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Direct TLC-MALDI Coupling Using a Hybrid Plate

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A new TLC-MALDI direct coupling method which recovers approximately 100% of the analyte is presented. The method makes use of a hybrid TLC-MALDI plate in which a silica layer and a MALDI layer are configured adjacently on a common backing. After TLC separation, the plate is rotated 90° and the separated analyte spots are eluted from the silica layer to the MALDI layer via capillary action of the MALDI layer. Signal-to-noise ratios are significantly improved over previously reported coupling methods. Low-femtomole detection limits have been demonstrated for small cyclic peptides, which are comparable to detection limits for standard MALDI measurements.

Thin-layer chromatography (TLC) directly coupled with mass spectrometry (MS)[1,2] greatly increases the information content and effective separation capabilities of TLC because chromatographically overlapping analytes can be fully resolved using MS detection. Our group has been actively involved in directly coupling TLC with matrix-assisted laser desorption/ionization (MALDI[3,4]). TLC-MALDI[5−8] takes advantage of the high sensitivity (femtomole−attomole range) of MALDI and its ability to ionize both low and high molecular weight compounds without significant fragmentation. When directly coupled with TLC, MALDI has been shown to have better spatial resolution and sensitivity than secondary ion mass spectrometry (SIMS)[9−12] and fast atom bombardment (FAB).[13,14] MALDI is more broadly applicable than direct laser desorption/ionization (LDI)[15−18] because fragmentation of larger analytes is reduced.

Several TLC-MALDI direct coupling methodologies have been evaluated by our group. Initially, a MALDI matrix solution was directly deposited onto the TLC plate, and after solvent evaporation and matrix/analyte cocrystallization, the TLC plate was analyzed by MALDI-MS. This direct "solution deposition" method had relatively low detection limits (2−4 ng) for small peptides; however, the method suffered from significant analyte spreading.[5] Analyte spreading was caused by convection driven by matrix crystallization.

To overcome the spreading problem and to improve sensitivity, a "pressing" method was established.[6] The idea was to separate the MALDI matrix crystallization step from the matrix deposition step. This is accomplished by first forming a MALDI matrix layer on a smooth inert substrate. The matrix layer is then transferred to the top surface of the TLC plate by pressing the matrix layer and TLC plate face-to-face. It is necessary to spray the TLC plate with an extraction solvent. The extraction solvent releases analyte from the stationary phase and also aids in the MALDI matrix layer transfer. Selection of the extraction solvent is based on the criteria that it extract the analyte from the stationary phase, that the solvent not completely redissolve the matrix layer, and that the solvent not cause undue spreading of the TLC analyte spot.

The pressing method is capable of providing picomole detection limits and has been applied for the analysis of antibiotics, pesticides, drugs of abuse,[7] peptides,[6] and DNA adducts.[19,20] The method has the potential of being useful for imaging 2-dimensional TLC plates, where hundreds of compounds could be separated and detected by the powerful combination of chromatography and MS.

However, the "pressing" method has some inherent sensitivity limitations. A study[8] of the pressing method coupling parameters revealed that the optimal analyte recovery is only approximately 22%. This low analyte recovery is due to lateral spreading of analyte and loss of analyte to the intraparticle porosity of the stationary phase. To some degree, the analyte recovery can be improved by using smaller MALDI matrix transfer substrates. This has the effect of reducing lateral analyte spreading but at the same time jeopardizes TLC-MALDI imaging capabilities.

