Analysis of Small Molecules by Ultra Thin-Layer Chromatography-Atmospheric Pressure Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

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The feasibility of ultra thin-layer chromatography atmospheric pressure matrix-assisted laser desorption ionization mass spectrometry (UTLC-AP-MALDI-MS) has been studied in the analysis of small molecules. Because of a thinner adsorbent layer, the monolithic UTLC plates provide 10–100 times better sensitivity in MALDI analysis than conventional high performance thin-layer chromatography (HPTLC) plates. The limits of detection down to a low picomole range are demonstrated by UTLC-AP-MALDI-MS. Other advantages of UTLC over HPTLC include faster separations and lower solvent consumption. The performances of AP-MALDI-MS and vacuum MALDI-MS have been compared in the analysis of small drug molecules directly from the UTLC plates. The desorption from the irregular surface of UTLC plates with an external AP-MALDI ion source combined with an ion trap instrument provides clearly less variation in measurements of *m/z* values when compared with a vacuum MALDI-time-of-flight (TOF) instrument. The performance of the UTLC-AP-MALDI-MS method has been applied successfully to the purity analysis of synthesis products produced by solid-phase parallel synthesis method. (J Am Soc Mass Spectrom 2005, 16, 906–915) © 2005 American Society for Mass Spectrometry

In recent years, improvements in thin-layer chromatography (TLC) instrumentation and methods as well as the introduction of the high-performance thin-layer chromatography (HPTLC) has increased the use of this simple, inexpensive, and efficient method. TLC allows simultaneous analysis of many samples on one plate, the plates are disposable, and therefore memory effects can be avoided, solvent consumption is low, and a number of nondestructive detection methods with appropriate derivatization reagents can be used in sequence. The modern HPTLC technique, combined with automated sample application and densitometric scanning, has proven to be sensitive, reliable, and suitable for the qualitative and quantitative analysis of pharmaceutical, environmental, toxicological, forensic, and food samples [1–9].

The development of miniaturized ultra-thin-layer chromatography (UTLC) [10] in addition to the development of other miniaturized analytical methods [11] is currently a hot topic in analytical chemistry. The UTLC method combined with UV or diode-array detection (DAD) provides faster elution times (1–6 min), lower solvent consumption (1–4 ml), and lower detection limits than those obtained when using the conventional TLC or HPTLC methods [12]. However, the weakness of UTLC when compared with HPTLC is reduced resolution caused by shorter elution distances and a smaller overall specific adsorption surface area [12].

Several methods, such as ultraviolet/visible (UV/

Published online April 20, 2005

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1044-0305/05/$30.00
doi:10.1016/j.jasms.2005.02.025

Received August 27, 2004
Revised February 4, 2005
Accepted February 28, 2005

VIS), fluorescence, DAD, mass spectrometry (MS), Fourier transform infrared (FTIR), and Raman spectroscopy have been applied for the in situ detection of analyte zones on a TLC plate [12–15], the most common of these being UV and fluorescence. In qualitative TLC, the identification of the compounds is based on either the color reactions of the separated sample zones or on the comparison of the R_F values of the analyte and a standard compound after visualization under a UV lamp. Quantitative TLC measurements are performed by densitometric scanning using either one or several wavelengths in absorbance or fluorescence mode. With densitometric measurements the analytes are identified by their corrected R_F values and by using UV/VIS-spectra of the analytes and standard compounds measured in situ. In those cases in which the standard compounds are not available (e.g., the screening of new natural agents or combinatorial chemistry samples), the identification of unknowns has to be performed using a specific technique, such as mass spectrometric detection.

The combination of TLC and MS has been a very active research area over the last few years [5–21]. TLC-MS has most frequently been performed as an off-line process in which the sample is scraped and extracted from the plate before MS analysis [5, 22, 23], or is analyzed as such with the use of various in situ techniques [15, 16, 19–21, 24–31], the most common of these being TLC-liquid secondary ion mass spectrometry (LSIMS), TLC-fast atom bombardment (FAB), TLC-matrix-assisted laser desorption/ionization (MALDI), and TLC-surface-assisted desorption/ionization (SALDI).

MALDI, as a simple and fast technique, has been found to be the most promising method for direct TLC-MS analysis [15, 16, 19, 24–29]. The operational parameters of TLC-MALDI-MS have been well characterized. For example, it has been observed that a lower analyte to matrix ratio for low mass molecules is needed compared to MALDI analysis of high mass molecules [26]. Different approaches on how to add the matrix have also been investigated, electrospraying the matrix on top of the separated analyte zones being one of the most promising techniques [26–29]. The use of different matrix compounds has also been compared [19, 20, 24–29]. Many of the matrices used in TLC-MALDI-MS cause interfering mass peaks at low mass numbers. However, it has been shown that the matrix background can be suppressed by using appropriate analyte-to-matrix molar ratio in MALDI measurements [23, 26, 32–35]. New potential matrices, producing low matrix background, have also been introduced [16, 20, 21, 27, 30, 31]. A disadvantage of the MALDI method has been the relatively poor repeatability in quantitative analysis. However, a recent study demonstrated good precision with an internal standard method [29] in which the internal standard was predeveloped over the plate. Furthermore, working with vacuum MALDI sources, as was done in all the TLC-MALDI papers published to date, makes the method somewhat risky since large amounts of chromatographic material are directly introduced inside the vacuum chamber of a mass spectrometer. This can be avoided by working with the recently introduced atmospheric pressure MALDI (AP-MALDI) [32, 36–38] source. Changing the sample plates is faster with AP-MALDI instruments than with vacuum MALDI instruments since pump down is not needed. Additionally, the ionization process in AP-MALDI compared with vacuum MALDI has been reported to be softer and therefore can produce more intact protonated molecule and less fragmentation than in vacuum MALDI [32].

