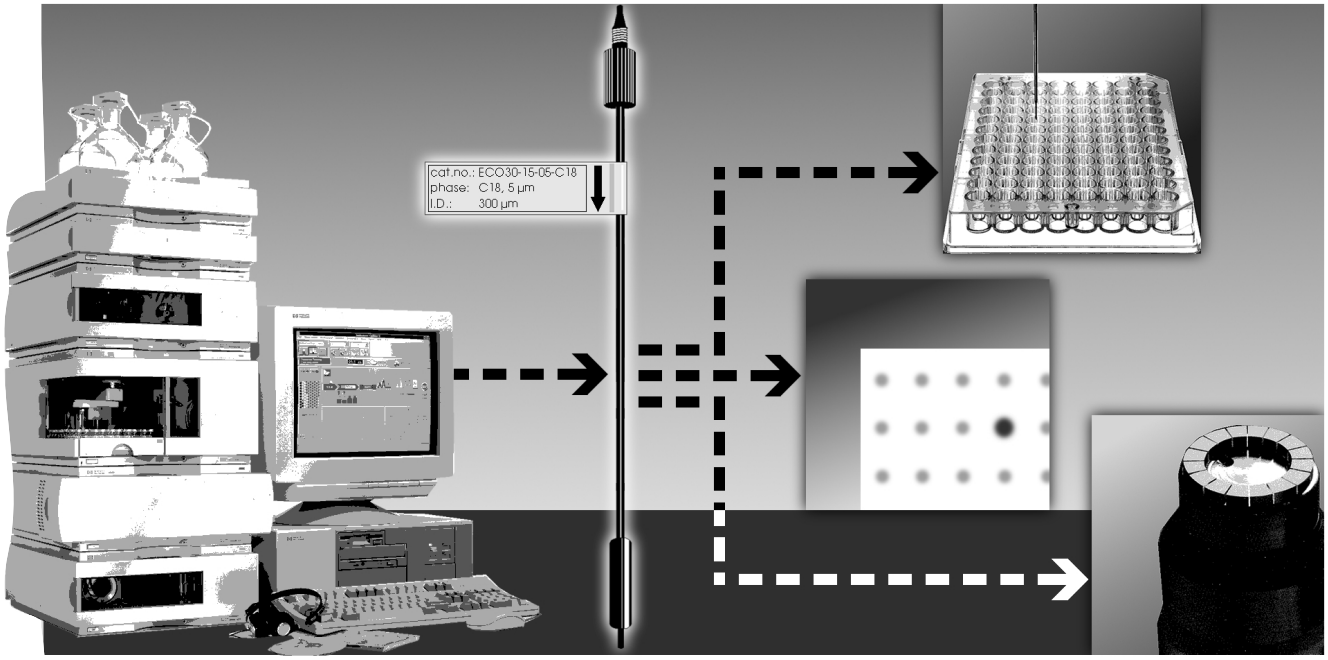
Microbore and Capillary LC with Automated Online Microfraction Collection

Technical Note



Introduction

With limited sample quantities where increased sensitivity is needed, peptide mapping on microbore or capillary LC columns is widely accepted as the preferred method for analysis. Typically columns of 1 mm or capillary columns with 300 µm or even 180 µm inner diameter are used at flow rates of 50, 4 and 2 µl/min, respectively.

A previous technical note[1] published by Agilent Technologies shows how the Agilent 1100 Series modules and systems for LC can easily be converted into a highly efficient capillary LC system using accessories provided by LC Packings (Amsterdam, The Netherlands). Full system flexibility is guaranteed and excellent results with respect to reproducibility and sensitivity are achieved.

Quantitative fraction collection has become a great challenge in the running of microbore or capillary LC separations in the low (microliter) to high (nanoliter) flow rate range. Automated online microfraction collection is a necessity in order to minimize sample loss in case further analysis of the collected fractions—by MALDI-TOF mass spectrometry or protein sequencing—is to be conducted.