(1) Poole, C. F.; Poole, S. K. *Anal. Chem.* **1994**, *66*, 27A−37A.
(2) Busch, K. L. Thin Layer Chromatography Coupled with Mass Spectrometry. In *Handbook of Thin Layer Chromatography*; Sherma, J., Fried, B., Eds.; Marcel Dekker: New York, 1991; p 183.
(3) Karas, M.; Hillenkamp, F. *Anal. Chem.* **1988**, *60*, 2299−2301.
(4) Hillenkamp, F.; Karas, M.; Beavis, R. C.; Chait, B. T. *Anal. Chem.* **1991**, *63*, 1193A−1202A.
(5) Gusev, A. I.; Rabinovich, Y. I.; Proctor, A.; Hercules, D. M. *Anal. Chem.* **1995**, *67*, 1805−1814.
(6) Gusev, A. I.; Vasseur, O. J.; Proctor, A.; Sharkey, A. G.; Hercules, D. M. *Anal. Chem.* **1995**, *67*, 4565−4570.
(7) Nicola, J. A.; Gusev, A. I.; Hercules, D. M. *Appl. Spectrom.* **1996**, *50*, 1479−1482.
(8) Mehl, J. T.; Gusev, A. I.; Hercules, D. M. *Chromatographia* **1997**, *46*, 358−364.
(9) Busch, K. L.; Mullis, J. O.; Chakel, J. A. *J. Planar Chromatogr.* **1992**, *5*, 9−15.
(10) Duffin, K. L.; Flurer, R. A.; Busch, K. L.; Sexton, L. W.; Dorsett, J. L. *Rev. Sci. Instrum.* **1989**, *60*, 1071−1974.
(11) DiDonato, G. C.; Busch, K. L. *Anal. Chem.* **1986**, *58*, 3131−3232.
(12) Doherty, S. L.; Busch, K. L. *Anal. Chim. Acta* **1989**, *218*, 217−229.
(13) Monaghan, J. J.; Morden, W. E.; Johnson, T.; Wilson, I. D.; Martin, P. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 608−615.
(14) Oka, H.; Ikai, Y.; Hayakawa, J.; Masuda, K.; Harada, K.; Suzuki, M.; Martz, V.; MacNeil, J. D. *J. Agric. Food Chem.* **1993**, *41*, 410−415.
(15) Fanibanda, T.; Milnes, J.; Gormally, *J. Int. J. Mass Spectrom. Ion Processes* **1994**, *140*, 127−132.
(16) Novak, F. P.; Hercules, D. M. *Anal. Lett.* **1985**, *18*, 503−518.
(17) Novak, F. P.; Wilk, Z. A.; Hercules, D. M. *J. Trace Microprobe Tech.* **1985**, *3*, 149−163.
(18) Kubis, A. J.; Somayajula, K. V.; Sharkey, A. G.; Hercules, D. M. *Anal. Chem.* **1989**, *61*, 2516−2523.
(19) Isbell, T. D.; Gusev, A. I.; Tarankenko, N. I.; Chen, C. H.; Hercules, D. M. *Rapid Commun. Mass Spectrom.*, submitted for publication.
(20) Isbell, T. D.; Gusev, A. I.; Tarankenko, N. I.; Chen, C. H.; Hercules, D. M. *Fresenius' J. Anal. Chem.*, submitted for publication.

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A newer method that can recover approximately 100% of the analyte was recently developed in our laboratory. The method makes use of a hybrid TLC-MALDI plate in which two juxtaposed zones, a TLC zone and a MALDI zone, are formed on a common plate backing. TLC separation is performed in one direction, followed by perpendicular elution of the analyte from the TLC zone to the MALDI zone. The present report describes the development and application of this method.

EXPERIMENTAL SECTION

Materials. Valinomycin (Val), cyclosporin A (CsA), and *N*-*t*-BOC-Phe-Lue-Phe-Lue-Phe (*N*-*t*-BOC-FLFLF, Sigma Catalog No. B5639) were purchased from Sigma Chemical Co., St. Louis, MO. 2,4,6-Trihydroxyacetophenone (THA), 2,5-dihydroxybenzoic acid (DHB), rhodamine B, and trifluoroacetic acid (TFA) were purchased from Aldrich Chemical Co., Milwaukee, WI. All solvents were of HPLC grade when available; otherwise, ACS grade was used. Solvents were purchased from Fisher Scientific, Pittsburgh, PA. Aluminum-backed silica gel TLC plates were obtained from Merck, Darmstadt, Germany (Catalog No. 1.0555). Polyester-backed, no-binder, nonindicator silica gel TLC plates were obtained from Macherey-Nagel, Düren, Germany (Catalog No. 804013). Stock solutions of Val and CsA were prepared by dissolution in acetone. *N*-*t*-BOC-FLFLF was dissolved in CH_3OH/0.1% TFA. Spotting solutions were prepared by serial dilution. The final concentrations of the spotting solutions were such that 1 μL of solution contained the desired amount of analyte, e.g., 100 pg/μL.

MALDI-TOF. All analyses were performed on a Voyager-DE STD MALDI-TOF mass spectrometer from PerSeptive Biosystems, Framingham, MA. The instrument is equipped with a CCD camera for visual observation of the sample. A joystick enables computer-controlled *x*,*y*-movement of the sample. The instrument is equipped with a nitrogen laser emitting at 337 nm. Samples were analyzed in the positive-ion mode using the reflectron, unless otherwise indicated. An acceleration voltage of 20 kV was used. Typically 48−128 single-shot mass spectra were averaged to give a composite mass spectrum. Samples were mounted on the sample stage using double-sided tape. "Fast-evaporation" MALDI samples were prepared by dissolving THA in acetone at 10 mg/mL. A 1−3 μL portion of matrix solution was applied to the sample target and allowed to dry and form a homogeneous matrix layer. Next, a solution of analyte, saturated with THA matrix, was deposited on top of the THA matrix layer.

TLC Separation. The test mixture of Val, CsA, and *N*-*t*-BOC-FLFLF was deposited from a 1:1 acetone/CH_3COOH solution containing 0.1% TFA onto the TLC plates using a micropipet. Multiple development, using two solvents (A and B), was used for separation. Solvent A was $CHCl_3$, which only moves *N*-*t*-BOC-FLFLF ($R_f = 0.8$). This is followed by solvent B, 3:1 ethyl ether/toluene, which moves only Val ($R_f = 0.8$). Solvent B is not developed as far as solvent A in order to prevent overlap of Val and *N*-*t*-BOC-FLFLF. A parallel zone using a higher 1.0 μg loading of each analyte was used for iodine visualization.