In this study, we present a novel UTLC-AP-MALDI-MS method which was tentatively introduced for the first time in our earlier work [39]. UTLC and HPTLC methods using both the UV and AP-MALDI-MS detection, and UTLC combined with AP- and vacuum MALDI-MS, are compared in the analysis of small molecules. Commercial, pharmaceutically interesting compounds as well as heterocyclic 1,2,3-triazoles produced by solid-phase combinatorial chemistry are used in the comparison.

Experimental

Reagents

In the experiments, two types of compound were used (Figure 1). The reference standards of midazolam, verapamil (as hydrochloride salt) and metoprolol (as tartrate salt) were obtained from Roche (Basel Switzerland), Sigma-Aldrich (Steinheim, Germany), and ICN Biomedicals (Aurora, OH), respectively. Five other compounds, which all are 1,2,3-triazoles (Figure 1), were selected from a combinatorial library synthesized in our laboratory by the solid-phase method described by Harju et al. [40]. α-Cyano-4-hydroxycinnamicëacid (α-CHCA), used as a matrix compound for MALDI-MS analysis, was purchased from Fluka Chemie (Buchs, Switzerland). All organic solvents were of analytical or chromatographic grade. Ethyl acetate was purchased from Merck (Darmstadt, Germany), acetonitrile and dichloromethane from Rathburn (Walkerburn, Scotland), and *n*-hexane and methanol from J. T. Baker (Deventer, Holland). Trifluoroacetic acid, acetic acid, and 25% ammonia solution were from Acros Organics (Geel, Belgium), Rathburn (Walkerburn, Scotland), and Riedel-de Haën (Seelze, Germany), respectively.

Sample and Matrix Solutions

All stock solutions of compounds were prepared by dissolving a compound into a concentration of 1 mg/ml, triazole 1 and 2 with dichloromethane/methanol (50:50 vol/vol), triazole 3 and 4, and metoprolol with methanol, and midazolam and verapamil with acetonitrile. The working solutions of the compounds were prepared by diluting a stock solution with the same solvent used to prepare the stock solution. The stock

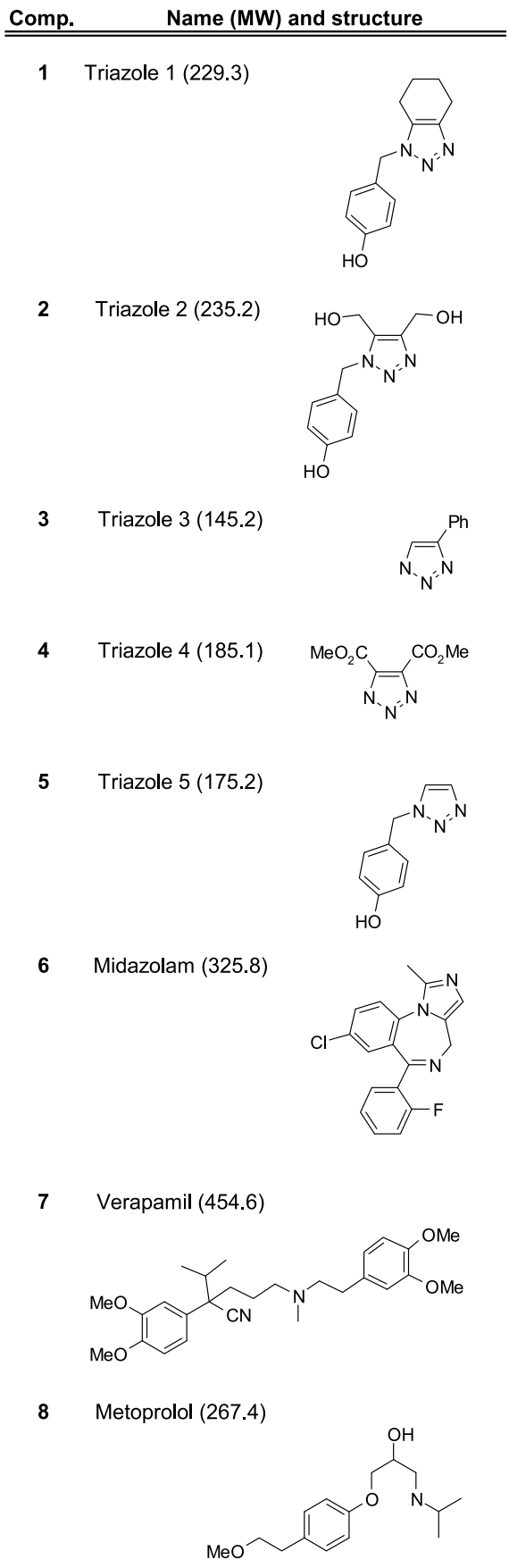


Figure 1. Structures and molecular weights (average masses) of the compounds used.

