Coupling of nano-LC to MALDI mass spectrometry – Real-time LC run mapping on a MALDI plate

Application

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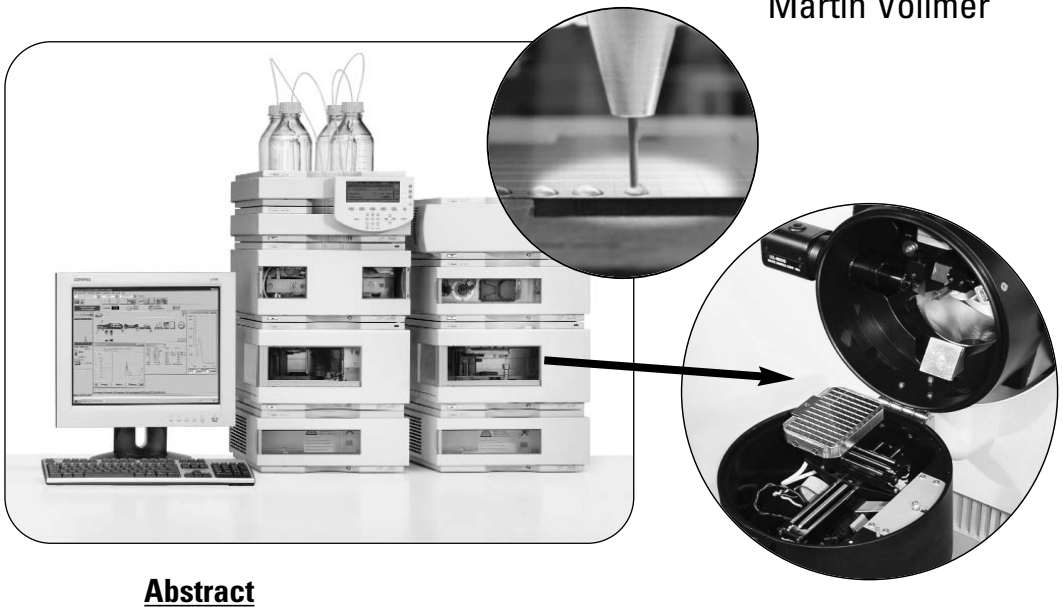

Abstract

An obstacle in using LC mass spectrometry instruments when analyzing complex proteome samples is the limited time available for data-dependent mass analysis. To overcome this drawback, the Agilent 1100 Series micro fraction collector with spotting capabilities was developed. The instrument is able to perform MALDI spotting in real time in order to deposit the LC run on the MALDI plate, and therefore couples the high resolution power of nano-RP-HPLC separation directly with MALDI mass spectrometry and decouples the aquisition of MS/MS from chromatographic separation and MS cycle time.
This Application Note describes an optimized method for spotting with online matrix addition and illustrates its use in the analysis of a complex proteome sample.

Agilent Technologies

Introduction

A wide variety of mass spectrometers, especially those with MS/MS capabilities, are used today as "work horses" in proteomics research. Their popularity is mainly due to technology, sensitivity, mass resolution and faster MS cycle times that have been achieved over the last few years. These instruments typically use nanoelectrospray, MALDI, or AP-MALDI ion sources[1] to generate ions for subsequent analysis.

The remaining hurdle for using MALDI instruments in the direct HPLC analysis of complex proteome samples is the lack of a routine, robust automated interface between the high-efficiency nano LC separation device and the MALDI plate. Theoretically speaking, the combination of offline LC separation coupled with MALDI MS would provide a method to "freeze" the LC separation on the MALDI plate. This would allow the MS/MS analysis to be performed with greatly relaxed constraints, i.e., ion suppression effects can be minimized, and a single spot can be examined for many minutes if needed. Decoupling the separation from the MS/MS analysis consequently provides the opportunity to independently optimize the separation performance and the MS/MS performance, thereby improving overall quality of the results.