TLC-MALDI Hybrid Method. Figure 1 shows a schematic of a hybrid TLC-MALDI plate. Silica was scraped from the plate using a glass microscope slide to expose the TLC backing for the MALDI matrix zone. The scraping step was conducted in a way that resulted in a gradual taper over a horizontal distance of 1 cm; i.e., the thickness was gradually decreased from the original 250 μm to zero over the distance of 1 cm. Finely ground MALDI matrix powder was dispersed into a mixture of toluene and ethyl ether (60:40 for THA and 80:20 for DHB). Roughly 5 g of matrix powder was swirled in 100 mL of the toluene/ether mixture. Compressed air was used to spray the dispersion/suspension from a glass reagent sprayer onto the exposed TLC backing. A shield (glass microscope slide) was placed over the silica zone to prevent the matrix from covering the silica layer. The matrix layer was applied after separation of the test mixture. In general, the organic-binder aluminum-backed TLC plates worked best. A solvent mixture of 2.5:1:1 $CHCl_3/CH_3OOH/CH_3OH$ with 0.1% trifluoroacetic acid was used to elute the separated analyte spots from the silica zone into the MALDI zone. A mixture of 1:1 CH_3OH/H_2O was deposited on the analyte spot following elution onto the THA MALDI matrix to improve analyte/matrix cocrystallization. This step was not necessary when DHB was used as the matrix or when Val was used as the test analyte with a THA matrix.

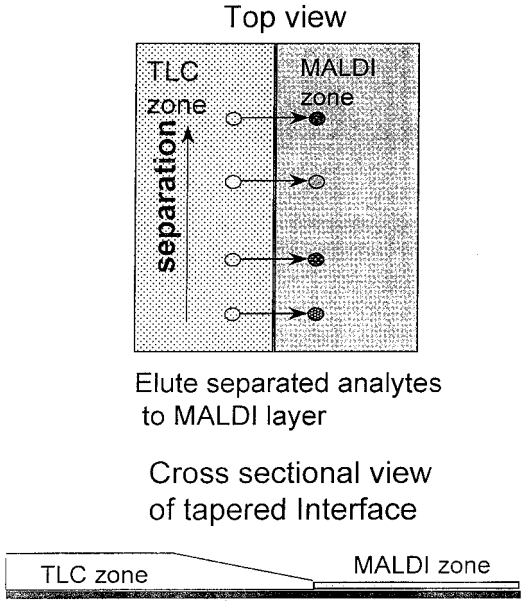

Figure 1. Schematic of the hybrid TLC-MALDI plate. In the TLC-MALDI technique, first TLC separation is performed in a conventional manner and then the plate is rotated 90° and the separated analyte spots are eluted out of the TLC zone and into the MALDI zone.

TLC-MALDI Pressing Method. The pressing method has been described previously;[6,8] here we discuss only relevant details. A 6 μL portion of a 10 mg/mL solution of THA in acetone was deposited onto 5 mm × 5 mm stainless steel substrates (0.5 mm thick) and allowed to evaporate and crystallize to form a homogeneous MALDI matrix film. The extraction solvent used was 60:40 CH_3COOH/H_2O with 0.1% TFA. The MALDI matrix layer was pressed face-to-face with the TLC plate at a pressure of 2.0 kg/mm^2 for 1 min.

Spectrofluorometry. Experiments were conducted to determine the percentage of analyte recovered by the MALDI phase. A 0.515 ng amount of rhodamine B was deposited onto the silica zone of a hybrid TLC-MALDI plate. Nonindicator TLC plates were used (see Materials). A solvent mixture of 2:1:1 $CHCl_3/CH_3OH/CH_3COOH$ with 0.1% TFA was used to elute rhodamine B from the silica zone to the MALDI zone (THA matrix). The analyte was then scraped off, along with the matrix, and dissolved in 300.0

μL of methanol. A spectrofluorometer (model PC-1, ISS, Champaign, IL) was used to measure the rhodamine B emission at 568.00 nm. An excitation wavelength of 543.00 nm was used. Entrance and exit slits were 0.1 μm. The calibration standards were prepared by serial dilution of a stock solution, 0.610 mg/mL, prepared by dissolution of 61.0 mg of rhodamine B in 100.0 mL of methanol. The concentrations of the standard solutions were 0.289, 0.360, 0.515, 0.686, and 0.915 μg/mL. The calibration curve was prepared by spotting 1.0 μL of each standard solution directly onto the matrix layer (THA). Each sample was then scraped off, along with the matrix, and dissolved into 300.0 μL of methanol.

