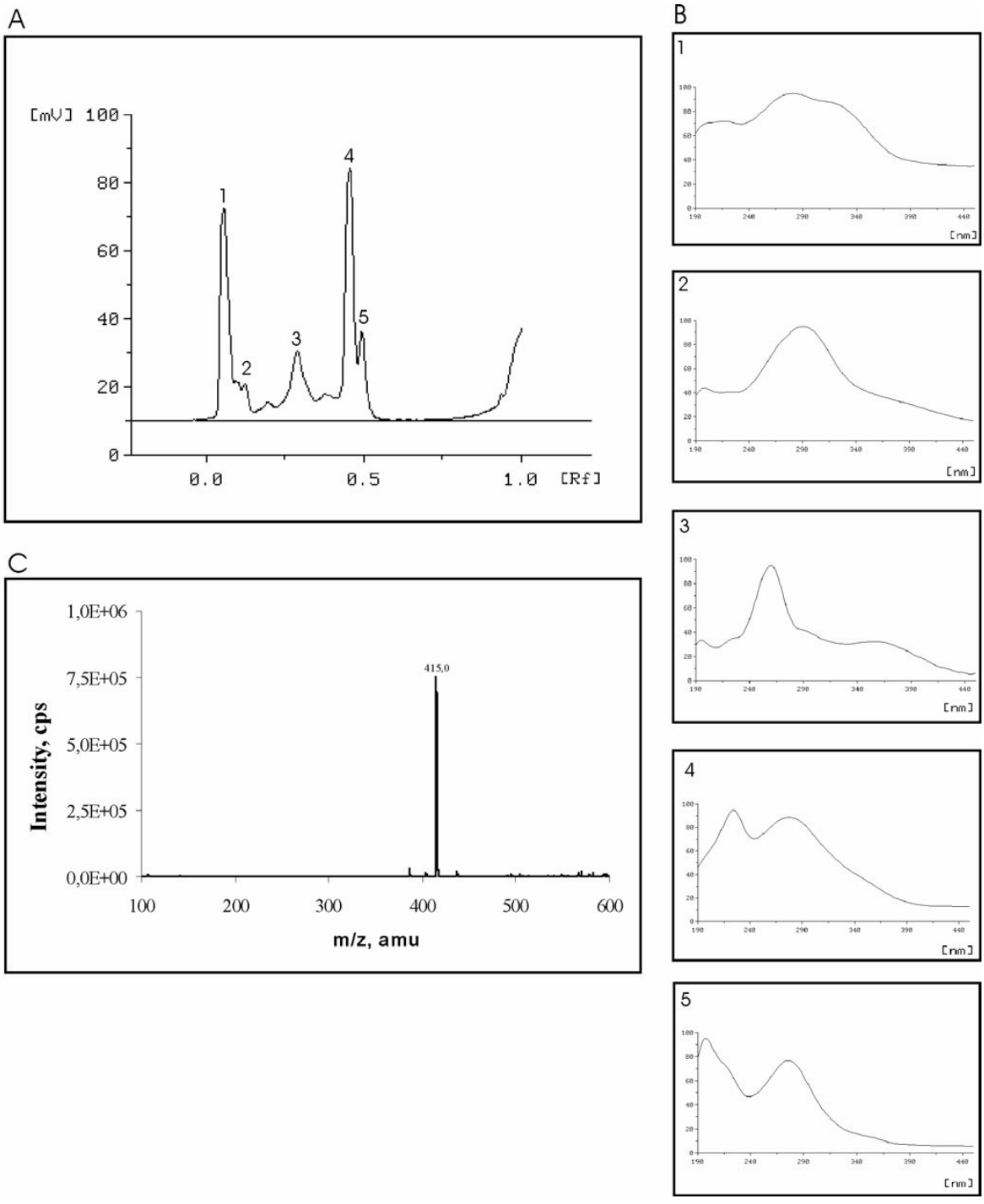


Figure 9. Identification of the synthesis product C8 (m/z 414) by HPTLC method. ***A.*** *Typical densitogram of the synthesis product (C8);* ***B.*** *in situ UV spectra of the peaks 1−5 (presented in A);* ***C.*** *ESI−MS spectrum of the zone of the synthesis target product (peak 4).*

Desorption electrospray ionization (V)

Desorption electrospray ionization (DESI) is a relatively new MS technique in which the analyses are routinely performed directly from the surface to be analyzed. Analyses take only a few seconds and no sample pretreatment is required. These features make DESI of great interest for in situ analyses. As the coupling of TLC with DESI–MS had earlier been achieved using conventional TLC plates [155, 156], we tested DESI with UTLC in the expectation that sensitivity would be improved due to the very thin adsorbent layer.

In the early stages of study V, comparisons were attempted between the UTLC and conventional HPTLC surfaces but satisfactory signals of the analytes could not be obtained from the HPTLC surface, probably due to the thicker adsorbent layer. UTLC, in turn, showed a long-lasting and stable signal. UTLC was especially compatible with DESI, since the monolithic surface was not abraded when sprayed, unlike the conventional HPTLC stationary phase composed of separate silica particles. The coupling of DESI and UTLC provided a sensitive and soft ionization technique: the LODs for analytes were 1–100 pmol (V), and only the protonated molecules (or M^+ for acetylcholine) of the analytes were observed in the spectra (Figure 2 in study V). None of the analytes showed fragments.

To determine the usefulness of UTLC as a means of separation coupled to DESI–MS, 1 μL of a mixture containing 100 μM each of six analytes was separated on a UTLC plate and analyzed by scanning the plate with the DESI sprayer. Figure 10 shows the extracted ion chromatograms and spectra for each analyte. The x-axis in the chromatograms shows the time used to scan the eluted plate from the site of sample application to the end of the elution front. The migration distance was 25 mm. The elution distance was shortest for acetylcholine, indicating its very strong affinity towards the polar UTLC surface instead of the non-polar solvent. The other, less polar compounds were eluted further by the mobile phase: dobutamine, midazolam and verapamil were satisfactorily separated, but the apparently least polar analytes, testosterone and diazepam, were not retained in the stationary phase at all under these conditions and were observed at the very end of the elution front, with overlapping signals. The spectra of all the analytes showed abundant protonated molecules $[M+H]^+$ (or M^+ in case of acetylcholine). In addition to this, midazolam and verapamil formed sodium adducts $[M+Na]^+$ and testosterone sodium and ammonium adducts (Figure 10).

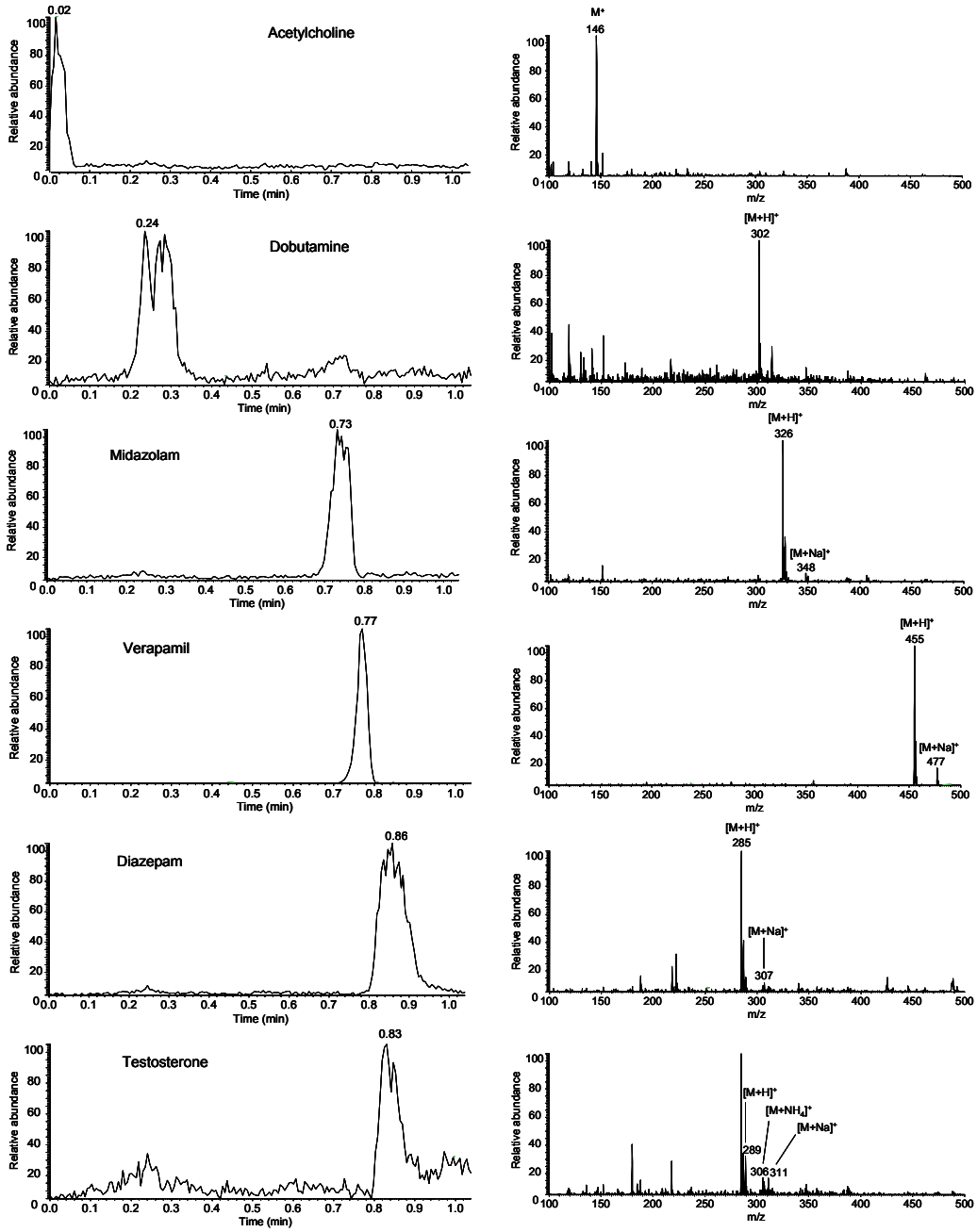


Figure 10. Separation and detection of a mixture of the analytes with UTLC–DESI–MS (V).