In order to achieve this goal, the Agilent 1100 Series micro fraction collection system with spotting enhancements[2] was developed. Using this instrument, the analyst is essentially able to perform spotting in real time mapping the LC run on the MALDI plate as well as to couple the high separation power of nano-RP-HPLC separation with high speed MALDI MS or MS/MS instruments. This Application Note outlines an optimized method for spotting with online matrix addition and describes the real time mapping of a highly complex proteome sample.

Experimental

Equipment

- Agilent 1100 Series nanoflow pump with micro vacuum degasser
- Agilent 1100 Series thermostatted micro well-plate autosampler
- Agilent 1100 Series diode-array detector with 80-nL cell
- Agilent 1100 Series thermostatted micro fraction collector with spotting accessories
- Agilent 1100 Series MSD Ion Trap XCT with AP-MALDI source

Accessories for matrix online addition

- KDS 200 syringe pump
- SGE 1-mL syringe
- Upchurch or valcro micro T-piece
- 125-µm capillary

Software

- Agilent ChemStation A 10.02
- Agilent Trap software 4.2
- Agilent software for the control of the AP-MALDI source
- Agilent Spectrum Mill MS Proteomics Workbench

Principle of spotting – the "liquid contact control mode"

In order to reliably deposit small volumes (nL to the lower µL range) onto the surface of a MALDI plate, a unique liquid deposit principle was developed (figure 1). During the entire spotting process, the spotter ensures that the capillary tip is in contact with the surface of the deposited liquid on the MALDI target plate. The initial droplet at the tip is pre-

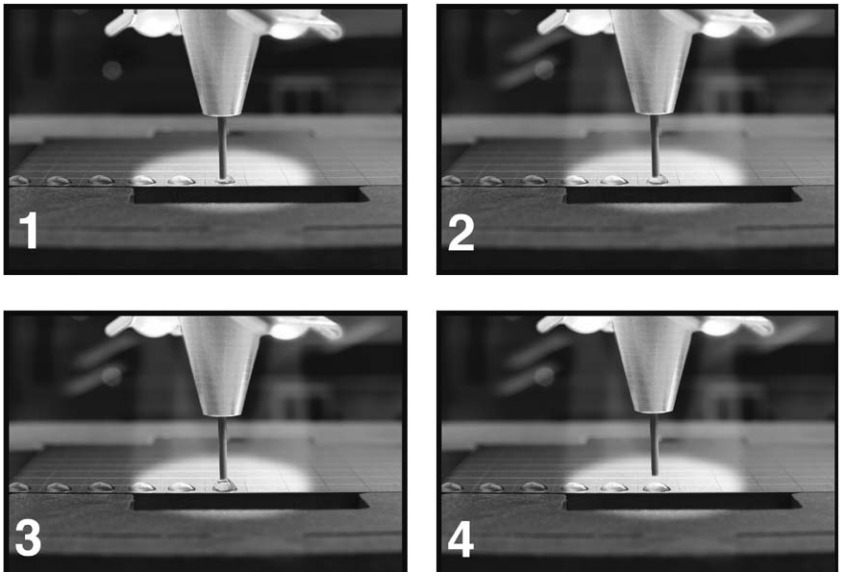

Figure 1
Liquid contact control mode for spotting.

cisely deposited on the plate at the start of the spotting process. If the liquid volume per spot is greater than 500 nL, the capillary tip starts to move continuously upwards, keeping contact with the droplet surface. When spotting of the current droplet is finished, the capillary tip moves upwards quickly, breaking contact between the tip and the droplet and ensuring that the droplet remains on the plate. This unique spotting action guarantees reproducible and precise deposition of low volume droplets on a broad variety of MALDI target plates from different manufacturers. In addition, cross contamination between collected spots is effectively prevented. For applications, which require an additional online matrix addition, it is possible to adjust the liquid contact control mode in the software for this higher flow onto the MALDI target plate.