RESULTS AND DISCUSSION

The sensitivity of any direct TLC-MS coupling method ultimately depends on the percentage of analyte recovered from the stationary phase. The TLC-MALDI "pressing" method previously established by our group has an upper limit of approximately 22% analyte recovery.[8] Therefore, at least 78% of the analyte is lost to the stationary phase. This approximation was arrived at by considering both the extraction efficiency of the coupling solvent and the total porosity of the stationary phase. Coupling solvents with extraction efficiencies between 40 and 60% are optimal; higher extraction efficiencies lead to excessive analyte spread. During the pressing step, the porosity is compressed by roughly 37%. Therefore, using a 60% extraction efficiency coupling solvent, only 22% (60% of 37%) of the analyte is forced from the TLC plate during the pressing step. A hybrid TLC-MALDI plate can overcome this limitation because a much greater percentage (vide infra) of the analyte can be directly eluted from the stationary phase to the MALDI phase. The hybrid plate takes advantage of the natural capillary action of a MALDI particle layer. It is therefore very consistent with TLC, which also uses capillary action.

There are some considerations which are necessary for constructing and using a hybrid TLC-MALDI plate (Figure 1). A uniform tapered interface between the two zones is critical for avoiding a distorted solvent front, which would cause spreading of the analyte spot. If the interface is not tapered and the silica layer is thicker than the MALDI layer, some analyte will remain in the silica layer and not be eluted into the MALDI layer. Only elution solvents which do not dissolve, or only partially dissolve, the MALDI matrix layer can be used. The elution solvent should have high extraction efficiency for the analyte in order to move all of the analyte from the silica zone to the MALDI zone. In some cases it may be necessary to apply a "postelution" solvent to the MALDI layer following elution of the analyte into the analysis zone. This will promote cocrystallization of the analyte and MALDI matrix, which is important for optimal signal intensity.

Dispersion Layer. To prepare a hybrid TLC-MALDI plate, a sprayed-on dispersion layer was used create the MALDI zone. The dispersion layer was a very light coating (≤25 μm thick) consisting of finely ground MALDI matrix particles. However, such layers have not been previously described in the MALDI literature. Therefore an evaluation of the dispersion layer was performed to determine how effective the layer is for obtaining MALDI spectra.

Figure 2 compares MALDI spectra of 4.5 pmol of Val obtained from a standard "fast-evaporation" [21,22] sample (Figure 2a) and a MALDI dispersion layer sample (Figure 2b). The fast-evaporation sample was prepared by first casting, from solution, a homogeneous MALDI crystal layer. Next, analyte solution was deposited onto the MALDI crystal layer. The dispersion layer sample was prepared in a similar manner, except that a sprayed-on suspension of MALDI particles dispersed in a nonsolvating solvent was used to create the MALDI layer. Analyte solution was then deposited onto the MALDI dispersion layer. The two spectra are comparable in signal intensity. The $[M + K]^+$ peak is about equal in intensity to the $[M + Na]^+$ peak. Others have reported that MALDI of Val produces primarily the $[M + Na]^+$ peak.[27] However, on the basis of solution complex studies, Val is known to have high selectivity for potassium ions.[28] It therefore seems understandable that, under certain conditions, MALDI could produce high-intensity $[M + K]^+$ peaks. It is possible that the relative intensity of the $[M + Na]^+$ and $[M + K]^+$ peaks may be dependent upon the matrix and solvent conditions used for sample preparation, as well as the sample loading. The signal-to-noise (S/N) ratios are 365 and 300 for the fast-evaporation and dispersion layer samples, respectively. The S/N ratio is determined by dividing peak height by RMS noise. An identical comparison was performed using 6.2 pmol of *N-t*-BOC-FLFLF as the test analyte (spectra not shown). The resulting S/N ratios were 374 and 197 for the fast-evaporation and

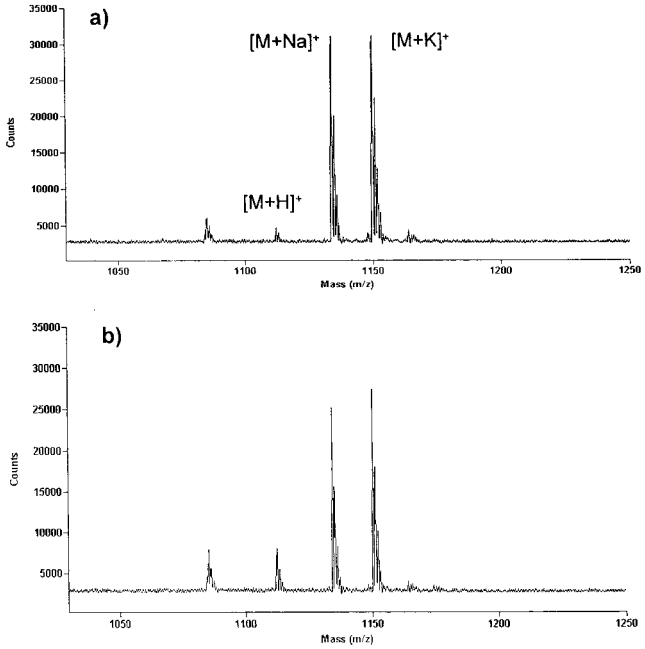

Figure 2. MALDI mass spectra for 4.5 pmol of Val obtained from a "fast-evaporation" sample (a) and from a MALDI matrix dispersion layer (b). THA was used as the matrix.

