Performance of high-throughput, high-resolution LC MALDI spotting as a tool for protein identification

The Agilent 1100 Series microfraction collection/ spotting system as the missing link between separation and detection in MALDI MS

Application Note

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Abstract

Combining MALDI MS/MS and nanoflow HPLC has the advantage that MS and MS/MS fragmentation is independent from separation. Therefore, selection of precursor ions from complex samples and in depth analysis of fragment ions is possible without any time constraint. To avoid tedious manual spotting procedures with low reproducibility we used the Agilent 1100 Series microfraction collection/spotting system to dispense acurately volumes from 100 nL to a few µL. In this Application Note we describe the performance of the system using a 7-peptide sample and a tryptic digest of four different proteins.

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Introduction

Matrix assisted laser desorption ionisation mass spectrometry (MALDI MS) and electrospray ionisation mass spectrometry (ESI MS) have become the techniques of choice to analyze peptide and protein samples during the past few years. While the ESI approach was basically used as a detection technique in LC-based workflows, MALDI was predominantly applied for peptide mass fingerprinting (PMF) after the sample had been subjected to 2D-gel electrophoresis (2DGE). The recent advent of MALDI Q-TOF and MALDI TOF/TOF instruments allow for relatively fast fragmentation of peptides in addition to accurate mass determination. These instruments provide an opportunity for the analysis of in gel digests or digests of whole proteomes. The main advantage of LC MALDI in comparison to ESI techniques is that separation is decoupled from MS analysis and subsequent peptide fragmentation. Therefore, precursor selection for MS/MS analysis can be performed individually without any time constraint and a more in depth analysis of complex samples is feasible. In addition, samples can be archived on-target enabling re-analysis for data confirmation, or guidance for further optimization of the separation steps.

The combination of LC and MALDI, however, requires instrumentation that is capable of spotting volumes eluting from a nano LC instrument. Volumes spotted are usually in the nanoliter range (100 nL to 1000 nL). Simultaneous matrix addition, in order to overcome tedious manual intervention, without sacrificing sensitivity forms another key feature of this approach.

With the Agilent 1100 Series microfraction collection/spotting system it is possible to dispense volumes acurately from 100 nL per spot to a few microlitres with spotting rates at user defined speed (three seconds to several minutes per spot). Matrix addition can be achieved by using the Agilent matrix addition kit. An external syringe pump delivers the matrix solution, which is channeled via a low dead volume T-piece into the eluent flow of the LC system. The contact control mode of the spotter takes into account the actual flow from the LC pump and the withdraw speed of the spotting capillary is calculated accordingly. This feature ensures accurate droplet deposition by minimizing carryover from spot to spot. If matrix is added by the external syringe pump an adjustment control factor can take this additional flow into account and the withdraw speed is adapted respectively.

The Application Note demonstrates the performance of the Agilent 1100 Series microfraction collection/spotting system in combination with the Bruker Daltonics Ultraflex TOF/TOF. Data is provided from analyses of model peptides and of digested protein mixtures in comparison to manually spotted control samples.

Experimental conditions

All experiments were performed using an Agilent 1100 Series microfraction collection/spotting system (table 1). Separation of peptide samples were performed using a Merck Chromolith 100 µm x 100 mm column with a

Component/module	Ordering number
Nanoflow pump with m-degasser	G2229A
Thermostatted m-wellplate sampler	G1378A
Diode array detector with 80 nL flowcell	G1315B with G1315B#012
Micro collector/spotter	G1364D
Thermostat	G1330B
MALDI carrier and calibration plate	5022-6541 and 5023-0208
Online matrix kit	G1364-68706
Syringe pump	KDS 200 from KDS scientific

Table 1
Components of the Agilent 1100 Series microfraction collection/spotting system.