5.2.3 MALDI–MS (II–IV)

The suitability of MALDI–MS with vacuum and atmospheric pressure (AP) ionization was tested for the analysis of small molecules (synthesis target compounds and reference standards of drugs) directly in situ from HPTLC and UTLC plates. The most important factors affecting the TLC–MALDI–MS analysis are discussed in this section along with the results.

Matrix

MALDI–MS analysis with use of matrix compound was required because the ionization efficiency of analytes from the UTLC and HPTLC plates without the matrix is very poor, or the ionization does not occur at all. After testing of a few common matrixes, such as dihydrobenzoic acid (DHB), sinapinic acid (SA), and α-Cyano-4-hydroxycinnamic acid (α -CHCA), α -CHCA was selected as matrix compound. α -CHCA provided good ionization efficiency for all the compounds studied (II–IV). The matrix was sprayed over the sample zone with a Linomat IV applicator device, i.e. with a spray-on technique by which the matrix could be deposited precisely in the center of the sample zone in the form of a narrow band. Spreading of the sample zone was not visually observable. The time required to apply the matrix onto one sample zone of the plate was only 15–30 seconds (1 µl applied with a flow rate of 4 µl/min), providing a rapid preparation of the plates for the MALDI–MS analysis. The effect of the amount of the matrix on sensitivity and selectivity was investigated in study III by applying 1 nmol of the analyte (midazolam and triazole 1) to the UTLC and HPTLC plates. The concentration of the α -CHCA solution in the optimization experiments was varied between 190 ng/µl and 13.3 µg/µl, so that the total amount of α -CHCA on the plate varied between 1 and 1000 nmol. The amount of optimal matrix was 10 nmol for UTLC (about 2.66 nmol/mm^2) and 100 nmol for HPTLC (about 22.2 nmol/mm^2). A smaller amount of matrix reduced the sensitivity, while a larger amount caused increased matrix background and decreased the selectivity.

Operational parameters

The effect of the dry gas (N_2) temperature on the ionization with AP–MALDI–MS was tested (III) because the temperature has been reported to affect the analyte–matrix dissociation; that is at low temperatures formation of analyte/matrix clusters or dimers can occur, and at high temperatures fragmentation of the molecular ion of the analytes [141]. Tests were made with one of the compounds (triazole 1) used in study III and drying gas temperatures between 100 and 250 ^{o}C. The absolute abundance of the protonated molecule doubled when the temperature was raised from 100 ^{o}C to 150 ^{o}C. A further rise in temperature from 150 ^{o}C to 250 ^{o}C increased the fragmentation and reduced the abundance of the protonated molecule. The temperature of the dry gas had no clear effect on the specificity since no additional peaks appeared, and the ratio of the relative abundances of the matrix ions and the analyte ions did not change significantly in the temperature range examined. The optimal temperature was 150 ^{o}C, which was used in further studies.

The target plate of the AP–MALDI system was maintained in a fixed position mode (II–IV). With this mode the matrix disturbances were strong during the first laser pulses, but the relative abundances of the analyte ions as compared with the matrix ions increased with the number of pulses (II, III). The same observation was made with vacuum MALDI–MS (III). This suggests that the analyte molecules were not efficiently diffused into the matrix, and the concentration of the analytes was higher on the surface of the UTLC plate than on the surface of the matrix. When the UTLC plate was used with 10 nmol matrix, the signal lasted for about 30 seconds in the fixed mode. When the amount of matrix was increased the signal lasted longer, and with 100 nmol the analyte ions were observed for a few minutes. Such a long-lasting signal allows sequential mass analysis including, for example, optimization of

the operational parameters and measurements of MS and different kinds of MS/MS spectra in positive and negative ion mode from the sample zone.

It has been shown that irregular surfaces such as polymer membranes and TLC plates can lead to decreased mass accuracy in vacuum MALDI–TOF–MS [97, 146]. This was observed in study III. Figure 11 shows the variation of m/z values for the triazole 1, measured from different sample zones on UTLC plates by vacuum MALDI–TOF–MS (Fig. 2A) with internal calibration and by AP–MALDI–ion trap–MS (Fig. 2B) with external calibration. Variation in m/z values was clearly less with AP–MALDI–ion trap–MS (± 0.08 u) than with vacuum MALDI–TOF–MS (± 0.32 u). This result is in accordance with the findings of earlier studies [97, 146]. AP–MALDI–ion trap–MS can be coupled to UTLC without compromise in the mass accuracy, remembering, of course, that the ion trap that was used is not a high resolution instrument. The relatively large error in mass accuracy for the TOF instrument most likely arises because, in the measurements of flight times of ions, the flight distance is less well defined for ions formed from a sample on an irregular surface, such as a TLC plate, than for ions formed from a sample mounted on a smooth stainless steel sample holder. Inaccuracy in the mass measurement therefore occurs [119]. This inaccuracy produced relatively large uncertainty in the measured mass values even though an internal standard was used. With an ion trap instrument, however, the position of ion formation does not affect the mass accuracy since in an ion trap instrument with an AP–MALDI ion source, ions are formed externally, collected inside the trap, and subsequently mass analyzed. In other words, the accuracy of the mass measurement is defined by the inherit operational stability of the instrument. Parallel results have been obtained by TLC–MALDI–Fourier transform (FT) MS using a reduced pressure ion source [98].

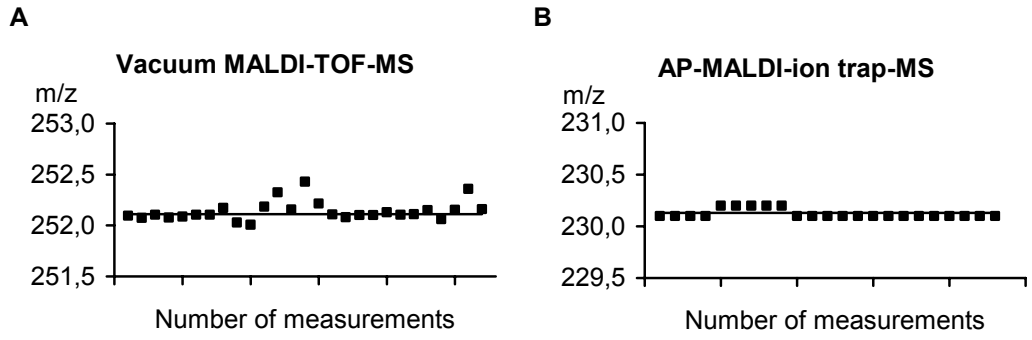


Figure 11. Replicate measurements of (A) triazole 1 $[M + Na]^+$ ions (m/z = 252.111) by UTLC–vacuum MALDI–MS (internal calibration mode), n = 27, and (B) triazole 1 $[M + H]^+$ ions (m/z = 230.129) by UTLC–AP–MALDI–MS (external calibration mode), n = 23. (■) measured mass, (—) calculated mass.

Spectra

The MALDI ionization of the analytes from the UTLC plate was studied in more detail in studies III and IV. Also the different formation of the ions in AP– (III–IV) and vacuum (III) MALDI was compared. The reference MALDI mass spectra of the compounds were measured by applying 1 nmol of the analyte and 10 nmol of the matrix to the UTLC plate (III, IV), and doing the measurement before the elution. The mass spectra produced by AP– and vacuum MALDI ion sources exhibited an abundant protonated molecule with minimal fragmentation (Table 9). Compounds containing a hydroxyl group (triazoles 1 and 2, and metoprolol, oxazepam, lorazepam) produced an abundant sodium adduct ion. The background disturbance caused by the matrix was relatively low, and background ions did not appear at the same m/z values as for the protonated molecules or sodium adducts of the compounds. This indicates that 10 nmol amount of the matrix is sufficient for effective ionization of the compounds. At the same time the amount is small enough to minimize any disturbance caused by the matrix. In the MS/MS experiments with benzodiazepines, the product ions (Table 9B) were mostly the same as those measured with an ion-trap instrument by Smyth et al. [161].