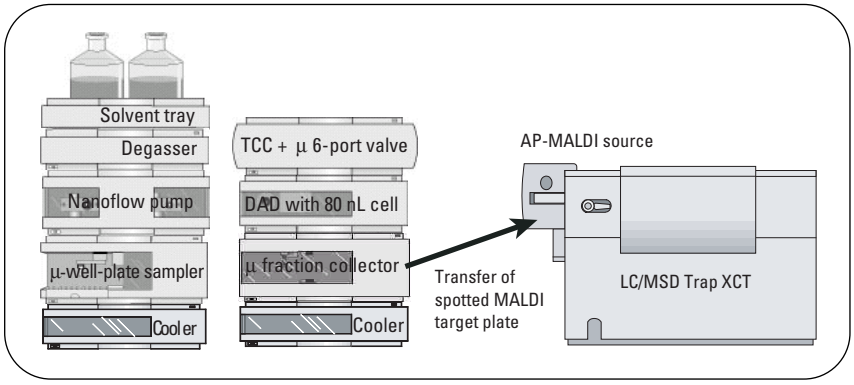

Figure 2
Micro fraction collection/spotting LC system with AP-MALDI LC/MSD Trap XCT.

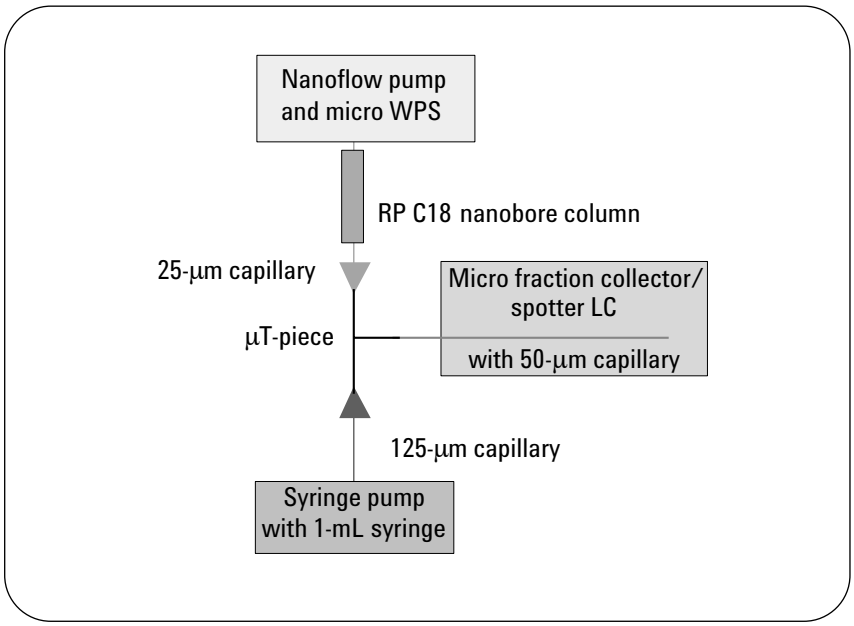

Figure 3
Diagram of the online matrix addition configuration.

MALDI sample preparation with the spotter

The dried droplet method is the preferred sample preparation procedure for MALDI mass spectrometry since its introduction by Karas and Hillenkamp[3]. With this procedure, the sample peptides or proteins are mixed with a matrix solution, and a small droplet of the mixture, usually 0.5 to 1.0 µL, is deposited on the MALDI target. The mass spectrometric analysis is then performed on the spot that remains after the droplet is dried at room temperature. Other methods have been developed, e.g. precoating the MALDI target plates with matrix or recrystallization of the dried residues of the droplet[4]. The dried droplet approach has also been adapted to special applications and unique MALDI targets have recently been developed, for example, for proteome analysis using α-Cyano-4-hydroxycinnamic acid (CHCA)[5] or for the analysis of a variety of sample compounds using different matrices[6].