(21) Vorm, O.; Mann, M. *J. Am. Soc. Mass Spectrom.* **1994**, *5*, 955−958.

(22) Vorm, O.; Roepstorff; Mamm, M. *Anal. Chem.* **1994**, *66*, 3281−3287.

(23) Muddiman, D. C.; Gusev, A. I.; Proctor, A.; Hercules, D. M.; Venkataramanan, R.; Diven, W. *Anal. Chem.* **1994**, *66*, 2362−2368.

(24) Wu, J.; Chatman, K.; Harris, K.; Siuzdak, G. *Anal. Chem.* **1997**, *69*, 3767−3771.

(25) Oka, H.; Ikai, Y.; Kondo, F.; Kawamura, N.; Hayakawa, J.; Harada, K.-I.; Masuda, H.; Suzuki, M. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 89−94.

(26) Busch, K. L.; Mullis, J. O.; Carlson, R. E. *J. Liq. Chromatogr.* **1993**, *16*, 1695−1713.

(27) Knochenmuss, R.; Dubois, F.; Dale, M. J.; Zenobi, R. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 871−877.

(28) Sobott, F.; Wattenberg, A.; Kleinekofort, W.; Pfenninger, A.; Brutschy, B. *Fresenius' J. Anal. Chem.* **1998**, *360*, 745−749.

Table 1. Fluorescence Data Obtained for Calibration Curve and the Test Analyte Spot

sample	concn,[a] nM	emission[c] (568.00 nm), cm^{-1}
blank	0.00	188.0
std 1	2.02	3599.5
std 2	2.69	4778.0
std 3	3.58	5914.5
std 4	4.78	7045.0
std 5	6.38	9393.5
test spot	*b*	5428.7

$y = 1409.8x + 588.6$; $r_o = 0.995$; std error of slope = 5.2%

[a] Each calibration curve standard was dissolved in 300.0 μL of CH_3OH prior to spectrofluorometry. [b] 0.515 ng of rhodamine B was spotted onto the silica zone and then eluted into the MALDI matrix zone. [c] Excitation wavelength = 543.0 nm.

dispersion layers, respectively. The results of this experiment suggest that the dispersion layer is capable of producing S/N ratios that are comparable, within a factor of 2, to the fast-evaporation sample preparation. Therefore, this type of MALDI layer should be well suited for TLC-MALDI coupling. It should also be noted that any solid MALDI matrix can in principle be used, as long as the matrix is pulverized into a fine powder. This is an advantage over the pressing method, which is restricted to only matrixes that form homogeneous crystal films. In addition to using the MALDI dispersion layer to create the MALDI zone, attempts to cast layers from solution were undertaken. This led to problems because the silica layer absorbed the matrix solution, resulting in obstruction of capillary flow due to crystallized matrix.

Analyte Recovery. The hybrid TLC-MALDI plate works by using capillary action to elute the analyte from one phase (silica gel) to a second phase (MALDI particles). If the plate is constructed properly, it should be possible to recover 100% of the analyte into the MALDI layer. Spectrofluorometry was used to quantitatively determine how much analyte was recovered. Use of MALDI to determine the analyte recovery was not possible because absolute MALDI signal intensities were not quantitative due to large variability.

To carry out the spectrofluorometric experiment, a test analyte (0.515 ng of rhodamine B) was spotted onto the silica layer and eluted onto the MALDI zone. Once on the MALDI zone, the rhodamine B along with MALDI matrix was scraped from the plate backing and dissolved in 300.0 μL of methanol. Table 1 gives the fluorescence data for the calibration curve and the test analyte spot. Linear regression yielded the following best-fit line: $y = 1409.8x + 588.6$. Using this equation, it was determined that 0.493 ng was recovered in the MALDI zone. This represents an analyte recovery of 95.7%, within experimental error of 100%. This demonstrates that, in certain cases, it is possible to achieve approximately 100% analyte recovery using the hybrid TLC-MALDI plate. However, it should be noted that this may not be the case for all analytes. In some cases, it may be expected that irreversible absorption may lead to reduction in analyte recovery, especially for low sample loadings. However, if a strong-elution solvent (elution from silica to MALDI zone) is used, high analyte recovery can be expected in most cases.