flow rate of 1 µL/min. A gradient from 3-90 % B was applied over 50 minutes (solvent A: water, 0.05 % TFA; solvent B: acetonitrile, 0.05 % TFA). Spotting frequency was set to 2 spots per min. The online matrix kit was used for the addition of an α-cyano-4-hydroxycinammic acid solution (1 mg/mL in water/acetonitrile/TFA 30/70/0.05 v/v/v). Matrix flow was delivered by a KDS200 syringe pump from KDS Scientific at 1 µL/min and channeled to the LC eluent via a low dead volume T-piece (Valco). Spotting of the matrix/eluent mixture was conducted at a spotting rate of two spots per minute starting ten minutes after gradient initiation. The spotter was run in the contact control mode, which ensures that the spotting capillary is withdrawn at the surface of the spot, thereby minimizing carryover from spot to spot. The adjustment control factor of the spotter was set to a value of two since the actual flow at the spotting capillary tip was doubled by the matrix flow. MS analysis was done on a Bruker Daltonics Ultraflex TOF/TOF with MS data accumulation of 400 laser shots per spot. Database search was performed using the Agilent SpectrumMill Proteomics workbench. Chemicals, peptides and proteins were of analytical grade or better and purchased either from Bachem or Sigma.

Results and discussion

The benefits of LC/MALDI spotting are especially obvious when the sample complexity is increased. The resolution capability of LC-based RP separations after a 2D gel electrophoresis, 1D SDS gel electrophoresis or even multidimensional LC workflow facilitate high quality spots on a MALDI target. Spotting frequency can be adapted to the peak width of the individual eluting compounds to achieve optimal resolution. High resolution of complex samples is a prerequisite for minimizing ion suppression of co-eluting compounds and thus for the detection of low abundant proteins. However, peptide sensitivity and signal to noise ratio (S/N) should not be negatively effected by the automated spotting and matrix addition procedure. In the set of experiments described, first the performance of the spotter was tested with a simple pre-defined peptide mixture using 50 fmol and 2 fmol for each peptide. The second experiment describes the analysis of a tryptic digest of four proteins resulting in a complex peptide mixture. The protein, hemoglobin, is a tetramer that contains two each of the sub-units α-chain and β-chain. All experiments were compared to a manually spotted, unseparated control sample as reference.

The analyzed peptide mixture contained seven different standard peptides (angiotensin III, angiotensin II, [Arg8] vasopressin, angiotensin I, glu1-fibrinopeptide, neurotensin and ACTH(18-39)). The peptide mixture was used in a concentration of 2 fmol/µL. The injection volume of the nano LC was varied to deliver the desired amount of sample into the system. In the 50-fmol experiment all peptides of the mixture were detected (table 2) with a good S/N ratio (>40).

Peptide mixture 50 fmol					
Peptide	**Retention time**	**Monoisotopic mass**	**Max. intensity**	**S/N**	**S/N manual control spots**
Angiotensin I	33.5	1296.7	1517	40	5
Angiotensin II	29.5	1046.5	1797	212	102
Angiotensin III	29	931.5	7705	773	613
Neurotensin	32.5	1672.9	8854	244	54
Glu1-Fibrinopeptide	28.5	1570.7	11156	999	682
[Arg8]-Vasopressin	24	1084.2	5728	1357	618
ACTH (18-39)	37.5	2465.2	3971	335	12.3
Peptide mixture 2 fmol					
Peptide	**Retention time**	**Monoisotopic mass**	**Max. intensity**	**S/N**	**S/N manual control spots**
Angiotensin I	33.5	1296.7			
Angiotensin II	29.5	1046.5	807	16,7	14
Angiotensin III	29	931.5	3111	91	63
Neurotensin	32.5	1672.9			
Glu1-Fibrinopeptide	28.5	1570.7	4115	173,1	49
[Arg8]-Vasopressin	24	1084.2	6314	252,2	119
ACTH (18-39)	37.5	2465.2			

Table 2
A 7-peptide mixture (50 fmol) analyzed by MALDI LC spotting MS.

Even with the 2-fmol injection four of seven peptides were found (angiotensin II, angiotensin III, glu1-fibrinopeptide and vasopressin). MS spectra of the spotted 2-fmol sample demonstrate the excellent S/N ratio obtainable at such low amounts (figure 1).
A complex sample with the four proteins α-amylase, carbonic anhydrase, myoglobin and hemoglobin (sub-units α and β) which were individually digested with trypsin and combined in equal ratios. 10 µL of a diluted solution of this mixture were injected for the experiments providing 100 fmol of each protein. The eluent was mixed with a matrix solution via the T-piece and dispensed in 1-µL droplets onto the target at a spotting frequency of two spots per minute. As a control the digest mixture, containing 100 fmol of each protein, was mixed with matrix and spotted manually onto the same target. The samples were then subjected to MALDI MS analysis. SpectrumMill data analysis showed that for all four proteins the number of identified peptides and the resulting sequence coverage was significantly increased using the Agilent 1100 Series microfraction collection/spotting system (table 3).