solution of the matrix (13.3 mg/ml) was prepared using the following method: 20 mg of α-CHCA was diluted with 2 parts of acetonitrile and 1 part of methanol containing 0.1% trifluoroacetic acid. The working solutions of the matrix were prepared by diluting the stock solution with acetonitrile.

UTLC and HPTLC Method

For planar chromatography, silica gel 60 F_{254} HPTLC plates (glass support) of 10 × 10 cm (Merck) and monolithic UTLC plates (glass support) of 3.6 × 6 cm (Merck) were used. The plates were prewashed once with acetonitrile before sample application. Sample solutions were sprayed as a thin rectangular band onto the adsorbent in amounts of 1 or 10 μl with a Linomat IV (Camag, Muttenz, Switzerland) at a flow rate of 4μl/min as 3-mm-long bands with 4 mm spaces. The total amount of samples on the plate was between 1 pmol and 10 nmol.

The mobile phase composition was optimized with the help of the PRISMA model [41–45]. Ethyl acetate-*n*-hexane (1:2 vol/vol) containing 2% acetic acid was used as the final mobile phase for triazoles, and ethyl acetate containing 0.5% ammonium hydroxide for drugs. The plates were eluted in a saturated chamber to the distance of 2 cm for UTLC and 5 cm for HPTLC. The elution time was 2–4 min for UTLC and 5–8 min for HPTLC. After elution, HPTLC plates were first detected visually under a UV lamp (Desaga, Heidelberg, Germany) and finally with a Camag TLC Scanner II (Muttenz, Switzerland) controlled by the CATS 3.17 program at λ = 222 nm for drugs and 228 nm for triazoles (D_2 lamp). The UTLC plates could only be detected using a TLC Scanner II owing to the lack of a fluorescent indicator. The densitometric measurements were performed in absorption and reflection modes. In situ UV spectra of the compounds were measured at wavelength range of 190–450 nm.

MALDI Instrumentation

The AP-MALDI mass spectrometry system consisted of an AP-MALDI ion source (Agilent Technologies, Germany) combined with an Esquire 3000plus ion trap instrument (Bruker Daltonics, Bremen, Germany). The AP-MALDI interface has been described in detail earlier by Doroshenko et al. [32]. After adding of the matrix, UTLC and HPTLC plates were attached to the face of an in-house-modified AP-MALDI target plate with double-sided conductive tape after cutting the plate to match the target plate. A nitrogen laser at 337 nm (10 Hz) was focused on the sample zone on a plate, the size of the laser spot being 0.5 mm [32]. The laser pulse energy was adjusted with an attenuator to 8.5 (arbitrary unit) providing an estimated pulse energy 264 μJ from the laser. The ions formed in the laser pulses were directed to the ion trap via extended capillary of the ion trap instrument. A potential of 2200 V was applied to

Table 1. R_F values and plate heights (H) of the compounds studied by the UTLC and HPTLC methods with UV-densitometry

		R_F		H (μm)	
Comp.	Name	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

the capillary. Nitrogen was used as a drying gas with a flow rate of 6 L/min and a temperature of 150 °C. The ion trap parameters were as follows: the accumulation time, 200 ms, the "averages" were set at 10, and the "rolling averaging" was "off." The voltages of the skimmer and capillary exit were 34 and 160 V, respectively. The mass spectra were recorded in the range of *m/z* 100–500. For MS/MS measurements, the cut-off value was set to *m/z* 100 and the fragmentation amplitude to 2.0. Other parameters were the same as in the MS mode. The instrument was calibrated using external calibration method and calibration mixtures provided by the instrument manufacturer. The resolution was calculated to be about 950 within the measured mass range.

Vacuum MALDI measurements were performed using a Bruker Autoflex MALDI-time-of-flight (TOF) instrument (Bruker Daltonics) operating with a nitrogen laser at 337 nm (5 Hz). The size of the laser spot was approximately 100–150 μm (private communication, Bruker Daltonics). After adding of the matrix, UTLC plates were attached to the face of an in-house-modified MALDI target plate with double-sided conductive tape after cutting the plate to match the target plate. MS instrument was operated in positive ion mode with the applied acceleration voltage of 20 kV. Attenuation value related to laser power in one pulse were 47% for drugs and 50% for triazoles. Averages of 50 pulses were recorded for MS spectra. The calibration was done by using $[M + H]^+$ (*m/z* 190) and $[M + Na]^+$ (*m/z* 212) ions of the matrix as internal calibration points. The resolution was calculated to be about 1200 within the measured mass range.