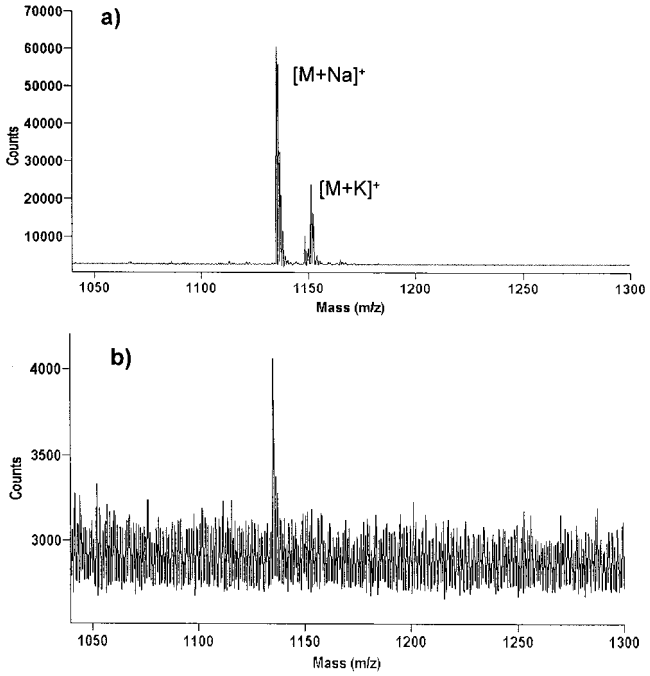

Figure 3. MALDI mass spectra for 4.5 pmol of Val obtained from a hybrid plate (a) and from a pressing method sample (b). THA was used as the matrix.

Use of a strong-elution solvent preserves the chromatographic resolution. However, streaking can occur if the interface is not properly constructed. Even when streaking occurs, multiple elution can refocus the analyte spot into a narrow band. Under optimal conditions, the width (coordinate parallel to interface) increases by at most 10%, as determined by the diameter of the spot before and after elution into the MALDI zone. Optimal conditions exist when a strong-elution solvent having low matrix solubility is used and a very uniform silica/matrix interface has been constructed.

Comparison of Coupling Methods. Several groups have reported direct TLC-MALDI coupling using different approaches. Mowthorpe et al.[29] electrosprayed a MALDI matrix solution onto the developed TLC plate. Guittard et al.[30] first blotted the developed TLC plate using a poly(vinylidene difluoride) membrane (PVDF). The MALDI matrix solution was then deposited onto the PVDF membrane. Both of these methods provided detection limits in the nanogram range. However, the pressing method described earlier in this report has provided the lowest detection limits to date (vide supra).

A comparison between the pressing and hybrid methods was undertaken to determine what level of improvement is possible. Equal loadings of a test mixture (Val, CsA, *N-t*-BOC-FLFLF) were deposited on TLC plates, separated, and coupled with MALDI using the two methods. Figure 3 compares MALDI spectra of 5 ng of Val obtained from a hybrid TLC-MALDI plate (Figure 3a) and a pressing method sample (Figure 3b). There is a dramatic improvement in signal intensity upon using the hybrid method.

(29) Mowthorpe, S.; Clench, M. R.; Cricelius, A.; Richards, D. S.; Parr, V.; Tetler, L. W. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 264−270.
(30) Carr, S. A.; Annan, R. S.; Huddleston, M. J.; McNulty, D.; Bergsma, D. *Proceedings of the 46th ASMS Conference on Mass Spectrometry and Allied Topics*, Orlando, FL, 1998; p 1363.

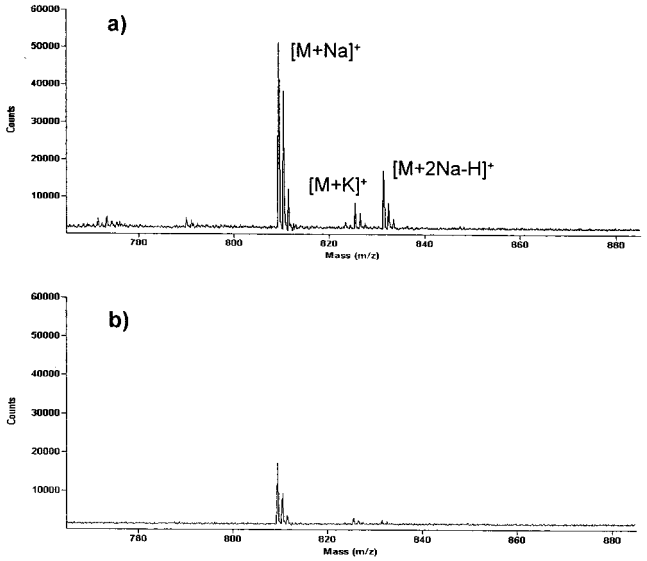

Figure 4. MALDI mass spectra of 64 pmol of *N*-*t*-BOC-FLFLF obtained from a hybrid plate (a) and from a pressing method sample (b). THA was used as the matrix.