Figure 12 illustrates as an example the AP– and vacuum MALDI mass spectra of triazole 1 and midazolam (compounds used in study III) measured from the UTLC plate after separation, and the AP–MALDI mass spectrum of triazole 1 (1 nmol) measured from the HPTLC plate after separation. In vacuum MALDI–MS, abundant matrix background ions (marked with an asterisk) were observed below m/z 250, the main ions being protonated α-CHCA (m/z 190), its sodium adducts $[M+Na]^+$ (m/z 212) and $[M-H+2Na]^+$ (m/z 234), a fragment ion $[M+H-H_2O]^+$ (m/z 172), and an unknown ion at m/z 198. AP–MALDI mass spectra showed the same matrix ions at mass range below m/z 250, but also matrix dimers $[2M+H]^+$ (m/z 379), $[2M+Na]^+$ (m/z 401), $[2M-H+2Na]^+$ (m/z 423), and $[2M-H+Na+K]^+$ (m/z 439), which were not observed with vacuum MALDI–MS. The collisional cooling in vacuum MALDI–MS is significantly less than that in AP–MALDI–MS, and dissociation of the dimers occurs in the vacuum MALDI–TOF experiments. However, all the analyte ions were visible with both methods. The matrix background is significantly lower with midazolam (Fig. 12E) than with triazole 1 (Fig. 12B), perhaps because the physical and chemical properties (proton affinity, hydrophobicity, and absorptivity at 337 nm) are more favorable for efficient ionization of midazolam than those of triazole 1. In addition, the extraction efficiency from the inner parts of the silica layer to the matrix during the addition of the matrix solution may be better with midazolam than with triazole 1. Comparison of the UTLC– (Fig. 12B) and HPTLC–AP–MALDI–MS (Fig. 12C) spectra of triazole 1 indicates that the matrix disturbances are less with the UTLC than the HPTLC plate. This is because the optimal matrix amount with the UTLC plates (10 nmol) is only one tenth that with the HPTLC plates (100 nmol).

Table 9. Molecular, fragment, and product ions of standard analytes measured before separation by A) UTLC–AP– and UTLC–vacuum MALDI–MS (III) and B) UTLC–AP–MALDI–MS and UTLC–AP–MALDI–MS/MS (IV). Sample amount was 1 nmol and matrix amount 10 nmol.

A

UTLC-AP-MALDI-MS				UTLC-Vacuum-MALDI-MS			
Comp.	**m/z (rel. abund)**			**Comp.**	**m/z (rel. abund)**		
	$[M+H]^+$	**$[M+Na]^+$**	**Other ions**		**$[M+H]^+$**	**$[M+Na]^+$**	**Other ions**
triazole 1	230 (100)	252 (25)	124[a] (36)	**triazole 1**	230 (48)	252 (100)	124[a] (83), 107[b] (10)
triazole 2	236 (100)	258 (79)	130[c] (84)	**triazole 2**	236 (-)	252 (100)	130[c] (30), 107[b] (20)
triazole 3	146 (100)			**triazole 3**	146 (100)		
triazole 4	186 (50)	208 (100)		**triazole 4**	186 (50)	208 (100)	
midazolam	326 (100)	-	-	**midazolam**	326 (100)	-	-
verapamil	455 (100)	-	303 (12)	**verapamil**	455 (100)	-	303 (63)
metoprolol	268 (100)	290 (15)		**metoprolol**	268 (100)	290 (8)	

[a] m/z 124 =$[C_6N_3H_{10}]^+$, [b] 107 = $[CH_2C_6H_4OH]^+$, [c] 130 = $[C_4N_3O_2H_8]^+$

B

	MS			MS/MS	
	m/z (rel. abund)			Precursor	
Comp.	**$[M+H]^+$**	**$[M+Na]^+$**	**Other ions**	**$[M+H]^+$**	**m/z (rel. abund) [product ion]**
midazolam	326 (100)	-	-	$[M+H]^+$; 326	291 (100) $[M + H - Cl]^+$; 244 (29); 209 (2)
diazepam	285 (100)	307 (13)	257 (7)	$[M+H]^+$; 285	257 (100) $[M + H - CO]^+$; 222 (60) $[M + H - CO - Cl]^+$ 228 (40) $[M + H - CO - CH_2NH]^+$; 193 (20) $[M + H - CO - CH_2NH - Cl]^+$ 182 (30); 154 (60)
lorazepam	321 (77)	343 (100)	303 (19); 275 (11)	$[M+H]^+$; 321	303 (100) $[M + H - H_2O]^+$; 275 (50) $[M + H - H_2O - CO]^+$; 208 (10)
oxazepam	287 (100)	309 (90)	269 (23); 241 (15)	$[M+H]^+$; 287	269 (100) $[M + H - H_2O]^+$; 241 (20) $[M + H - H_2O - CO]^+$
N-desalkyl-flurazepam	289 (100)	311 (11)	261 (6)	$[M+H]^+$; 289	261 (100) $[M + H - CO]^+$; 226 (80) $[M + H - CO - Cl]^+$; 140 (94)
triazolam	343 (100)	365 (30)	-	$[M+H]^+$; 343	308 (100) $[M + H - Cl]^+$; 315 (25); 279 (4)
nitrazepam	282 (100)	-	236 (21)	$[M+H]^+$; 282	236 (100) $[M + H - NO_2]^+$

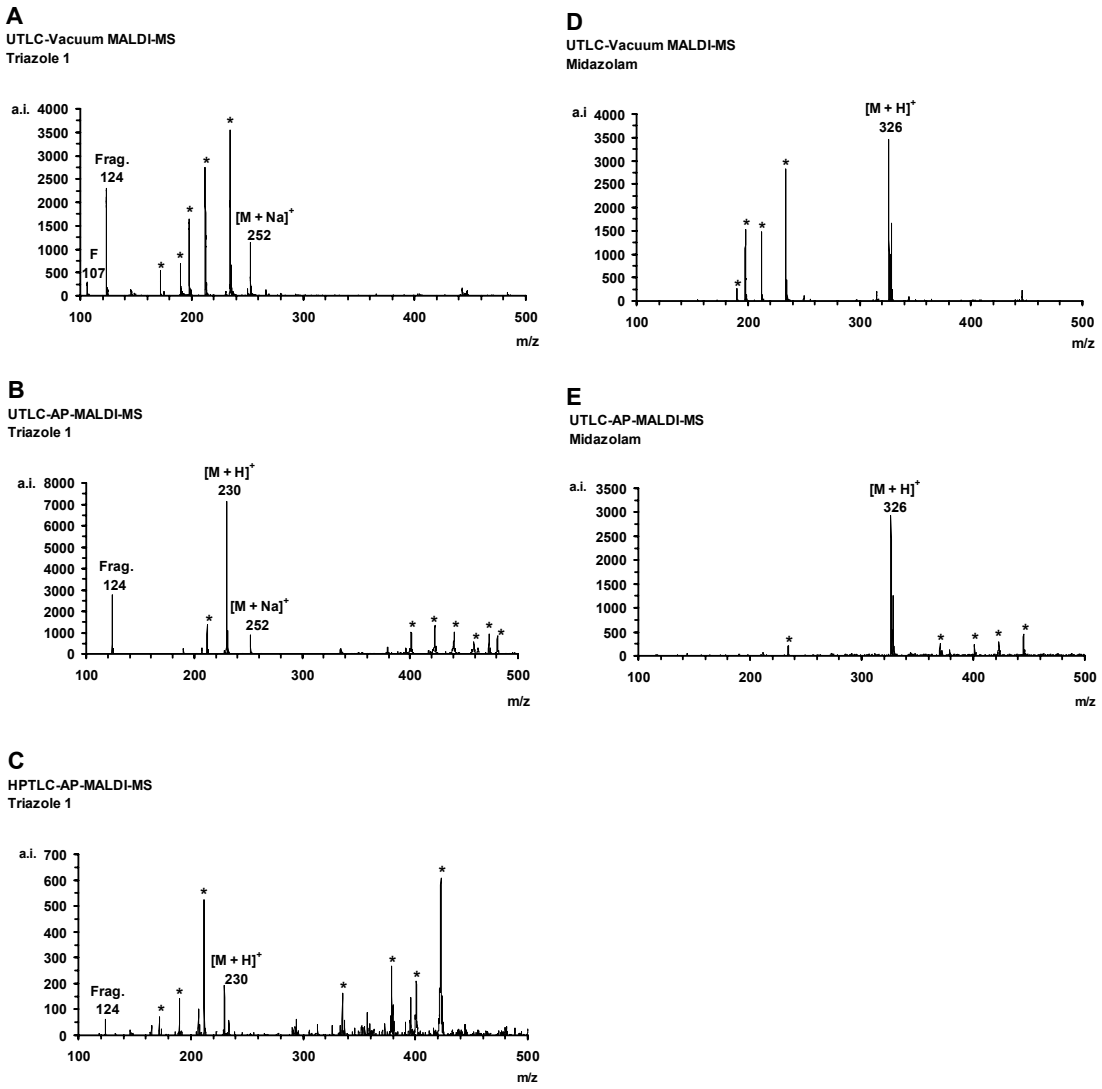
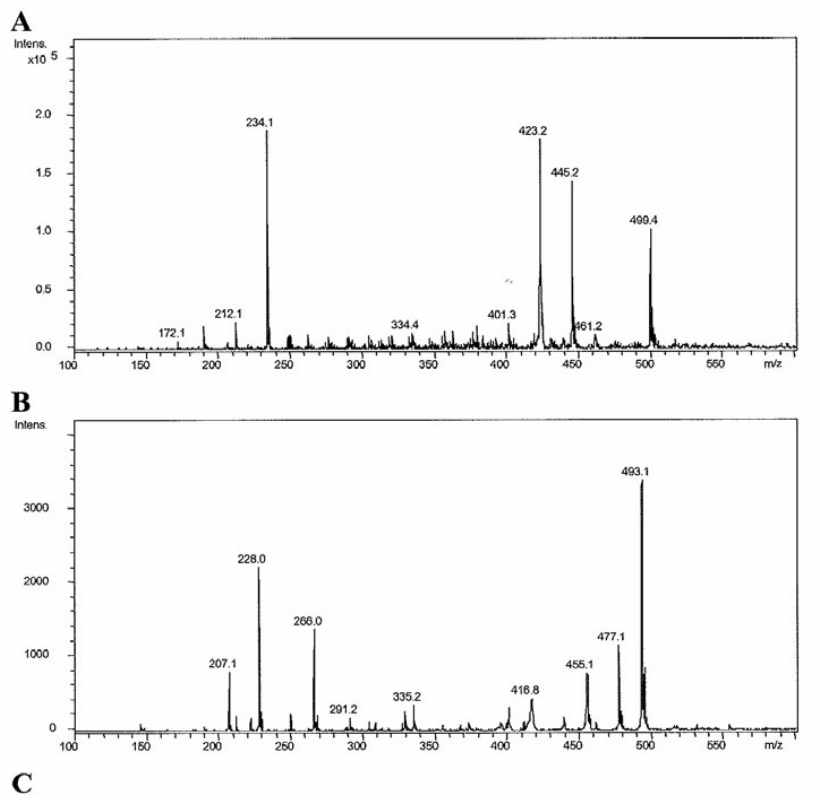


Figure 12. Mass spectra of triazole 1 (A–C) and midazolam (D–E) measured after separation, by UTLC–vacuum MALDI–MS (A and D), UTLC–AP–MALDI–MS (B and E), and HPTLC–AP–MALDI–MS (C). Sample amounts were 1 nmol and matrix amounts 10 nmol (UTLC) and 100 nmol (HPTLC). The main matrix ions are marked with an asterisk (*). For analyte fragment ions (F), see Table 9A.