To obtain CHCA/sample crystals comparable to the dried droplet method using an Agilent 1100 Series micro fraction collection/ spotting system (figure 2), an online matrix addition system was connected to the LC system (figure 3). For the sample separation, this configuration uses an Agilent 1100 Series nanoflow pump operating at a flow rate of 800 nL/min with a 100 µm i.d. nanobore column. With this flow rate, it is possible to use a diode-array detector equipped with an 80-nL flow cell to monitor or to optimize the separation of the sample components. The matrix solution is added to the sample using a syringe pump connected to a micro-volume

T-connector that is located between the column or DAD outlet and the micro fraction collector/spotter inlet (figure 3). To achieve a drying and crystallisation behaviour comparable to the manual dried droplet method, two parameters of the spotting system have to be optimized. One parameter is the solvent composition[7], which has the greatest influence on the crystallization behaviour. The solvent composition, which changes during gradient elution of the separated peptides, causes large variations in the number and size of crystals formed on the MALDI spots. Therefore, the solvent for the matrix solution was chosen to minimize the negative effects on crystallization caused by the changing composition of the column effluent. Different matrix solutions were evaluated and ranked by their influence on the resulting protein identification after analysis with AP-MALDI ion trap MS/MS. For these experiments a 25-fmol BSA digest was separated and spotted together with CHCA dissolved in different solutions. From these experiments, a mixture of 2 mg/mL CHCA dissolved in 50 % 2-propanol/49 % water/1 % acetic acid resulted in the best protein identification with the highest sequence coverage (25 fmol BSA digest: Score 38.60, 7 peptides, 9 % AA coverage).

The second parameter, which showed great influence on the crystallization, was the speed of solvent evaporation from the spot[8]. This parameter can be controlled directly by regulating the temperature of the micro fraction collection/spotting device. Our experiments suggest that best crystallization may be achieved by maintaining the plate at 20 °C. A spot, created at this temperature resulted in the identification of the highest number of peptides/proteins after MS/MS analysis with AP-MALDI ion trap MS. This is demonstrated for 100 fmol of bovine serotransferrin digest in a complex mixture of ten protein digests, each present at 100 fmol (table 1).

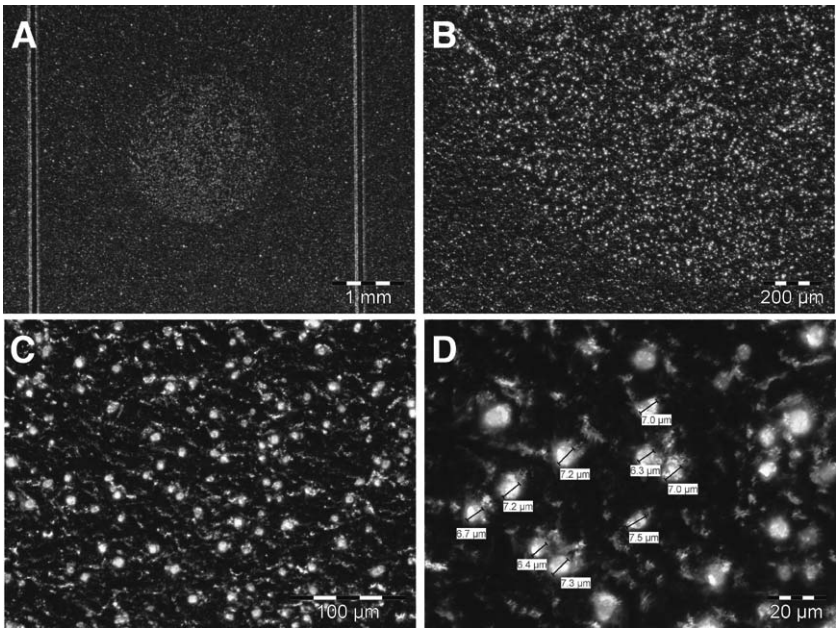

Figure 4
CHCA crystals in the spot area, formed with the optimized online matrix addition process.