Results and Discussion

Mobile Phase and UV-Detection

The preoptimization of the mobile phase with UTLC plates was carried out with the help of PRISMA model [41–45] using UV densitometric detection. The optimal solvent composition for triazoles (Compounds 1–5) was ethyl acetate-*n*-hexane (1:2) containing 2% acetic acid, and for the drug substances (Compounds 6–8) ethyl acetate containing 0.5% ammonium hydroxide. With these eluents, the R_F values of the compounds studied were between 0.1 and 0.97 with UTLC and between 0.03 and 0.54 with HPTLC plates, (Table 1) providing good separation efficiency. The R_F values obtained by UTLC are higher because the total surface area is smaller, i.e., the adsorbent layer is thinner and the specific surface area is smaller in UTLC (10 μm and about 350 m^2/g) than in HPTLC plates (0.2 mm and about 500 m^2/g) [12]. Furthermore, the plate heights (H) were in most of the cases higher with UTLC plates than with HPTLC plates (Table 1). The elution time was about two times shorter with UTLC (2–4 min) than with HPTLC (5–8 min) and the solvent consumption in the elution of one UTLC plate was 3 ml, which was about three times less than with HPTLC. All these UTLC results are parallel to the results reported earlier by Hauck and Schulze [12].

The relative standard deviations (RSD) of the R_F values were between 1.7 and 3.1% (Table 2) indicating good repeatability of the separation with UTLC. The quantitative repeatability of the UTLC-UV measured as peak heights or areas were acceptable, RSDs being below 9% (Table 2). The limits of detection (LODs) measured using a UV densitometer (S/N = 3) were about 1–10 times lower with UTLC than HPTLC for most of the compounds studied (Table 3). Although

Table 2. Repeatability of UTLC-UV and UTLC-AP-MALDI-MS methods (n = 5) as mean, standard deviation (±SD), and relative standard deviation (RSD %). Sample amount on plate was 0.1 nmol and matrix amount was 10 nmol

	UV			AP-MALDI-MS
Compound	R_F	Peak area	Peak height	Abs. abund. of $[M + H]^+$
Triazole 1				
Mean ± SD	0.29 ± 0.005	185.3 ± 15.3	11.7 ± 0.4	540 ± 136
RSD %	1.7	8.3	3.8	25.1
Midazolam				
Mean ± SD	0.53 ± 0.01	480.0 ± 29.2	22.6 ± 0.8	1830 ± 399
RSD %	1.9	6.0	3.7	21.8
Metoprolol				
Mean ± SD	0.16 ± 0.005	110.4 ± 6.9	7.4 ± 0.4	975 ± 216
RSD %	3.1	6.2	5.7	22.1

Table 3. Limits of detection (LODs) of UTLC/HPTLC-UV and UTLC/HPTLC-AP- and vacuum MALDI-MS (S/N = 3)

	Method					
Compound	UV non-eluted (pmol)	UV eluted (pmol)	AP-MALDI-MS non-eluted (pmol)	AP-MALDI-MS eluted (pmol)	Vacuum MALDI-MS non-eluted (pmol)	Vacuum MALDI-MS eluted (pmol)
Triazole 1						
UTLC	38	69	4	12.5	10	33
HPTLC	23	79	280	500	—	
Triazole 2						
UTLC	88	154	85	100	90	
HPTLC	25	75	750	2140	—	
Triazole 3						
UTLC	33	84	30	300	16	
HPTLC	68	539	500	750	—	
Triazole 4						
UTLC	42	79	100	400	90	
HPTLC	266	819	6700	>10000	—	
Midazolam						
UTLC	1	25	0.5	4.8	4	5
HPTLC	4	326	30	300	—	
Verapamil						
UTLC	7	66	0.5	1.3	3	
HPTLC	9	622	22	300	—	
Metoprolol						
UTLC	49	54	4	6.4	4	
HPTLC	25	345	31	600	—	

UTLC-UV provides a fast and repeatable analysis method, the specificity of the method is not good enough for detailed structural characterization of the compounds. Therefore, the capability of AP-MALDI-MS for the identification of compounds directly from UTLC and HPTLC plates was studied.

UTLC/HPTLC-MALDI-MS

For MALDI-MS analysis, the use of a matrix was required since the ionization efficiency of the compounds studied from the UTLC and HPTLC without the matrix was very poor. α-Cyano-4-hydroxycinnamic acid (α-CHCA) was selected to be as a matrix compound, since it provided good ionization efficiency for the compounds studied. The matrix was sprayed over the sample zone with a TLC applicator device (Linomat IV), by which the matrix could be deposited precisely in the center of the sample zone in the form of narrow bands. The spreading of the sample zone was not visually observable. The application time for the matrix onto one sample zone of the UTLC plate was only 15 s (1 μl applied with a flow rate of 4 μl/min) providing a very rapid preparation of the UTLC plates for MALDI-MS analysis. In conclusion, the Linomat spray-on technique provides the same advantages obtained by using the electrospraying technique reported by Mowthorpe et al. [26].