The effect on the AP–MALDI–MS spectra when the Dragendorff dyeing reagent [162] was used for visualization of the benzodiazepines on the UTLC plate was investigated in study IV. The test was performed by applying 1 nmol of the analytes and 10 nmol of the matrix to the UTLC plate and then spraying the plate with Dragendorff reagent. This reagent contains potassium iodide, and the MS spectrum of the neat α-CHCA matrix and this reagent (Figure 13B) showed potassium adducts and dimers of the matrix (m/z 228, 266, 417, 455, 477, and 493) instead of the ions at m/z 212, 234, 423, 445, and 499 that are observed without the dye (Figure 13A). However, the MS spectra of the benzodiazepine derivatives measured after

dyeing showed abundant protonated molecules (as without the reagent), although their absolute abundances were about 10–100 less with than without dyeing. Also, the background disturbances due to the Dragendorff reagent were increased. The results show that the Dragendorff reagent suppresses the ionization of the benzodiazepines and can be used only where the concentrations of these analytes are relatively high (100–500 pmol).



C

Neat matrix		Neat matrix with Dragendorff reagent	
m/z	**ion**	**m/z**	**ion**
172	$[M - H - H_2O]^+$		
190	$[M + H]^+$	207	unknown composition
212	$[M + Na]^+$	228	$[M + K]^+$
234	$[M - H + 2Na]^+$	266	$[M - H + 2K]^+$
401	$[2M + Na]^+$	417	$[2M + K]^+$
423	$[2M - H + 2Na]^+$	455	$[2M - H + 2K]^+$
445	$[2M - 2H + 3Na]^+$	477	$[2M - 2H + Na + 2K]^+$
499	unknown composition	493	$[2M - 2H + 3K]^+$

Figure 13. MS spectra of neat α-CHCA matrix (A) without and (B) with the Dragendorff dyeing reagent. Matrix amount was 10 nmol. (C) Main matrix background ions in mass spectra.

Limits of detection and repeatability

The limits of detection (LODs) for analytes with UTLC and HPTLC plates and AP–MALDI–MS and vacuum MALDI–MS detection were investigated in studies III and IV. LODs were determined using a signal-to-noise ratio of 3, and with one of the characteristic analyte ions (molecular, sodium adduct, or fragment ion) as signal and background ions nearby the analyte signal as noise. The results are presented in Table 10.

LODs achieved in study III with UTLC–AP–MALDI–MS (Table 10A) after elution were 10–400 pmol for triazoles (1–4) and 1–7 pmol for the drug substances (6–8). The LODs with HPTLC–AP–MALDI–MS were 500–10000 pmol for triazoles (1–4) and 300–600 pmol for drug substances (6–8). These results show, that with AP–MALDI–MS, the UTLC plates provide about 10–100 times better sensitivity than the HPTLC plates. The same was true when the measurements were performed from the application zone (i.e. before elution). The better sensitivity with the UTLC plates can be attributed to the thinner adsorbent layer on the plate from which it follows that the number of sample molecules per surface area is significantly higher than on the HPTLC plate. Furthermore, with UTLC plates the analyte molecules are more efficiently extracted from the inner parts of the adsorbent onto the surface. The laser pulse is capable of ionizing compounds efficiently only at the surface of the adsorbent. Spreading of the zone during the elution reduced the sensitivity: the LODs measured from the application zone were about 2–10 times lower than those measured after elution. This result suggests that sample application with a narrower band might lead to lower LODs especially with the UTLC method. The LODs obtained with AP–MALDI–MS and vacuum MALDI–MS were mostly at the same level.

The LODs measured in study IV are presented in Table 10B. In the 2D case, the LODs for benzodiazepines after separation with UTLC and with AP–MALDI–MS detection were 2.0–66.7 pmol in pure solvent and 7.9–97.0 pmol in SPE-purified urine (Table 10B). Clearly, in MS mode, endogenous compounds in urine disturb the analysis. LODs were 2–10 times higher for urine samples. The endogenous compounds do not, however, suppress the ionization, since the LODs measured by MS/MS were mostly at the same level for benzodiazepines in pure solvent and in SPE-purified urine. The LODs measured by MS/MS were mostly below 10 pmol, and about ten times lower than those measured by MS detection (Table 10B).

The quantitative repeatability of the MALDI method was tested with UTLC–AP–MALDI–MS (III) by applying 0.1 nmol of analytes (triazole 1, midazolam, and metoprolol) and 10 nmol of the matrix to five parallel UTLC plates. The relative standard deviations were about 22–25% (III, Table 2) suggesting that the method is more suitable for semi-quantitative analysis than for analyses in which high quantitative accuracy is required. Nevertheless, accurate quantitative results could be obtained with UV densitometry. Quantitative accuracy in MALDI measurements can be also improved by using the internal standard calibration method [96, 137, 138].

Table 10. Limits of detection (S/N = 3) with A) UTLC/HPTLC–AP–MALDI–MS and UTLC/HPTLC vacuum MALDI–MS (III) and B) UTLC–AP–MALDI–MS and UTLC–AP–MALDI–MS/MS (IV).

A

Compound	AP-MALDI-MS		Vacuum MALDI-MS	
	non-eluted (pmol)	eluted (pmol)	non-eluted (pmol)	eluted (pmol)
Triazole 1				
UTLC	4	12.5	10	33
HPTLC	280	500	-	
Triazole 2				
UTLC	85	100	90	
HPTLC	750	2140	-	
Triazole 3				
UTLC	30	300	16	
HPTLC	500	750	-	
Triazole 4				
UTLC	100	400	90	
HPTLC	6700	>10000	-	
Midazolam				
UTLC	0.5	4.8	4	5
HPTLC	30	300	-	
Verapamil				
UTLC	0.5	1.3	3	
HPTLC	22	300	-	
Metoprolol				
UTLC	4	6.4	4	
HPTLC	31	600	-	

B

	AP-MALDI-MS (pmol)		AP-MALDI-MS/MS (pmol)	
Compound	**Standard**	**Urine spiked**	**Standard**	**Urine spiked**
Midazolam	4.6	8.6	0.2	0.4
Diazepam	11	7.9	1.3	1.4
Lorazepam	22	52	6.4	6.4
Oxazepam	67	97	2.0	1.3
N-Desalkyl-flurazepam	8.3	91	7.9	84
Triazolam	2.0	9.7	6.0	1.7
Nitrazepam	7.5	97	19	21

MALDI–MS applied to fast identification of analytes separated by HPTLC and UTLC
As shown above, MALDI–MS is a fast and relatively simple in situ technique for use with TLC. This section describes two applications of MALDI–MS in this work. In both, MALDI–MS was applied for the identification of target compounds in crude synthesis mixtures (studies II and III). HPTLC or UTLC plates were used for the separation and AP–MALDI–MS for detection. Additionally, UTLC–AP–MALDI–MS was applied to the identification of benzodiazepines in urine (IV). This application is described in section 5.3.

The aim of study II was to determinate the suitability of PLC for the purification of the target compounds from the crude synthesis products for bioactivity and preliminary ADME tests. Analytical HPTLC plates were used to optimize the mobile phase for establishment of the PLC method, as usual. After HPTLC separation, the plate was scanned with a UV densitometer. Since the samples were crude products, several impurity compounds were seen in the densitogram (Figure 14A). To determine the zone of the target product, the identification was carried out in situ on the HPTLC plate, with AP–MALDI–MS, after the addition of matrix to the zones of the compounds indicated by the UV densitometer. Because application of the matrix to one zone of the plate required only 30 s, plates could be rapidly prepared for MALDI–MS analysis, and analysis of the desired zones could be done quickly. All measured AP–MALDI mass spectra exhibited an abundant protonated molecule and sodium adduct ion with no fragment ions (Figure 14B), allowing adequate identification of the target compounds. Once the zone of the target product on the plate had been confirmed on the basis of the MALDI–MS identification, the assessment of the semi-quantitative purity, as a percentage, could be calculated from the UV densitometry data. And finally, exactly the right zone could be isolated from the eluted PLC plate.