The homogeneity and uniformity of the crystals from these matrix solutions, which is important to obtain good MS and MS/MS spectra[9], was verified by means of a microscope. Figure 4 shows the crystals obtained from the optimized matrix solution mentioned above. The crystallization is nearly uniform over the whole spot (figures 4A, 4B) and the crystals are essentially homogenous in their size (figure 4C). The diameter of the crystals was between 6–8 µm (figure 4D).

Temperature [°C]	Peptides found	Summed score	% AA Coverage
10	2	17.33	3
20	6	44.51	9
30	3	22.80	6

Table 1
Results for 100 fmol serotransferrin, deposited using different MALDI plate temperatures.

Results and discussion

The coupling of HPLC to MALDI-MS is achieved by depositing droplets of the column effluent on a MALDI target plate. With this method, ion suppression may occur if too many components are present in a single spot. That means that some compounds might go undetected in this portion of the sample. Therefore, it is crucial that the spotting instrument is able to deposit spots with a speed comparable to the chromatographic resolution. This time-dependent effect is demonstrated by spotting a moderately complex mixture of digested proteins onto a MALDI target using different spotting rates (figure 5). The chromatograms and the corresponding spots clearly indicate that the effective resolution was increased with increasing spotting rate. The fractions shown in the chromatogram in figure 5A were spotted with a spotting rate of 1 spot per 2 minutes. It is obvious that in each spot more than one peak was collected. The same holds true for the chromatogram and the resulting spots shown in figure 5B, where a spotting rate of 1 spot per minute was applied. However, at 2 spots per minute, roughly one peak is collected per spot (figure 5C). Since proteomics samples of high complexity often have many coeluting peptides represented by a single peak, it makes sense to increase the spotting rate per minute to retain the full resolution of the LC run on the MALDI plate. The Agilent 1100 micro fraction collector/spotter is capable of spotting at a maximum rate of 20 spots per minute. The capability of such high spotting rates ensures the conservation of resolution from separations of complex proteome digests even at high flow rates and steep gradients. To demonstrate the full capability of this methodology, a complex digested proteome sample from the cytosol of human lymphocyte cells was separated on a RP nano-bore column and spotted on MALDI targets at different spotting rates with matrix online addition. The applied method is outlined in table 2.

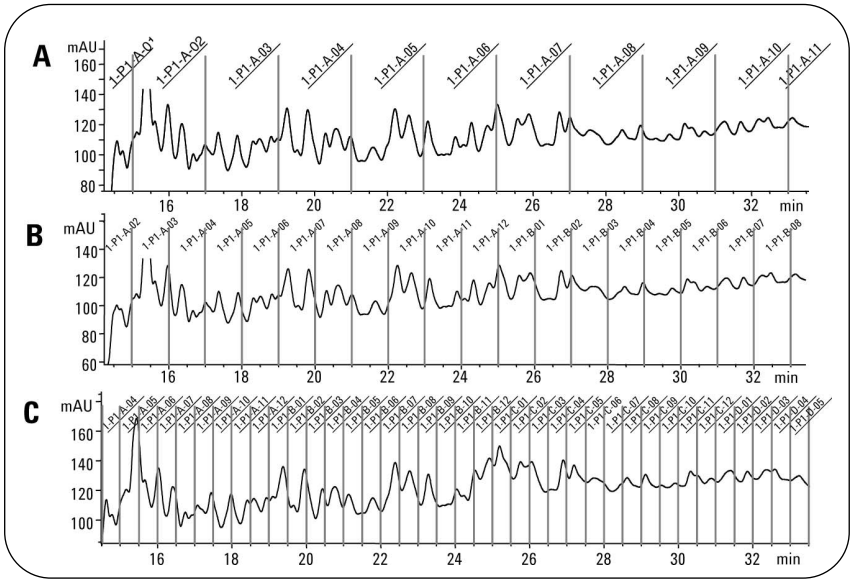

Figure 5
Spotting of a moderately complex digested protein mixture using different spotting rates
A) 0.5 spots/min B) 1 spot/min C) 2 spots/min.