The matrix amount in MALDI-MS and TLC-MALDI-MS has been shown to have a significant effect on the sensitivity, repeatability, and matrix background [23, 26, 33–35, 46, 47]. The effect of the matrix amount on sensitivity and selectivity was studied by applying 1 nmol of midazolam and triazole 1 onto the UTLC and HPTLC plates. The concentration of the α-CHCA solution was varied in the optimization experiments between 190 ng/μl and 13.3 μg/μl, thus the total amount of α-CHCA on the plate varied between 1–1000 nmol. The optimal matrix amount was 10 nmol for UTLC (about 2.66 nmol/mm^2) and 100 nmol for HPTLC (about 22.2 nmol/mm^2). The lower amount of matrix reduced sensitivity and the higher amount caused increased matrix background and therefore decreased selectivity.

The effect of the dry gas (N_2) temperature on the ionization with AP-MALDI-MS was tested because it has been reported that the temperature affects the analyte-matrix dissociation process in AP-MALDI-MS, i.e., at low temperatures, formation of the analyte/matrix clusters/dimers has been observed, whereas high temperature can cause fragmentation of molecular ion of the analyte [32]. The tests were made between 100–250 °C using 1 nmol of triazole 1 on the UTLC plate. The absolute abundance of the protonated molecule doubled when the temperature was raised from 100 to 150 °C. The rise in temperature from 150 to 250 °C increased fragmentation and reduced the abundance of the protonated molecule. The temperature of 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

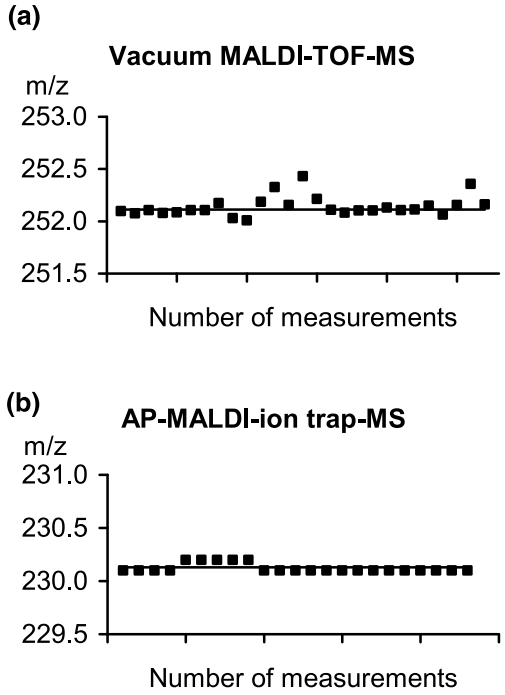


Figure 2. Replicate measurements of (**a**) triazole 1 $[M + Na]^+$ ions (m/z = 252.111) by UTLC-vacuum MALDI-MS (internal calibration mode); n = 27, and (**b**) triazole 1 $[M + H]^+$ ions (m/z = 230.129) by UTLC-AP-MALDI-MS (external calibration mode); n = 23. (filled square) = measured mass, (line) = calculated mass.

when the temperature was raised from 100 to 250 °C. The optimal temperature was 150 °C, which was selected for the further studies.

The target plate of the AP-MALDI system used in this study was maintained in a fixed position mode. With this mode the matrix disturbances were strong during the first laser pulses, but the relative abundances of the analyte ions compared with the matrix ions increased along with the number of pulses. The same observation was made with vacuum MALDI-MS. This suggests that the analyte molecules were not diffused thoroughly 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 using the UTLC plate with a matrix amount of 10 nmol, the signal lasted for about 30 s in the fixed mode. By increasing the matrix amount, the signal lasted longer and, for example, with 100 nmol the analyte ions were observed for a few min. The long-lasting signal allows sequential mass analysis including, for example, optimization of operation parameters, measurements of MS, and different kinds of MS/MS spectra in positive and negative ion mode from sample zone.

Figure 2 shows the variation of m/z values for triazole 1 measured from different sample zones on the UTLC plates by vacuum MALDI-TOF-MS (Figure 2a) using internal calibration, and by AP-MALDI-ion trap-MS (Figure 2b) using 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). These results are in accordance to the earlier studies in which it has been shown that irregular surface materials, such as polymer membranes and TLC plates, can lead to decreased mass accuracy by vacuum MALDI-TOF-MS [48,49]. However, AP-MALDI-ion trap-MS provides the coupling of UTLC without compromising in mass accuracy, taking into account that the used ion trap is not a high resolution instrument. Parallel results have been obtained by TLC-MALDI-Fourier transform (FT) MS using an external ion source [50].