The situation in the application of study III was nearly the same as that in study II; the sample was a synthesis sample (target compound: triazole 5, mw 175.2), and assessment of the purity of the target compound in the crude product was desired. A UTLC plate was used for the separation. The UV densitogram of the eluted plate (Figure 15A) showed two distinct peaks with R_F values of 0.10 (A) and 0.46 (B). The AP–MALDI–MS spectrum of peak B (Figure 15D) revealed an abundant protonated molecule of triazole 5 (m/z 176), which in MS/MS analysis produced the product ion $[CH_2C_6H_4OH]^+$ (m/z 107) (Figure 15E). This confirmed that the product was triazole 5. The ion m/z 107 was a common fragment ion for triazoles containing the phenolic functionality. The MS spectrum of the by-product (peak A, Fig. 15B) showed an extraordinary ion at m/z 369, which did not appear in the spectrum of the matrix. The product ion spectrum of ion m/z 369 (Figure 15C) showed an ion at m/z 107, which was also recognized in the product ion spectrum of triazole 5. This suggests that peak A represents a synthesis by-product. After identification of the zone of the target compound with AP–MALDI–MS, the purity percentage of triazole 5 was calculated on the basis of the UV densitometry data.

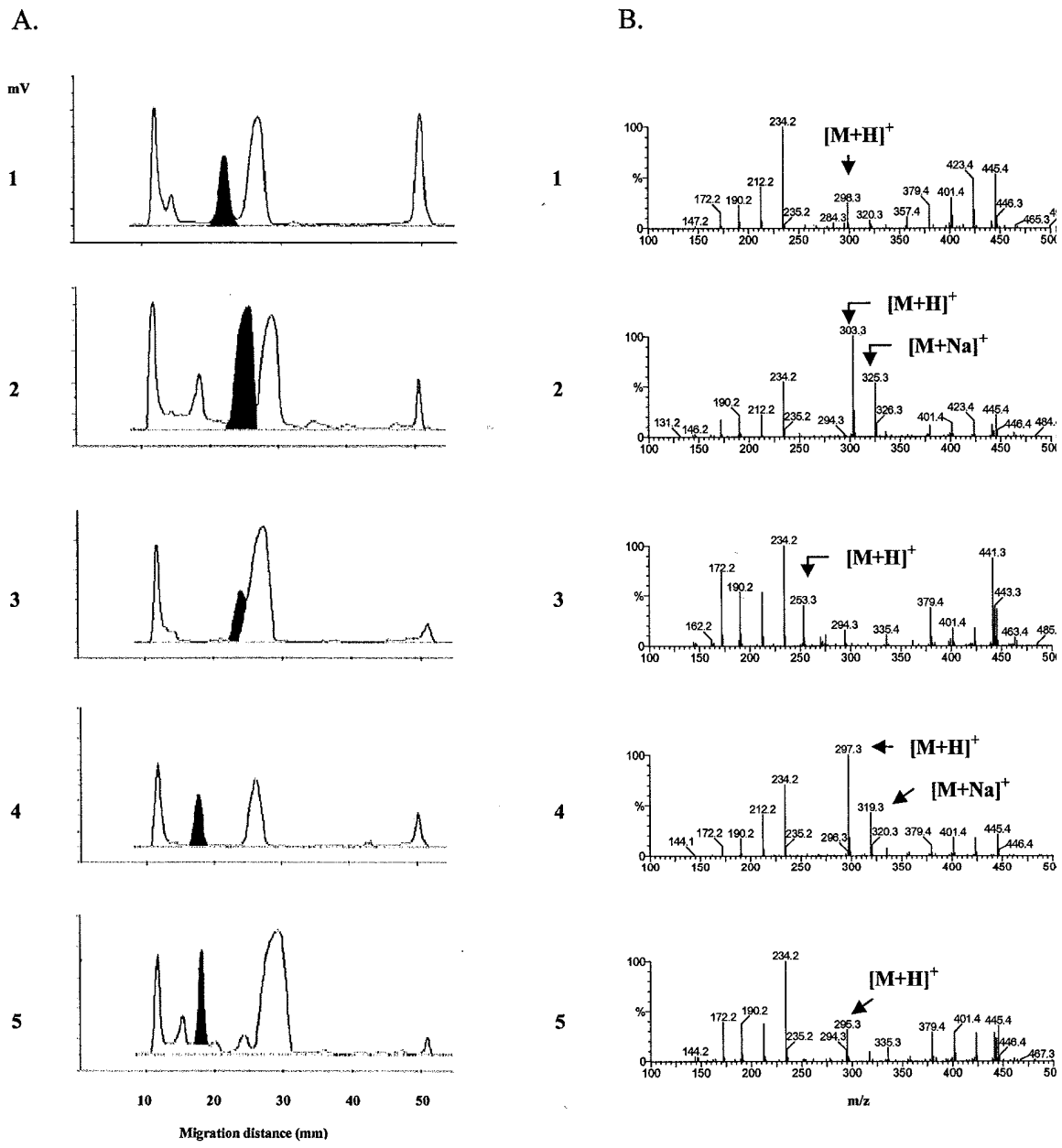


Figure 14. A. UV densitograms of crude synthesis products (1–5) measured in situ on the HPTLC plate. The target compounds are marked in black. B. AP–MALDI–MS spectra of the target compounds (marked in black in Fig. 14A) measured directly from the eluted HPTLC plate. The protonated molecules and sodium adduct ions are indicated with arrows. Other ions in the spectra were background ions of the α-CHCA matrix. The amount of the matrix on plate was 100 nmol.

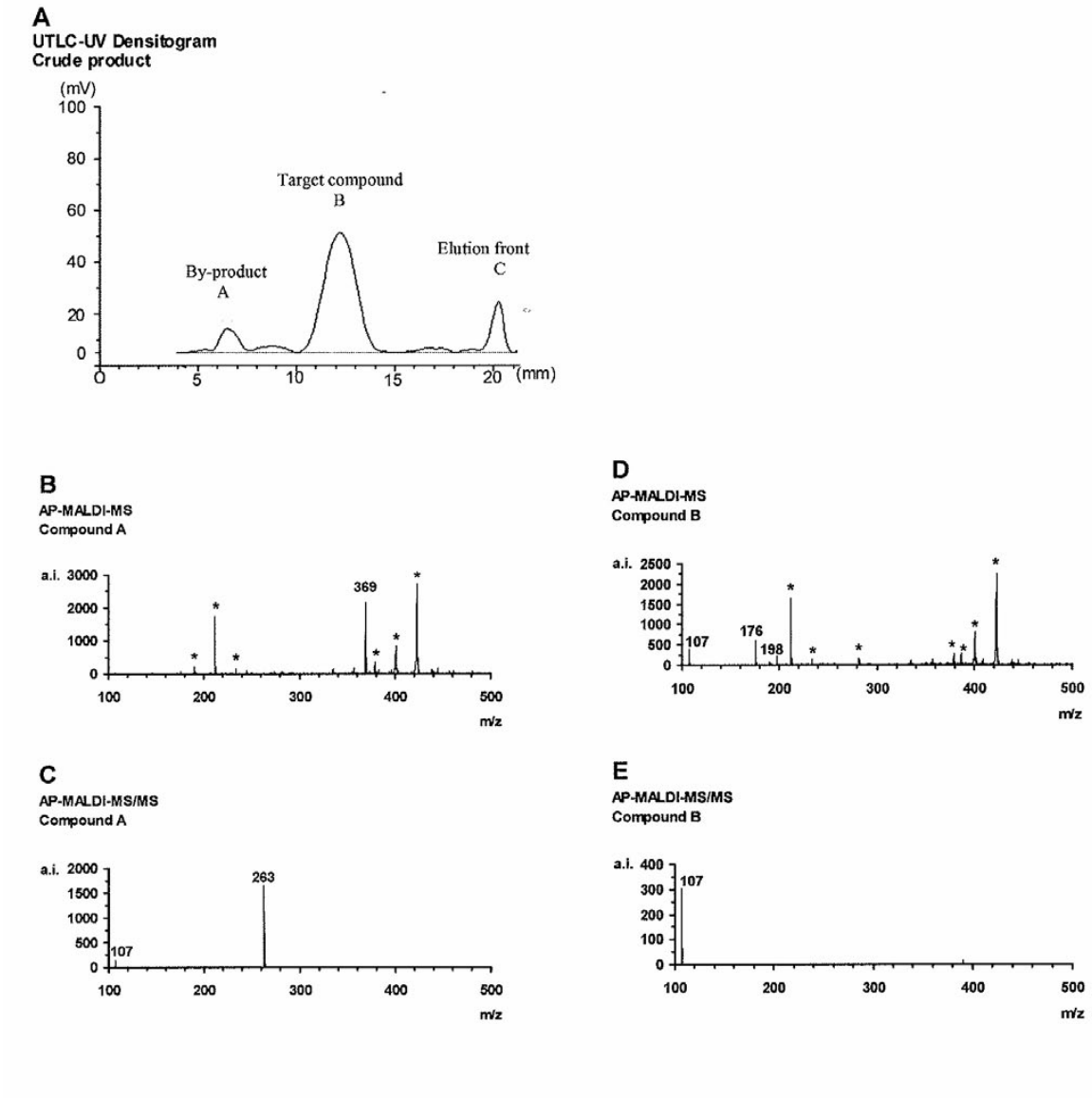


Figure 15. Identification of target compound (triazole 5) and a by-product in the crude synthesis product. ***(A)*** *UTLC–UV densitogram of the crude synthesis product and* ***(B–E)*** *AP–MALDI–MS spectra of the separated compounds:* ***(B)*** *MS spectrum of compound A (by-product),* ***(C)*** *MS/MS spectrum of ion m/z 369 of compound A,* ***(D)*** *MS spectrum of compound B (target product; m/z 176, m/z 198, and m/z 107 are* $[M + H]^+$, $[M + Na]^+$, *and fragment ion* $[CH_2C_6H_4OH]^+$ *of compound B, respectively)* ***(E)*** *MS/MS spectrum of ion m/z 176 of compound B.*

5.3. Two-dimensional (2D) UTLC–UV/VIS and UTLC–AP–MALDI–MS system (IV)

The properties of the UTLC plates (see Table 5) set limits on the separation efficiency with UTLC. In general, the separation efficiency in TLC can be increased by applying two-dimensional separation, but the localization of the analytes on the plate by UV densitometric scanning is then more complicated, especially if the analyte concentration is low. The methodology associated with two-dimensional separation and detection using UTLC plates is presented and discussed in this section.