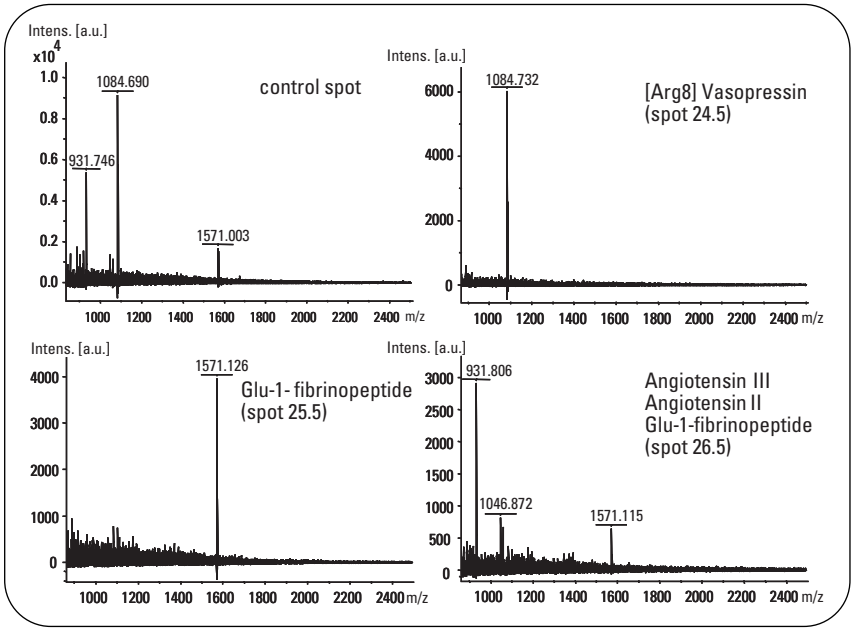

Figure 1
Manual control spot and selected spots of an LC-MALDI experiment extracted from a 2-fmol peptide mixture at 24.5 min, 25.5 min, and 26.5 min run time.

Protein	Identified peptides		Amino acid sequence coverage	
	Spotting	Control	Spotting	Control
α-Amylase	27	13	43	28
Carbonic anhydrase	5	1	27	8
Hemoglobin α	8	2	46	21
Hemoglobin β	10	3	53	24
Hemoglobin g	4	2	26	17
Hemoglobin ε.2 chain	2	1	17	8
Hemoglobin ε 4 chain	4	2	30	14
Myoglobin	16	7	72	45

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Myoglobin	16	7	72	45

Table 3
Analysis of a 4-protein digest by LC MALDI spotting.

The number of identified peptides using the LC MALDI spotting process in comparison to the control sample was increased by up to four times (figure 2). The sequence coverage obtained for all proteins was significantly higher using MALDI spotting with values between 50 % and 200 % above the control sample (figure 3). A comparison of the LC spotted protein mixture and the control sample is illustrated in figure 4 and shows improved spectra quality with respect to peak intensity and signal to noise (S/N). The fact that the number of identified peptides as well as protein sequence coverage increased significantly using LC MALDI spotting in the 4-protein mixture indicates that this will be even more pronounced with a "real" proteome sample, where the initial complexity of the samples are potentially several orders of magnitude higher. These results clearly indicate that the Agilent 1100 Series microfraction collection/spotting system provides an excellent tool for the acquisition of high quality, high throughput data in a LC MALDI workflow for complex proteome samples.