Spectra

The AP- and vacuum MALDI mass spectra of the compounds studied were measured by applying 1 mol of the analyte and 10 nmol of the matrix on the UTLC plate (Table 4). The measured mass spectra produced by both sources exhibited an abundant protonated molecule and only the compounds including the hydroxy group (triazole 1 and 2, and metoprolol) produced an abundant sodium adduct ion. The spectra of triazoles 1 and 2 and verapamil also showed some fragment ions. The appearance of $[M + Na]^+$ in addition to formation of the fragment ions was somewhat stronger with vacuum MALDI-MS than with AP-

Table 4. Main analyte ions in mass spectra measured by UTLC-AP- and UTLC vacuum MALDI-MS before elution. Sample amount was 1 nmol and matrix amount 10 nmol

UTLC-AP-MALDI-MS				UTLC-Vacuum-MALDI-MS			
	m/z (rel. abund)				m/z (rel. abund)		
Comp.	$[M + H]^+$	$[M + Na]^+$	Other ions	Comp.	$[M + H]^+$	$[M + Na]^+$	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 (-)	258 (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 = $[C_4N_3O_2H_8]^+$

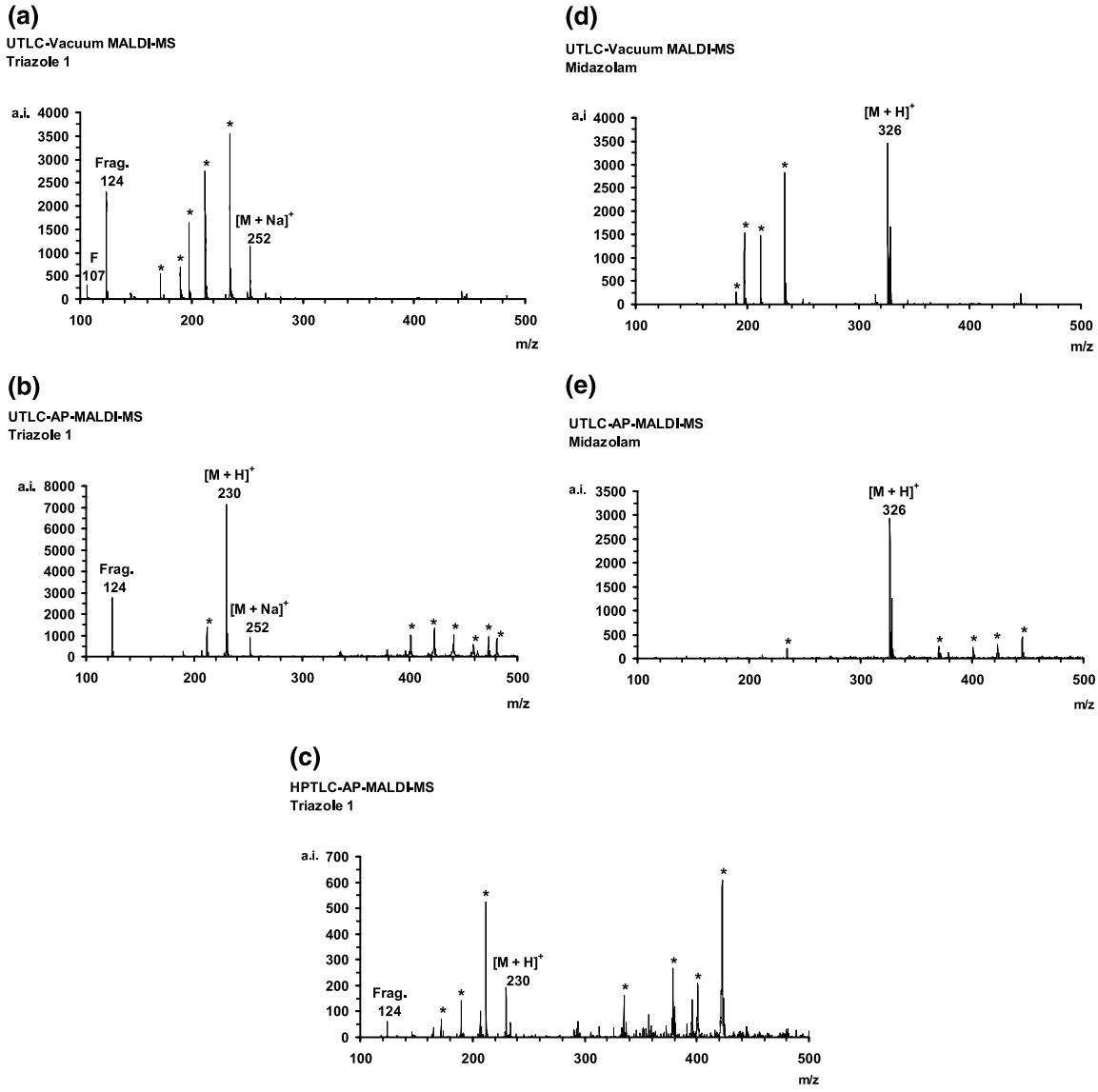
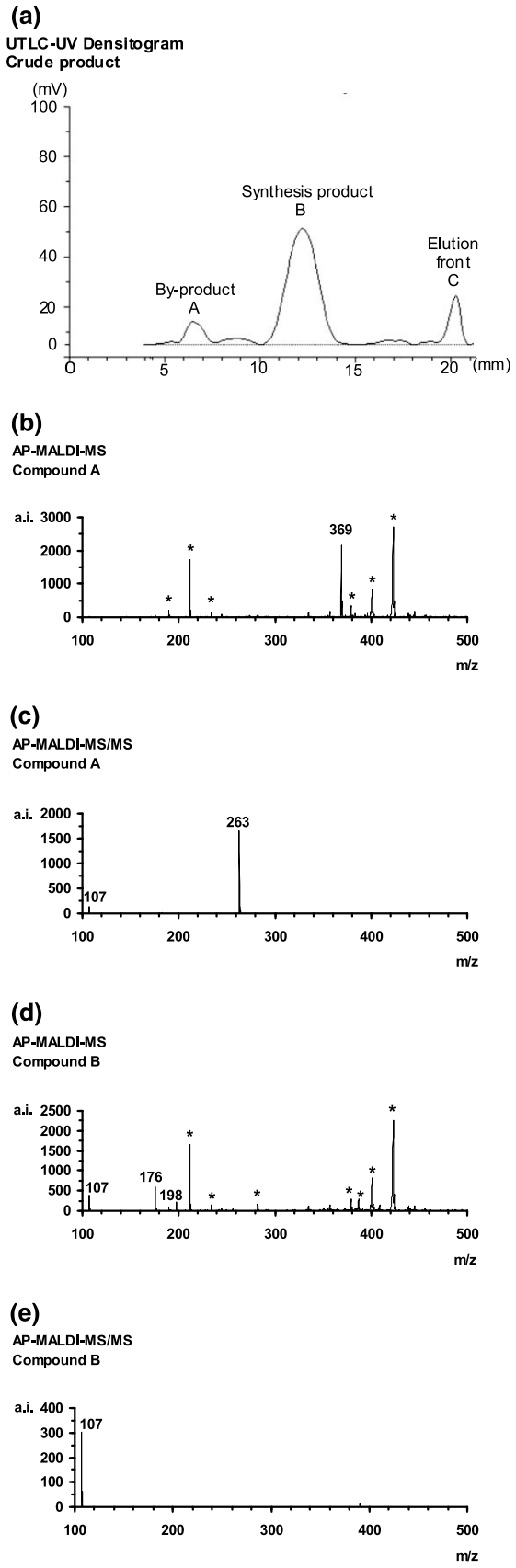