5.3.1 Separation and UV/VIS detection

Six reference standards of benzodiazepines were used in the development of the 2D UTLC method. The commercial UTLC plates lack a fluorescent indicator, and to obtain adequate data to specify the exact location of the benzodiazepines on the eluted plate, the feasibility of visual and UV densitometric detections was tested. With the Dragendorff reagent [162] used for dyeing, the benzodiazepines could be rapidly visualized as derivatives down to 100 and 500 pmol (i.e. 30–150 ng). While this level is adequate for method development, it is too high for bioanalysis. The feasibility of UTLC–UV densitometry was then tested. The whole eluted area (20 x 20 mm) with equidistant spacing of 1 mm was scanned. The UV densitogram of the first dimension showed three zones: 1, 2, and 3 in Figure 16 A-B. After 2D separation, all six standard compounds were separated (Figure 16 A and C-F). Zone 1 was separated into two zones (1.1 and 1.2) and zone 2 into three zones (2.1, 2.2, and 2.3). Note that the zones 2.1 and 2.3 were actually already separated in 1D separation, but zone 2.2 was situated between them and only one broad peak (peak 2) was seen after the 1D separation (Figure 16B). After 2D elution the zone 2.2 was separated from the zones 2.1 and 2.3 and the 1D separation of 2.1 and 2.3 became visible even though their R_F values in the 2D separation were the same. Zone 3 included only one compound (3). The UV densitometer provided lower limits of detection (about 30–100 pmol, Table 1 in study IV) and higher specificity than did the Dragendorff reagent. However, localization of the separated zones after 2D separation requires time-consuming (about 5–10 minutes) scanning of the whole eluted area of the UTLC plate, and the interpretation of the data, as well.

The repeatability of the separation by 2D UTLC was determined with the reference standard samples and an SPE-purified urine samples spiked with benzodiazepines. Urine had only a minimal downward effect on the migration: the distances were 6.7–20.5 mm for the reference standards and 6.2–19.1 mm for the SPE-purified urine samples (Table 11). Standard deviations of all samples were between 0.2 and 1.4 mm, and the relative standard deviations between 1.2 and 11.7%, indicating good repeatability for the separation (Table 11).

In conclusion, although the 2D UTLC separation with UV densitometric detection provides LODs down to pmol range, the specificity, and in many cases the sensitivity, is not sufficient for trace analysis of benzodiazepines in biological samples. As a next step, therefore, the feasibility of AP–MALDI–MS for the detection of benzodiazepines on the UTLC plate was studied. Since the 2D UTLC separation is repeatable, it is sufficient to determine the positions of the zones by dyeing or UV densitometry only once. After that, the AP–MALDI–MS analysis can be done merely by adding the matrix and focusing the laser to the correct place or zone on the plate.

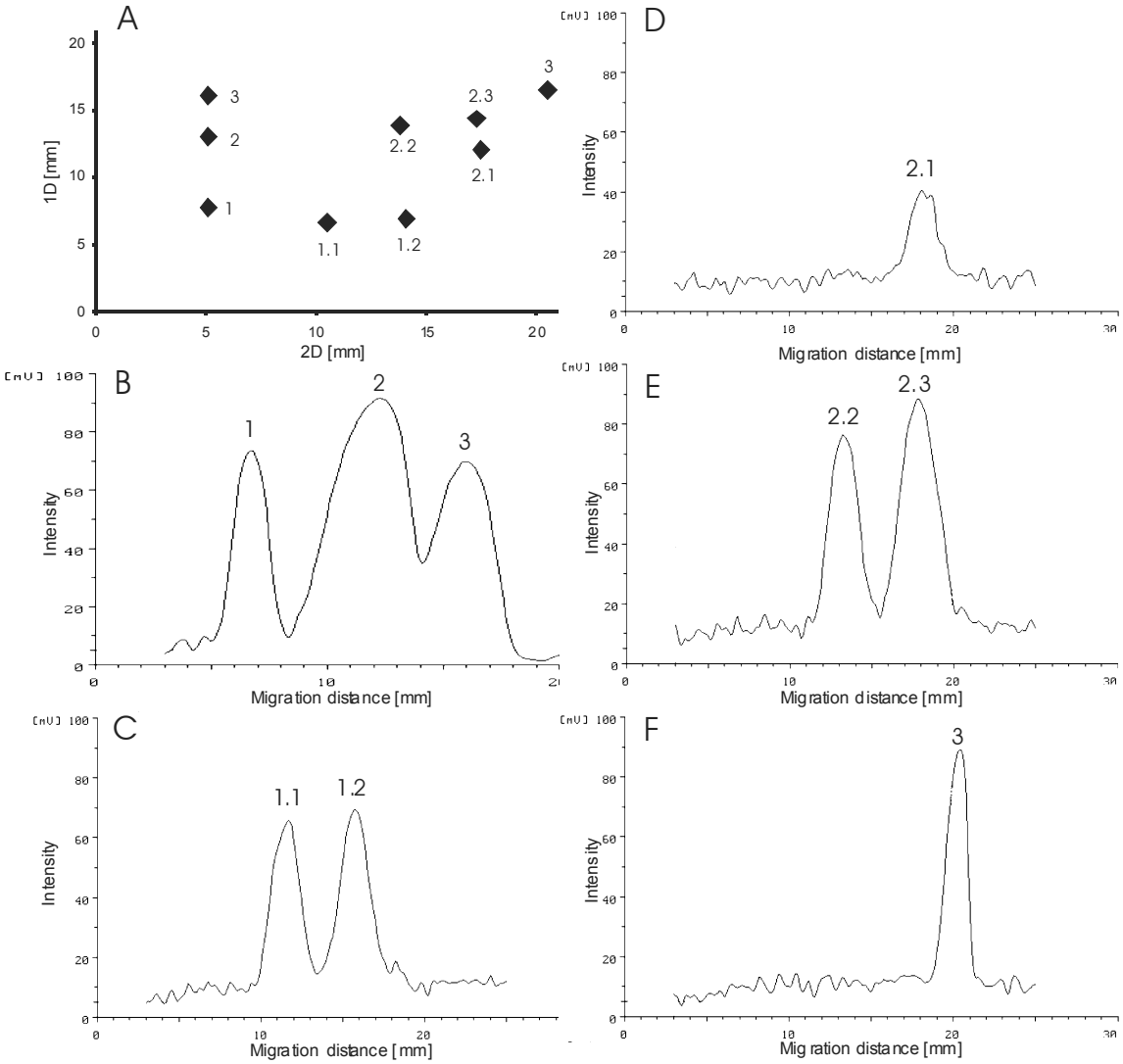


Figure 16. A) Illustrative presentation of benzodiazepine zones on a UTLC plate after 1D and 2D separations. B–F) UV densitograms of separated benzodiazepines measured from a UTLC plate after 1D separation (B) and 2D separation (C–F).

Table 11. Repeatabilities of migration distances (mm) of benzodiazepine standards and of benzodiazepines spiked in urine (n = 5) after 1D and 2D separations, as mean, standard deviation (SD), and relative standard deviation (RSD %). The y- and x-axes represent the migration distances on the UTLC plate after 1D separation (y-axis) and after 2D separation (x-axis).

Reference standards

	diazepam	nitrazepam	oxazepam	N-desalkyl-flurazepam	midazolam	triazolam
y-axis (in mm)						
mean	16.6	14.4	13.9	12.1	6.9	6.7
SD	0.4	1.0	1.0	1.2	0.4	0.2
RSD %	2.3	6.7	7.3	9.9	5.4	3.7
x-axis (in mm)						
mean	20.5	17.3	13.8	17.5	14.1	10.5
SD	1.3	0.9	0.7	0.8	1.4	0.7
RSD %	6.2	5.4	4.9	4.8	9.7	7.2

Spiked in urine

	diazepam	nitrazepam	oxazepam	N-desalkyl-flurazepam	midazolam	triazolam
y-axis (in mm)						
mean	16.1	13.6	13.0	11.6	6.3	6.2
SD	0.2	1.0	0.8	1.1	0.4	0.4
RSD %	1.2	7.1	6.0	9.2	6.3	6.5
x-axis (in mm)						
mean	19.1	16.5	13.7	16.4	12.5	9.0
SD	0.9	1.0	1.0	0.9	1.3	1.0
RSD %	4.8	6.4	7.5	5.2	10.4	11.7

5.3.2 AP–MALDI–MS detection

AP–MALDI–MS and AP–MALDI–MS/MS spectra were measured for six SPE-purified urine samples spiked with benzodiazepines (300 pmol) and separated by 2D UTLC. The MS spectra (Figure 17) showed more or less the same ions as those measured for the pure reference samples (Table 9), but sodium adducts of the analytes were more abundant in the spiked urine samples despite the SPE purification. Likewise, the MS/MS spectra showed the same product ions in the urine and reference standard samples, indicating that endogenous compounds in urine do not significantly disturb the analysis in MS/MS mode. This finding was further investigated by measuring the LODs (presented in section 5.2.3 and Table 10). The LODs for benzodiazepines showed that the endogenous compounds in urine do in fact disturb the analysis in MS mode: the LODs were 2–10 times higher in urine. But they do not suppress the ionization since the LODs in MS/MS mode were mostly at the same level for benzodiazepines in pure solvent and in SPE-purified urine.