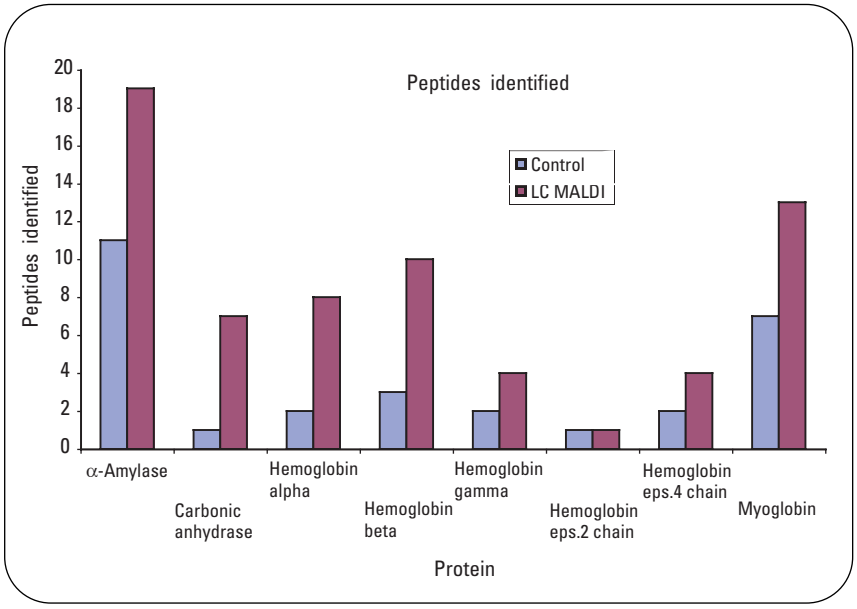

Figure 2
Comparison of peptide ID in a 4-protein mix between LC MALDI spotting and control sample.

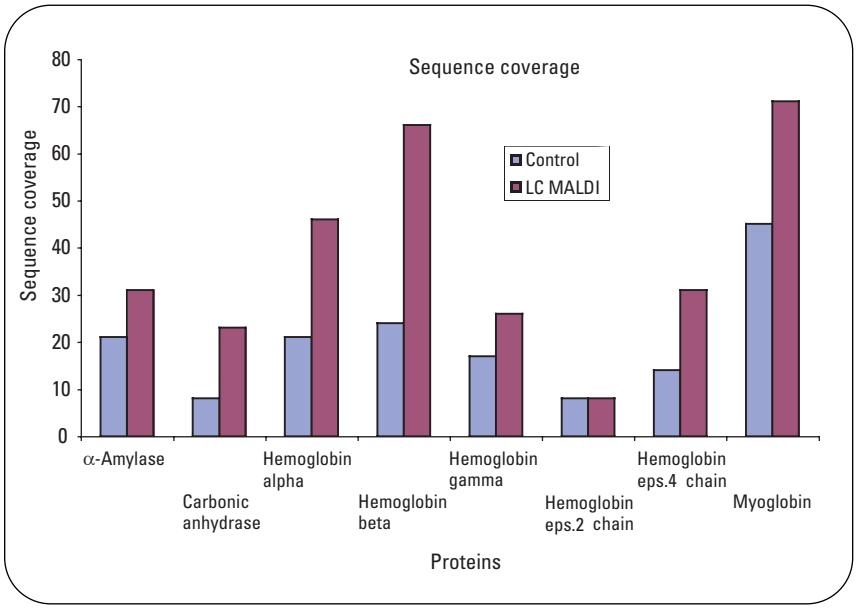

Figure 3
Comparison of sequence coverage in a 4-protein mix between LC MALDI spotting and control sample.