Table 2. Signal-to-Noise Ratios from Spectra Obtained by Using the Hybrid and Pressing TLC-MALDI Coupling Methods

	S/N		
analyte	hybrid plate	pressing method	loading, ng
Val	943	9.5	5.0
CsA	479	24	10
N-*t*-BOC-FLFLF	575	128	50

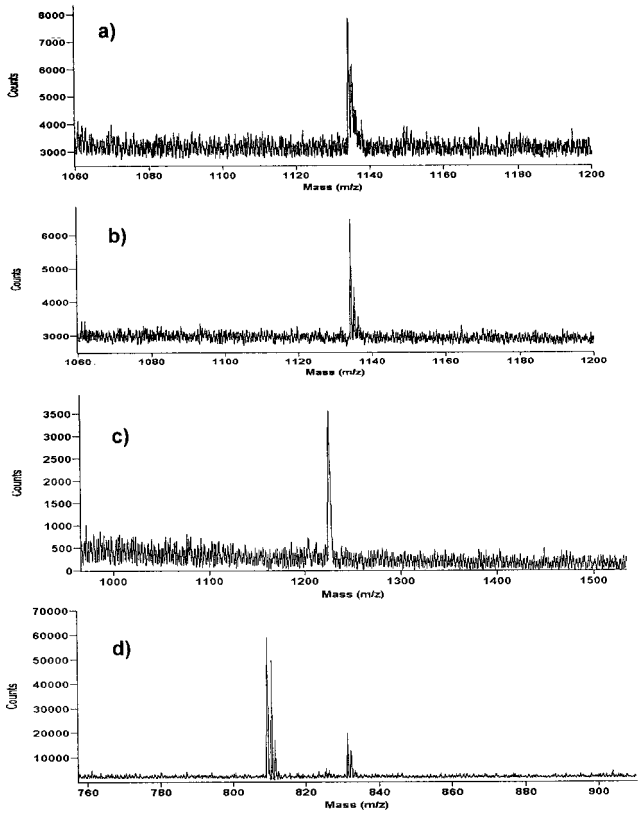

Figure 5. MALDI mass spectrum for 90 fmol of Val obtained from a fast-evaporation sample (a). MALDI mass spectrum for 90 fmol of Val obtained from a hybrid TLC-MALDI plate (b). MALDI mass spectrum for 0.83 pmol of CsA obtained from a hybrid TLC-MALDI plate (c). MALDI mass spectrum for 6.3 pmol of *N*-*t*-BOC-FLFLF obtained from a hybrid TLC-MALDI plate (d). THA was used as the matrix.

Clearly, the hybrid method recovers more analyte in the MALDI matrix than the pressing method. The S/N ratio is increased by a factor of 99. It should be realized that it was possible to elute Val from the silica layer to the MALDI layer and then collect a mass spectrum. This was not the case for the two other test analytes, CsA and *N*-*t*-BOC-FLFLF. Both CsA and *N*-*t*-BOC-FLFLF required the addition of a "postelution" solvent to be spotted onto the MALDI layer. This step was necessary to improve analyte incorporation into the THA matrix. When DHB was used as the MALDI matrix a postelution solvent was not necessary; however, for the method comparison experiments, THA was used as the MALDI matrix because DHB does not form a homogeneous crystal layer, as required by the pressing method.

Figure 4 shows spectra for 50 ng samples of *N*-*t*-BOC-FLFLF obtained from each of the two coupling methods. At this analyte loading level, respectable signals are obtained from both samples. The hybrid method provides a 4.5-fold greater S/N ratio than the pressing method. For a 10 ng loading of CsA (spectra not shown), the hybrid method yielded a 20-fold increase in S/N ratio. Table 2 lists the S/N ratios obtained from each method. These results indicate that the hybrid method offers a significant improvement in signal intensity over the pressing method.

Detection Limits. The hybrid method offers the capability of recovering more analyte than any of the previously reported direct TLC-MALDI coupling methods. However, the hybrid method is only suitable for one-dimensional TLC separation, unlike the other methods which allow for two-dimensional TLC. The hybrid method still allows parallel development because multiple plates can be spotted and developed simultaneously. The advantage of the hybrid method over the previous methods is improved detection limit. Therefore, experiments were conducted to evaluate the detection limits of hybrid TLC-MALDI and compare them with standard MALDI detection limits. Low loadings of each analyte were spotted onto the silica layer and eluted onto the MALDI layer. The same loading was applied to fast-evaporation MALDI layers and MALDI dispersion layers.

Parts a and b of Figure 5 show spectra of 100 pg obtained from a fast-evaporation sample and a hybrid TLC-MALDI sample, respectively. The spectra have similar signal intensities; however, the hybrid method actually produced a slightly higher S/N ratio. Signals were obtained from most locations on the hybrid sample, thus making it easier to accumulate multiple laser shots in order to average spectra. It may be possible in some cases for the hybrid method to perform better than standard MALDI because the elution step can compress the analyte into a tighter band. This is known in the TLC literature as the zone-refocusing mechanism and is used to an advantage in multiple-development techniques.[1] Parts c and d of Figure 5 show spectra for 1.0 ng of CsA and 5.0 of ng *N*-*t*-BOC-FLFLF obtained from hybrid TLC-MALDI samples.