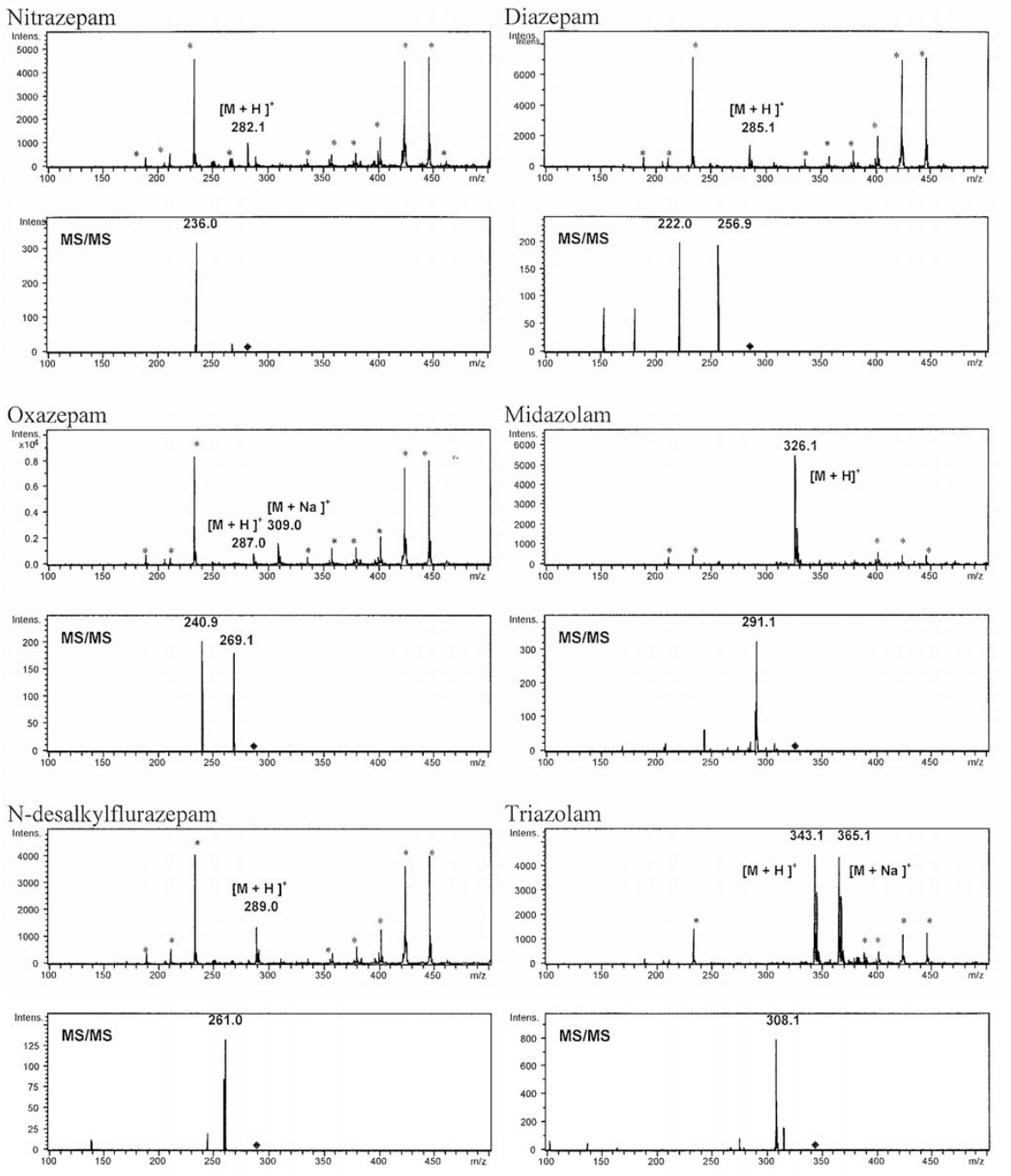


Figure 17. MS and MS/MS (of M + H^+) spectra of benzodiazepines (spiked in urine) recorded from a 2D eluted UTLC plate. The main matrix ions are marked with an asterisk (*).

5.3.3 Screening of authentic urine sample

The 2D UTLC–AP–MALDI–MS method was applied in the screening of an authentic urine sample obtained after intake of a single dose of 10 mg of diazepam (Diapam®). Since diazepam has a rather long (about 20–100 hours) half-life, the urine was collected 53 hours after the intake. Diazepam is mainly metabolized to N-desmethyldiazepam (MW=270), temazepam (MW=300), and oxazepam (MW=286), and their glucuronides. The metabolites, too, have long half-lives: temazepam and oxazepam about 15–20 hours, and N-desmethyldiazepam as much as 30–200 hours. First, 20 µl of enzymatic hydrolyzed and SPE-purified authentic urine sample was applied to the UTLC plate. After 2D separation, MALDI matrix was added to the plate at the known positions of the eluted zones. Because the 2D UTLC separation was highly repeatable and the width of the eluted benzodiazepine zones on the plate was about 2–4 mm (in x and y dimensions), and wider than the standard variations of the migration distances (0.2–1.4 mm), the addition of the matrix and focusing of the laser to the right locations in the AP–MALDI–MS analysis could be done without prior detection of the zones by dyeing or UV densitometry. Two possible metabolites of diazepam were detected in the authentic urine sample by 2D UTLC–AP–MALDI–MS. The MS spectra in Figure 18 show ions at m/z 323 (Fig. 18A) and 271 (Fig. 18B) along with the isotope peak of chlorine. Neither ion is seen in the MS spectra of blank urine. The ions correspond to the sodium adduct of temazepam and the protonated molecule of N-desmethyldiazepam, respectively. These are the expected findings after intake of diazepam.

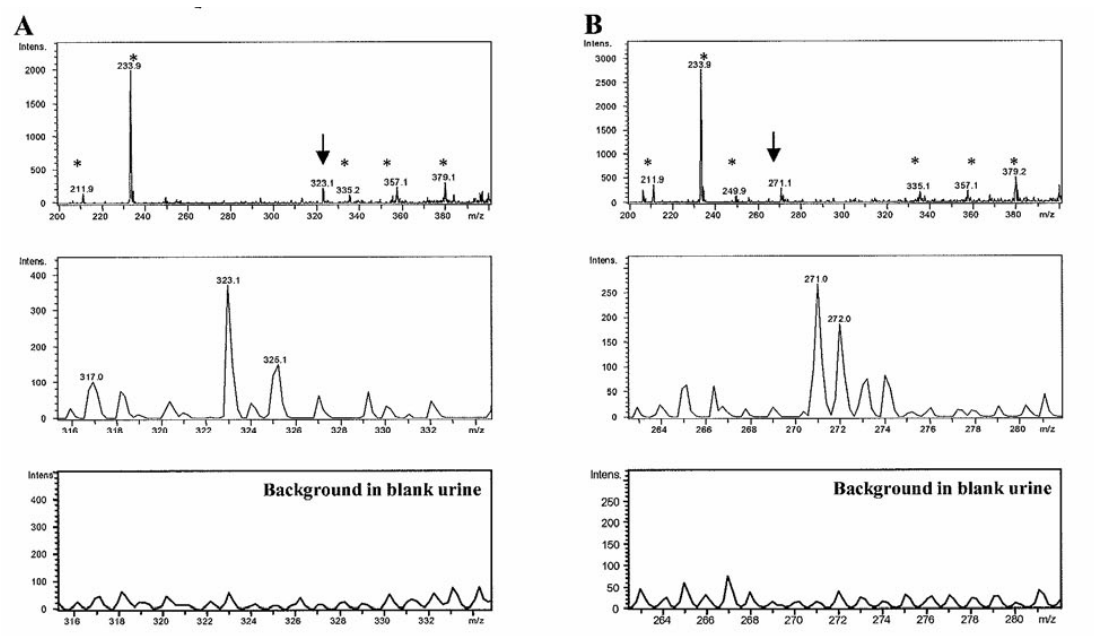


Figure 18. Findings from an authentic urine sample after intake of diazepam. MS spectra were measured from a 2D-eluted UTLC plate by AP–MALDI–MS. A) Sodium adduct of temazepam (marked with arrow), and the magnification of the chlorine isotope pattern of the ion at m/z 323, and the background in blank urine. B) Protonated molecule of N-desmethyldiazepam (marked with arrow), and the magnification of the chlorine isotope pattern of the ion at m/z 271 and the background in blank urine.