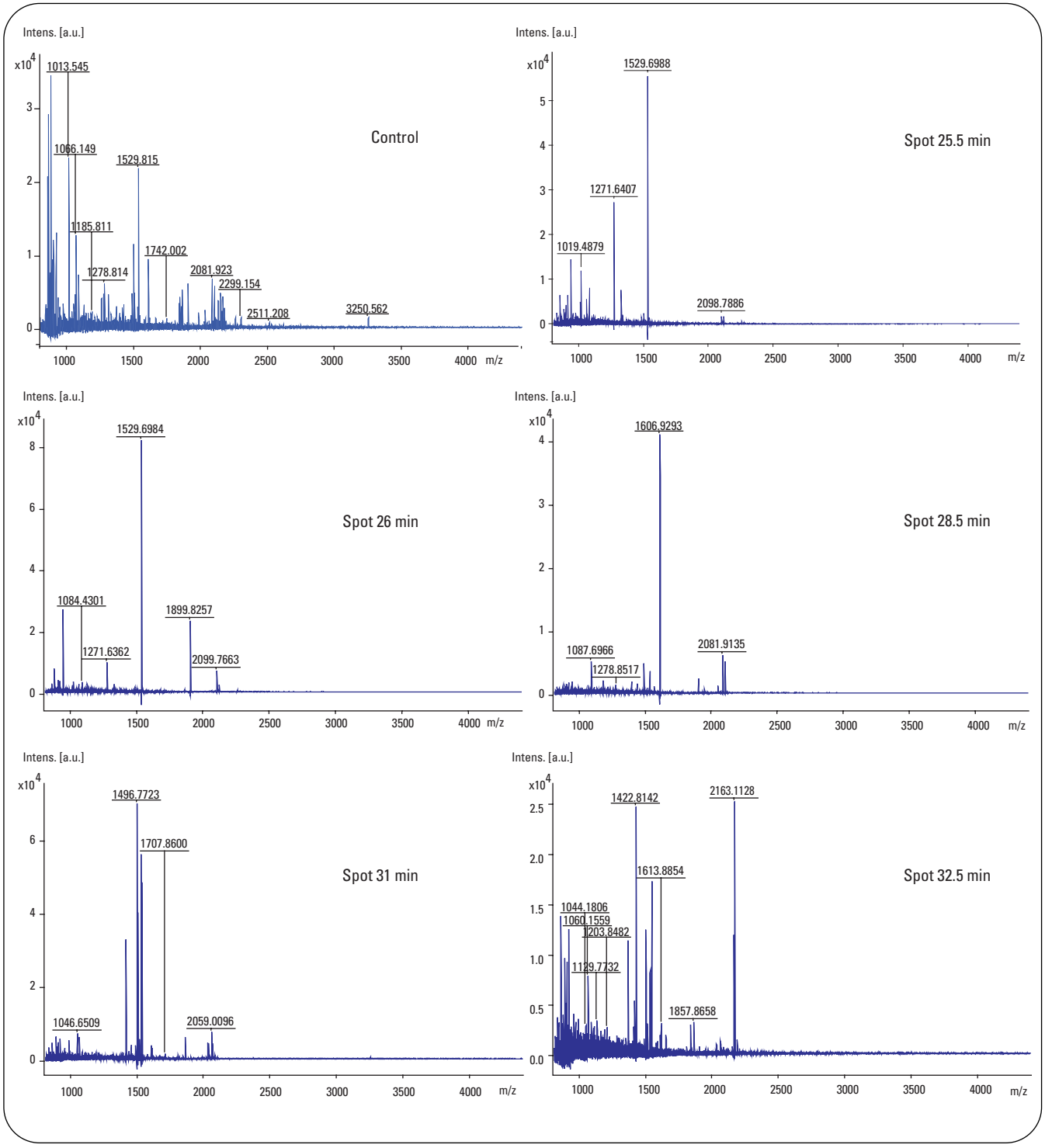

Figure 4
Selected spots from LC MALDI spotting of the 4-protein digest and from manually spotted control sample.

Conclusions

The Agilent 1100 Series microfraction collection/spotting system enables excellent proteome sample analysis in a MALDI MS and MS/MS workflow. It provides a tool that is capable of spotting droplets precisely from 100 nL upwards which is not possible with a manual procedure. The Agilent 1100 microfraction collection/spotting system described represents an ideal interface between high resolution LC separation and MALDI MS and MS/MS analysis. Connecting LC separation and MALDI in combination with small droplet sizes, allows high sensitivity detection of low abundant proteins. Due to high chromatographic resolution, less ion suppression is observed which will finally lead to detection of a larger number of proteins. It was demonstrated that the automated spotting and matrix addition properties of the instrument result in high sensitivity, and reproducible high quality MS data. Especially for samples of higher complexity, the outlined LC MALDI spotting technology proves to be by far superior and much more convenient in comparison to a manual spotting process. Thus it represents a missing link between high resolution separation and high throughput MALDI MS analysis.

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