Figure 3. Mass spectra of triazole 1 (**a**–**c**) and midazolam (**d**) and (**e**) measured by UTLC-vacuum MALDI-MS (**a**) and (**d**), UTLC-AP-MALDI-MS (**b**) and (**e**), and HPTLC-AP-MALDI-MS (**c**). Sample amounts are 1 nmol and matrix (α-CHCA) amount was 10 nmol (UTLC) and 100 nmol (HPTLC). The main matrix ions are marked with an asterisk. For analyte fragment ions (F), see Table 4.

MALDI-MS, which indicates a more energetic ionization process of vacuum MALDI-MS under operational conditions used in this study since $[M + Na]^+$ ions are often more stable than $[M + H]^+$. Also, the stabilization of the protonated molecule by collisional cooling was more efficient in AP-MALDI-MS than in vacuum MALDI-MS.

Figure 3 illustrates as an example the AP- and vacuum MALDI mass spectra of triazole 1 and midazolam (1 nmol) measured from the eluted UTLC plate and AP-MALDI mass spectrum of triazole 1 (1 nmol) from the eluted HPTLC plate. In vacuum MALDI-MS, the abundant matrix background ions (marked as an asterisk) were observed below *m/z* 250. AP-MALDI mass spectra showed the same matrix ions at a mass range below *m/z* 250 but also matrix dimers, which were not observed with vacuum MALDI-MS. The dimers are rapidly stabilized by collisional cooling in AP-MALDI-MS and they can be transferred into the ion trap. The collisional cooling in vacuum MALDI-MS is significantly less than in AP-MALDI-MS leading to dissociation of the dimers in the vacuum MALDI-TOF experiments. However, all the analyte ions were visible using both methods. The matrix background is significantly lower with midazolam (Figure 3e) than with triazole 1 (Figure 3b). This might be because the physical and chemical properties, such as proton affinity, hydrophobicity, absorbtivity at 337 nm of midazolam, are more favorable for efficient ionization than those of triazole 1.



On the other hand, the extraction efficiency from the inner parts of the silica layer to the matrix during the addition of the matrix solution might be better with midazolam than with triazole 1. The comparison between UTLC (Figure 3b) and HPTLC-AP-MALDI-MS (Figure 3c) spectra of triazole 1 indicates that the matrix disturbances are less with the UTLC than with the HPTLC plate. This is because the optimal matrix amount with UTLC plates (10 nmol) is ten times less than with HPTLC plates (100 nmol).