Nanoflow pump	
Column flow:	800 nL/min
Primary flow:	500-800 µL/min
Solvent:	A) water + 0.05% TFA, B) ACN + 0.05% TFA
Gradient:	0 min 5 % B, 10 min 5 %B, 12 min 15 % B, 52 min 65 % B, 55 min 85 % B, 60 min 85 % B
Stop time:	70 min
Post time:	15 min
Injector	
Injection volume:	0.25 µL. Bypass after 10 min
Column	
ZORBAX:	300 SB-C18, 150 mm x 0.1 mm, 3.5 µm
Diode-array detector	
210 nm, ± 8 nm, ref. 360 nm	
Flow cell:	80 nL
Micro fraction collector/spotter	
Time-based spotting onto Agilent AP-MALDI plates between 14–34 minutes during the LC run with spotting rates of 1, 2, 4, 8, and 16 spots per minute. The liquid contact control adjustment was set to 2.	
Matrix addition	
Matrix flow (syringe):	800 nL/min
Matrix:	2 mg/ml CHCA in 50 % iPrOH/49 % H_2O/1 % AcOH
AP-MALDI ion trap MS/MS	
Capillary:	3200 V
Dry gas:	5.0 L/min
Dry temperature:	300 °C
Mode:	AutoMS^2
Number of precursors:	30
Smartfrag:	on
Frag. ampl.:	1.50 V

Table 2
Methods used for LC separation, spotting and MALDI-MS/MS analysis

In this experiment, spotting rates of 1, 2, 4, 8 and 16 spots per minute were used to evaluate the resolution of the LC separation on the MALDI plate and its effect on the quality of the data. These spotting rates resulted in 20, 40, 80,160 and 320 spots, respectively (table 3). It is obvious that with an increasing number of spots, the number of identified proteins, identified peptides and quality spectra significantly increased. For instance, the number of identified proteins increased from 5 to 81 for an increasing spot number from 20 to 320. The protein sequence coverage also increased significantly. This result clearly demonstrates the importance of carefully mapping the highly resolved LC separation of a complex proteome sample into a large number of spots on a MALDI plate to obtain the maximum number of identified proteins. The 26 proteins with the highest score identified from the 320 spot experiment are summarized in table 4. Table 5 illustrates the distribution and the summed precursor abundance of identified peptides assigned to the protein beta-Actin over the different spots. This result was obtained from the 160-spot experiment using a spotting rate of eight spots/minute. The distribution of the peptides indicate that the pep tides are eluted in a standard time

Number of spots	nL/spot *	Proteins identified	Peptides identified	Number of quality spectra	Proteins identified with >1 peptide
20	800	5	6	9	1
40	400	12	14	19	1
80	200	30	39	61	6
160	100	57	75	124	9
320	50	81	112	415	17

*LC effluent only; same amount of matrix solution was added online

Table 3
Data quality as a function of spotting rate.

Protein score	Number of peptides identified	Number of quality spectra	% AA Coverage	Protein name
70.06	6	71	21	beta Actin
59,36	8	80	11	Polyubiquitin
31.67	4	8	8	Heat shock 71 kD protein
29.85	3	7	9	alpha Tubulin
29.53	3	20	9	beta Tubulin
26.82	2	26	8	Glycerinaldehyde 3-phosphate dehydrogenase
23.33	2	2	16	Histocompatibility antigen HLA-Dr
21.08	2	2	7	Aldolase A
19.79	2	8	6	Phosphoglycerat kinase
19.51	2	20	20	Histone H2B
17.71	2	13	9	Nucleolar phosphoprotein B23
16.39	2	3	9	Protein kinase c inhibitor protein
16.27	2	5	8	Triosephosphate isomerase
13.45	2	7	21	Profilin I
11.89	1	2	2	Heat shock 90 kDA protein
10.55	2	2	3	Hypothetical
10.39	2	2	2	Heat shock 70 kDA protein
9.13	1	2	4	L-Lactate dehydrogenase A
8.93	1	4	2	hypothetical
8.70	1	1	4	Hyaluronan-binding protein 1
8.50	1	4	3	Calregulin
8.45	1	2	20	MHC class II antigen
8.40	2	2	4	Lymphocyte cytosolic protein
8.36	1	1	6	Transforming protein RhoA
8.24	1	1	3	Ribonucleoprotein G
8.14	1	3	9	Ribonucleoprotein A1

Table 4
Results for the experiment where 320 spots were depositied on the MALDI plate.