6. SUMMARY AND CONCLUSIONS

Summary of studies I–V

Modern HPTLC combined with UV densitometric detection was shown to be an easy and low-cost method for assessment of the quality of a small and focused combinatorial library obtained by solid- and liquid-phase syntheses (I, II). HPTLC offered rapid screening of the quality of the libraries by visual detection under a UV lamp. More detailed information was easily obtained by HPTLC densitograms and in situ UV spectra. Although the target compounds could in many cases be identified by in situ acquisition of UV spectra, the specificity of UV detection is limited and identification is more reliably achieved by MS. For MS identification of the target compounds on the plate, a "cleave and analyze" method with FIA–ESI–MS and in situ MALDI–MS was used. For the "cleave and analyze" step, a new method was developed to scrape and transfer the HPTLC sample zone for MS analysis. This scraping method is simple and low-cost and requires only 2–3 minutes per sample. ESI offered very soft ionization, and the spectrum showed only an abundant protonated molecule with minimal fragmentation. AP–MALDI–MS, instead, enabled the identification of products on the plate in situ, with no need for scraping the plate. The AP–MALDI–MS analysis was thus faster and easier to perform. AP–MALDI–MS produced abundant protonated molecules and sodium adducts with minimal fragmentation. The matrix background, which may disturb the analysis, could be minimized by reducing the amount of the matrix sprayed on the separated zones.

The performance of HPTLC was also compared with HPLC, which is a dominant technique in purity assessment (I, II). In a combinatorial library containing 19 samples, the percentage purities of the target compounds measured by HPTLC and LC–MS (r^2 = 0.8404) and by HPTLC and LC–UV (r^2 = 0.8053) were in good agreement. Relatively, the purities of five crude isoflavone products determined by HPTLC correlated well with purities determined by HPLC (r^2 = 0.8795). The results obtained by HPTLC indicate that continuous use of the LC–MS apparatus is unnecessary for such semi-quantitative purity analysis as demonstrated in this work. While LC–MS offers valuable structural information, which is impossible to obtain by HPTLC–UV, our sensitive, low-cost, rapid, and simple HPTLC method offers a powerful tool for purity analysis.

Besides HPTLC, preparative-layer chromatography was applied for purification of the target compounds from crude synthesis samples (II). PLC was shown to be an easy and efficient method for the purification of the synthesized products in sub-milligram amounts. PLC with 20 x 20 cm plates enabled simultaneous purification of six samples. A new isolation device, which integrated scraping, filtering, and extraction, enabled rapid and easy isolation of the target compounds from the PLC plate and facilitated purification.

A novel UTLC–AP–MALDI–MS method was introduced for the analysis of small drug molecules (III). The UTLC method was compared with the HPTLC method with UV and AP–MALDI–MS detection, and also AP–MALDI–ion trap–MS was compared with vacuum MALDI–time-of-flight–MS. The advantages of UTLC over HPTLC included faster separations and reduced solvent consumption. The use of MS provided enhanced specificity

over UV detection, and significantly, as much as 10–100 times improved sensitivity was achieved with UTLC–AP–MALDI–MS when compared with HPTLC–AP–MALDI–MS. The UTLC–AP–MALDI–MS method was shown to be good enough for the identification of small drug molecules in crude synthesized samples if the samples are relatively simple and pure. More complex samples require the use of MS/MS mode. Additionally, AP–MALDI–ion trap–MS provided better repeatability in mass accuracy than did vacuum MALDI–TOF–MS. Thus, the combination of UTLC–AP–MALDI–ion trap–MS provided improvement over the conventional HPTLC–vacuum MALDI–MS methods, preserving at the same time many of the advantages of the TLC.

As the resolution is more limited with UTLC than with HPTLC, two-dimensional UTLC–AP–MALDI–MS was attempted for the first time, and shown to be well suited for complex bioanalyses (IV). The method was tested in the determination of benzodiazepine spiked in urine. The relative standard deviation of the position of the zones on the UTLC plate was below 10%, indicating good repeatability of the separation. The limit of detection down to picomole range made possible the detection of benzodiazepine metabolites in an authentic urine sample. This level of sensitivity in bioanalysis can be achieved only with the use of UTLC plates; as shown in our work, the sensitivity with HPTLC plates is about 10–100 times worse. The 2D UTLC–AP–MALDI–MS combination provided an easy and rapid method for the screening of an authentic urine sample.

Finally, the combination of UTLC plates and DESI–MS was introduced (V), and the feasibility of DESI–MS was tested for the analysis of drugs directly from the UTLC plate. Comparisons with surfaces earlier used with DESI–MS, polymethyl methacrylate (PMMA) and polytetrafluoroethylene (PTFE) for example, showed that similar or improved sensitivities can be achieved with UTLC plates. UTLC can also be utilized as a chromatographic separation method before DESI–MS detection. The compatibility of UTLC with DESI–MS was excellent thanks to the thin adsorbent layer of the UTLC plates, which gives high sensitivity with DESI–MS, and also the monolithic structure of the plates. This structure is not as easily broken by the spray as is the conventional HPTLC surface, which is made up of separate silica particles. Other advantages of UTLC are the fast and uniform drying of the sample, which further enhances the sensitivity and stability of the DESI signal.

Comparison of methods and techniques

The methods and techniques explored in this work are compared in Table 12. The observations are mostly based on my own experiences during the experimental work but also on reports in the literature. The three different plates are listed in the columns, and the compared items in vertical rows. A few comments are added in parentheses.

HPTLC was the most suitable method for separation. The achievable resolution in proportion to capacity was clearly the best with HPTLC plates. Also the method development, i.e. the optimization of the mobile phase composition, was simplest for the HPTLC plate since the effect of different solvents on the separation could be rapidly observed under a UV lamp. In the case of PLC, the mobile phase composition was first optimized by using an HPTLC plate

Table 12. Comparison of methods and techniques explored in this work (I–V). The more appropriate or easier to use the method or technique, the more plus signs are assigned.

	PLC	HPTLC	UTLC
Separation			
Method development	++	+++	++
Elution time	+ (longest)	++	+++ (fastest)
Resolution	+	+++	+ (can be increased by 2D elution)
Capacity	+++	++ (up to micrograms)	+ (up to nanograms)
Detection			
UV/VIS	+++	+++	only with derivatization
UV densitometry	+++	+++	++
FIA-ESI-MS	-*	+ (scraping and extraction required)	-*
DESI-MS	-*	+ (satisfactory signals)	++
AP-MALDI ion trap-MS	-*	++	+++
vacuum MALDI-TOF-MS	-*	+	++
LODs (UV)	-*	++ (~ 80 - 800 pmol)	++ (~ 25 - 225 pmol)
LODs (MALDI-MS) (MALDI-MS/MS)	-* -*	+ (~ 300 - >10000 pmol) -*	++ (~ 1.3 - 400 pmol) +++ (~ 0.2 - 84 pmol)
LODs (DESI-MS)	-*	-*	+++ (~ 1 -100 pmol; non-eluted)
Other			
Analysis of "dirty" samples	+	+++	++
Parallel samples on one plate	~ 1- 6	~ 20-40	~ 8
Ease of use	++	+++	++

-* not applied

(as usual), and then the method was scaled up for PLC. This takes time and complicates the system. Likewise in UTLC, preliminary optimization of the mobile phase was performed using the HPTLC plate; UTLC plates lack a fluorescent indicator and the success of a separation cannot be determined under a UV lamp. The UV densitometer can be used for optimization purposes, but it is rather slow. However, UV densitometric detection was feasible for analyses on all the plates, and the scanning was fast and appropriate for achieving the information desired. The sensitivity of UTLC–UV and HPTLC–UV was good: the LODs for the compounds investigated were in picomole range. MS detection in situ, using MALDI or DESI technique, was simple to perform as detection takes place directly on plate, and the sensitivities with MALDI–MS and DESI–MS were of the same magnitude. Because DESI has been introduced most recently, however, MALDI is more common and more often applied for TLC analysis. It is worth adding that adequate information was also achieved with FIA–ESI–MS, but then pretreatment before MS analysis was needed.

UTLC plates are the method of choice with MS detection. Due to the thinness and monolithic character of the adsorbent layer, the LODs are lower with UTLC than with HPTLC plates, for both MALDI and DESI. As a minor point, the cutting of the plate to match the MALDI target plate is significantly easier for the UTLC than the thicker HPTLC plate. However, use of the HPTLC plate and MALDI–MS is well suited if the working concentration of the analyte is high enough, for example in nanomole range. In a comparison of AP–MALDI–ion trap–MS and vacuum MALDI–TOF–MS, the AP–MALDI system is more user friendly, changing the sample plates is faster since pump down is not needed, and the desorption from the irregular surface of the TLC plates provides clearly less variation in measured m/z values with external calibration mode. Finally, HPTLC is better suited than PLC or UTLC for the analysis of “dirty” and complex samples. The number of samples that can be analyzed in parallel on one plate is highest with the HPTLC plate. However, UTLC is preferred if MS analysis or working in low concentration range is required. PLC is more appropriate for purification and isolation purposes.

Final conclusions

Overall, thin-layer chromatography combined with ultraviolet and mass spectrometric detection provides good alternative to liquid chromatography–mass spectrometry. In particular, UTLC–MS has the potential to provide high sensitivity. Although the separation efficiency was lower with UTLC than with HPTLC, this disadvantage would partly be overcome if an appropriate sample applicator and detection instruments were developed for the UTLC plates. Neither is yet available. The introduction of commercial UTLC plates with fluorescent indicator would assist in the method development. Also, the introduction of UTLC plates exactly matching the dimensions of the MALDI target plate would facilitate the experimental work. In addition to the work done here with reference standards, crude synthesized products, and urine samples, UTLC and MS could be applied to many other tasks. One useful development would be the combination of 2D UTLC separation and in situ MS imaging with different ionization techniques. This combination could prove valuable in metabolomics and in the analysis of peptides in proteomics.

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