Limit of Detection and Repeatability

The limits of detection (LODs) (Table 3) with UTLC-AP-MALDI-MS (S/N = 3) 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 500–10,000 pmol for triazoles (1–4) and 300–600 pmol for drug substances (6–8). These results show that with AP-MALDI-MS UTLC plates provide about 10–100 times better sensitivity than HPTLC plates. This holds also when the measurements were performed from the application zone (i.e., before elution). The better sensitivity with UTLC plates can be attributed to a thinner adsorbent layer of the UTLC plates. It follows that the number of molecules per surface area is significantly higher on the UTLC plate than on the HPTLC plate. Furthermore, with UTLC plates the analyte molecules are extracted from the inner parts of the adsorbent onto the surface more efficiently than with HPTLC. The laser pulse is capable of ionizing the compounds efficiently only from the surface of the adsorbent. The spreading of the zone during the elution reduced sensitivity, as the LODs measured from the application zone were about 2–10 times lower than those measured after elution. This suggests that the 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.

Quantitative repeatability of the UTLC-AP-MALDI-MS was studied on five different plates after elution by using 0.1 nmol of triazole 1, midazolam, and metoprolol, and 10 nmol of the matrix. The relative standard deviations were about 22–25% (Table 2) showing that the method is likely to be more suitable for semi-quantitative than for analysis in which high quantitative accuracy is required. Nevertheless, accurate quantitative results can be obtained using UV densitometry.

Figure 4. The identification of synthesis product (triazole 5) and by-product in crude product. (**a**) UTLC-UV densitogram of a synthesis sample 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 (*m/z* 176, *m/z* 198, and *m/z* 107 are $[M + H]^+$, $[M + Na]^+$, and fragment ion $[CH_2C_6H_4OH]^+$ of the synthesis product, respectively, (**e**) MS/MS spectrum of ion *m/z* 176 of Compound B.

Application

The UTLC-AP-MALDI-MS method was applied to the identification of the synthesis product (triazole 5, mw 175.2) and possible by-products in a crude product. The compounds were separated using ethyl acetate-*n*-hexane 1:2 containing 2% acetic acid as an eluent (Figure 4). The UV densitogram clearly shows two peaks with R_F values of 0.10 (A) and 0.46 (B) in Figure 4a. The AP-MALDI-MS spectrum of synthesis product (Peak B, Figure 4d) reveals an abundant protonated molecule of triazole 5 (*m/z* 176), which produces in MS/MS analysis (Figure 4e) a product ion $[CH_2C_6H_4OH]^+$ (*m/z* 107) confirming that the product is triazole 5. The ion *m/z* 107 is a common fragment ion for triazoles containing the phenolic functionality. The MS spectrum of by-product (Peak A, Figure 4b) shows an extraordinary ion at *m/z* 369, which does not exist in the spectrum of the matrix. The product ion spectrum of ion *m/z* 369 (Figure 4c) showed an ion at *m/z* 107 which is also recognized in the product ion spectrum of triazole 5. This suggests that Peak A represents a synthesis by-product. After identification of the synthesis product with AP-MALDI-MS, the purity percentage of triazole 5 was calculated to be 80% in a crude product based on UV densitometry measurement.

Conclusions

We have reported herein the feasibility of a novel UTLC-AP-MALDI-MS method for the analysis of small drug molecules. The UTLC method has been compared with the HPTLC method with UV and AP-MALDI-MS detection and UTLC-AP-MALDI-MS has been compared with UTLC-vacuum MALDI-MS. The UTLC-AP-MALDI-MS analysis of crude synthesis sample produced by combinatorial chemistry has also been applied. The advantages of UTLC over HPTLC include faster separations and reduced solvent consumption. The use of MS provides enhanced specificity over UV detection and UTLC-AP-MALDI-MS significantly improved sensitivity when compared with HPTLC-AP-MALDI-MS. The applicability of UTLC-AP-MALDI-MS has been shown to be good enough for the identification of small drug molecules in relatively simple samples in MS mode. In more complex samples, the use of MS/MS is necessary. In conclusion, UTLC-AP-MALDI-MS provides improvements to the present (HP)TLC-vacuum MALDI-MS methods, preserving at the same time many of the advantages of the TLC, such as fast and parallel analysis, a disposable stationary phase that avoids memory effects, and the possibility to use other analytical techniques before MALDI-MS analysis.

Acknowledgments

The authors gratefully acknowledge C. Sauber (Ph.D.) and F. Mandel (Ph.D.) for technical support, and Agilent Technologies for providing the AP-MALDI ion source. They also thank K. Vuorensola (Ph.D.), P. Östman (M.Sc.), and T. Rantanen (M.Sc.) for helpful discussions and technical assistance in MALDI experiments, and M. Baumann (Dos.) for providing MS instruments used in this study. The financial support of the National Technology Agency of Finland (TEKES) is gratefully acknowledged.

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