#	Sequence	MH+ Matched (Da)	Spot 5	Spot 20	Spot 21	Spot 23	Spot 41	Spot 42	Spot 43	Spot 44	Spot 47	Spot 48	Spot 49	Spot 50
			Summed precusor abundance											
1	(R)HQGVMVGMGQK(D)	1171.6		14100	12300	21200								
2	(K)IWHHTFYNELR(V)	1515.7					26600	20500	14000	10000				
3	(K)QEYDESGPSIVHR(K)	1516.7	39600											
4	(K)SYELPDGQVITIGNER(F)	1790.9									14300	1580000	9110	6670
5	(R)VAPEEHPVLLTEAPLNPK(A)	1954.1					292000	158000	84900	30900				

Table 5
Distribution and summed precursor abundance of the peptides identified for the protein beta-Actin in the different spots.
Spotting rate: 8 spots/min, Spot volume: 200 nL/spot, MS/MS Score 61.42, % sequence coverage: 18.

frame for peptides and that they are found in consecutive fractions in the series of spots. The fragmentation pattern from the AP-MALDI MS/MS experiments for the peptides of two identified proteins are examples of the high quality data obtained from the HPLC spotter/AP-MALDI trap system (figures 6 and 7). The first example (figure 6) shows the AP-MALDI MS from the spot containing the identified peptide at m/z 1675.4 from Aldolase A (figure 6A) and the corresponding MS/MS spectrum (figure 6B). The assigned MS/MS fragments of the peptide identified for Aldolase A in the fragmentation pattern (figure 6C) were obtained by Spectrum Mill analysis (Hit # 8, Score 21.08, 2 peptides, 7 % sequence coverage). The second example (figure 7) represents the MS/MS fragmentation pattern of a peptide identified for Lymphocyte cytosolic protein 1 (Hit # 23, Score 8.40, 2 peptides, 4 % sequence coverage). For both examples, MS/MS spectra show a good fragmentation pattern with y- and b-series ions sufficient for reliable protein identification.

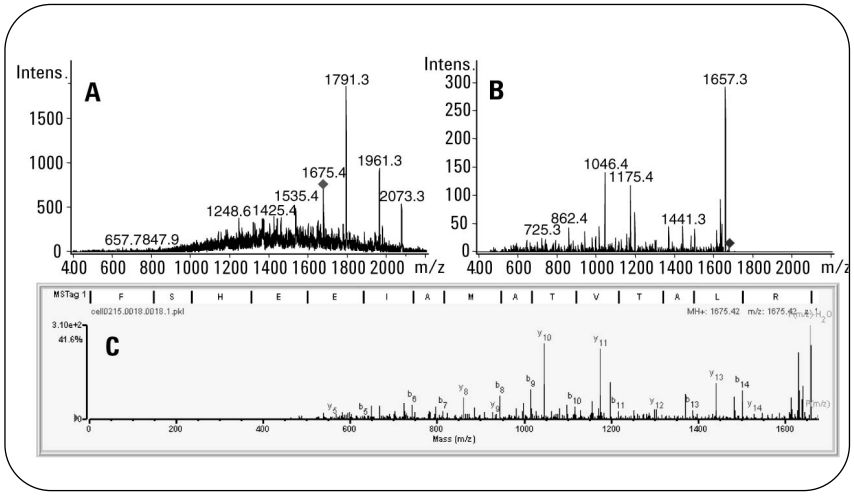

Figure 6
A) AP-MALDI MS of the spot containing an ion at m/z = 1675.4.
B) MS/MS spectrum of precursor m/z = 1675.4.
C) MS/MS fragmentation assignments of this peptide, identified as Aldolase A (Hit 8, Score 21.08, 2 peptides, 7 % sequence coverage).