Table 3 lists estimated detection limits for the three compounds tested. Estimated detection limits were determined by extrapolating from the measured S/N ratio to an S/N ratio of 3, assuming linear behavior. Referring to Table 3, for all three analytes, the

Table 3. Estimated Detection Limits

analyte	detection limit, fmol (ng) standard MALDI[a]	dispersion layer	hybrid TLC-MALDI	loading, ng
Val	13.5 (15)	21 (24)	11 (13)	0.1
CsA	11 (14)	30 (36)	40 (42)	1.0
N-t-BOC-FLFLF	30 (23)	78 (61)	116 (91)	5.0

[a] Fast-evaporation sample preparation.

standard MALDI preparation produced lower estimated detection limits than the dispersion layer. This could be due to analyte spreading because of the capillary action inherent in the dispersion layer. In the fast-evaporation MALDI preparation, the solvent was chosen so that surface tension allowed a bead of analyte solution to be deposited onto the matrix crystal layer. As the solution bead evaporated, the analyte remained concentrated in a small circular area. It was impossible to apply the analyte solution as a bead when the dispersion layer was used because the matrix particles disrupted the surface tension of the bead. As a result, the analyte solution was spread out over a larger area, thus reducing the signal intensity. For both CsA and *N-t*-BOC-FLFLF, the hybrid method yielded higher detection limits than either standard MALDI or the dispersion layer. These two analytes required the addition of a postelution solvent, which was deposited onto the MALDI zone using a syringe. As described above, this caused spreading because of the inherent capillary action of this type of dispersion layer. A better means of depositing the postelution solvent is required; a finely atomized spray might result in less analyte spreading.

Hybrid Plate Design Configurations. The hybrid TLC-MALDI coupling method offers some unique advantages over previously reported methods. Since the driving force that recovers the analyte from the stationary phase is the same driving force used in TLC, i.e., capillary action, no special equipment is necessary to use the plates. Ordinary TLC development chambers can be used. The method is also compatible with on-plate concentration methods which have been discussed in the TLC-FAB literature.[25] Busch et al.[26] have described a concentration method in which small triangular wedges are cut along the edge of the TLC plate. The analyte is then eluted into the vertex of a triangle, thus concentrating the analyte into a much smaller volume. This type of strategy used in conjunction with a hybrid TLC-MALDI plate may lead to further enhancement in sensitivity.

One of the problems with the current hybrid design is that the silica zone is directly adjacent to the organic MALDI matrix. This configuration places restrictions on the selection of the elution solvent (elution from the silica zone to the MALDI zone). As stated previously, the elution solvent must not only overcome the interactive forces between the analyte and stationary phase but also must have limited solubility for the MALDI matrix. For some analytes, e.g., polar peptides, it may be impossible to identify such an elution solvent.

This problem might be surmounted by using a multiphase hybrid plate. A three-zone plate could be prepared having an inert zone located between the silica (or other type of stationary phase) and MALDI zones. Figure 6 shows an illustration of a proposed multiphase plate. Whatman Ltd.[31] supplied Linear-K plates with a proprietary preabsorbent strip made of inert material. A similar strategy could be used to prepare multiphase TLC-MALDI plates. Two solvents would then be used to elute the analyte from the silica to the MALDI layer. The first solvent would move the analyte from the silica to the inert layer, followed by the second solvent which would move the analyte to the MALDI layer. Such a plate would offer much greater versatility for different analytes and MALDI matrixes.

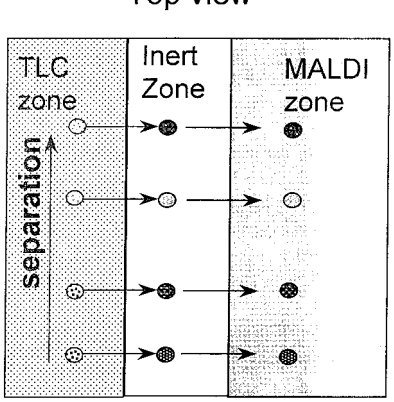

Figure 6. Illustration of the proposed multiphase hybrid TLC-MALDI plate. An inert zone is formed between the silica and MALDI zones.

CONCLUSIONS

A new TLC-MALDI direct coupling method utilizing a hybrid TLC-MALDI plate has been developed and demonstrated using a mixture of peptides. The method takes advantage of capillary action to elute the separated analyte from the stationary phase directly into the MALDI phase. Comparison between the hybrid method and the previously reported pressing TLC-MALDI coupling method has shown that the hybrid method provides significant improvement in the S/N ratio. This finding is consistent with the fact that the hybrid method recovers approximately 100% of the analyte, as determined by spectrofluorometry. Because of the much greater analyte recovery by the hybrid method, low detection limits can be achieved. For the cyclic peptides tested, the detection limits ranged from 11 to 116 fmol.

A number of design configurations could be implemented to improve the versatility and performance of hybrid TLC-MALDI plates. These include multiphase plates, which would place less restriction on the choice of elution coupling solvents. Hybrid TLC-MALDI plates should also be suitable for on-plate concentration methods aimed at improving sensitivity.

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(31) Whatman Ltd., Clifton, N.J. (LINEAR-K preabsorbent strip, Catalog No. 4856821).