Conclusions

This Application Note describes a method for coupling of a nano-LC to an AP-MALDI LC/MSD trap system by depositing nanoliter amounts of column effluent onto a MALDI target using the Agilent 1100 Series micro fraction collection/spotting system. For that purpose, an optimized spotting method was developed using simultaneous online matrix addition. The capability of the method was evaluated by the analysis of a complex proteome sample obtained from the cytosol of lymphocyte cells. The developed spotting method to map the LC run on a MALDI plate yielded in dramatic increases in the number of proteins identified and the quality of the data.

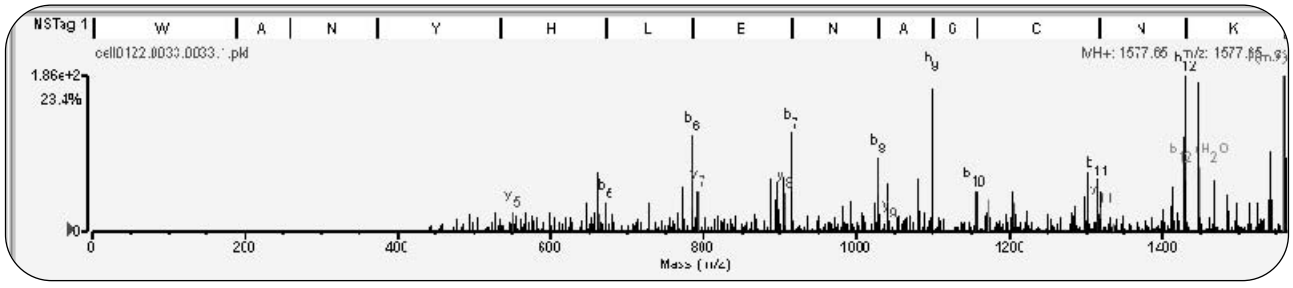

Figure 7
MS/MS peptide fragmentation pattern of the identified peptide from Lymphocyte cytosolic protein 1 (Hit 26, Score 8.40, 2 peptides, 4 % sequence

References

1.
Miller C. A., Yi D., Perkins P. D., *Rapid Commun. Mass Spectrom. 17: 860-868*, **2003.**

2.
"Agilent 1100 Series LC Micro Collection/Spotting System", *Agilent Product Brochure*, *Publication Number 5989-0718EN*, **2004.**

3.
Karas M., Hillenkamp F., *Anal Chem. 60*, *2299*, **1988.**

4.
Kussmann M., Nordhoff E., Rahbek-Nielsen H., Haebel S., Rossel-Larsen M., Jakobsen L., Gobom J., Mirgorodskaya E., Kroll-Kirstensen A., Palm L., Roepstorff P. , *J. Mass Spectrom. 32*, *593-601*, **1997.**

5.
Gobom J., Schürenberg M., Mueller M., Theiss D., Lehrach., Nordhoff E., *Anal. Chem. 73*, *434-438*, **2001.**

6.
Nordhoff E., Schürenberg M., Thiele G., Lübbert Ch., Kloeppel K.-D., Theiss D., Lehrach H., Gobom L., *Int. J. Mass Spectrom 226*, *163-180*, **2003.**

7.
Cohen S.L., Chait B.T., *Anal. Chem. 68*, *31-37*, **1996.**

8.
Vorm O., Roepstorff P., Mann M., *Anal. Chem. 66*, *3281-3287*, **1994.**

9.
Önnerfjord P., Ekström S., Bergquist J., Nilson J., Laurell T., Marko-Varga G., *Rapid Comm. Mass Spectrom. 13*, *315-322*, **1999.**

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