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This technical note describes how the Agilent 1100 Series LC system, used for microbore separations or converted to a highly efficient capillary LC system, is coupled to an automated system ("ProBot", BAI, Bensheim, Germany, or in North America from LC Packings) for online microfraction collection of nanoliter or microliter fractions. The system comprises a fixed holder for the collection capillary and a plate for sampling devices which can be moved along the x, y and z-axes through PC software provided by BAI or LC Packings. Applications are shown for microfraction collection onto Agilent MALDI targets or into standard 384-well microtitre plates.

Experimental microbore LC

Peptide mapping on 1.0 mm id columns was performed using a standard Agilent 1100 Series LC system. This method is described in a previous application note.[2]

Capillary LC

For capillary LC an Agilent 1100 Series LC with binary pump, vacuum degasser, variable wavelength detector, thermostatted column compartment and autosampler was used. The system was controlled through an Agilent ChemStation.

The conversion of the Agilent 1100 Series LC system to a capillary LC using the LC Packings accessories, such as a microflow processor, nanoliter flow cells and microcolumns, is described in detail in a previous technical note.[1] All accessories can be ordered from LC Packings, Baarsjesweg 154, NL-1057 Amsterdam, Netherlands or 80 Carolina Street, San Francisco, CA 94103, USA.

Installation of the microfraction collector

The "ProBot" system is supplied by BAI (Heimrod-Strasse, 64625 Bensheim, Germany) or in the USA by LC Packings (80 Carolina Street, San Francisco). The microfraction collector is placed underneath the variable wavelength detector to minimize the delay volume between the detector cell and the collection capillary. The fused-silica outlet capillary from the nanoliter detector flow cell is then introduced and fixed in the fraction collection arm of the "ProBot" system. After hardware installation, the software for controlling the microfraction collector—supplied by BAI on diskette—is loaded on the Agilent ChemStation. Before programming the parameters for collection onto the various collection devices, a starting point where the first fraction should be spotted has to be defined. This is programmed by moving the collection arm electronically to the first position of the collection device and saving the x, y and z-coordinates. Then, depending on whether round or linear collection devices are used (Agilent MALDI targets, microtitre plates or membranes) the following parameters have to be programmed.

Programming of round devices

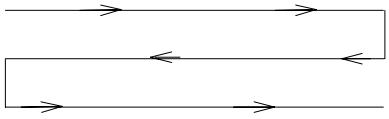
(for example the Agilent MALDI targets, 10 positions, placed in a homemade target holder). See figure 1.

- Enter the radius of the round target in millimeters into "Radius 1". (Where an Agilent target is used, "Radius 2" and "Radius 3" can be left blank as these have only single circle of target positions. These need to be programmed for Bruker MALDI targets which have 3 circles).
- Enter the distance from target to target in millimeters. For example, in the case of our homemade target holder "Next target" = 20 mm.
- Enter the number of spots on one target into "Spots outer". This will be either 10 or 16 for the Agilent MALDI target or for the Bruker 14, 8 and 4.
- Enter the total number of targets into "Target".
- Enter the speed of the arm in mm/s (maximum is 20 mm/s) into "Speed".
- Enter the size of the fractions in seconds into "Segm Time". For example 30 s means 1 µl at a flow rate of 2 µl/min.
- Enter the z-value in millimeters into "Z to Z". This describes how far the arm goes up during movement from one spot to another.

Programming of linear devices

(for example microtitre plate, 384 wells). See figure 2.

- “X to X”: distance from well to well in millimeters.
- “Y to Y”: distance from row to row.
- “Z to Z”: how far the arm goes up during movement from well to well.
- “X-points”: total number of wells in x direction.
- “Y-points”: total number of wells in y direction.
- “Speed”: speed of arm in mm/s (recommended setting is 20 mm/s—this is also the maximum setting).
- “Segm time”: size of fractions in seconds, for example 30 s means 2 µl at a flow rate of 4 µl/min.
- click on box “ZigZag”. This chooses the shortest direction for fraction collection. For example:



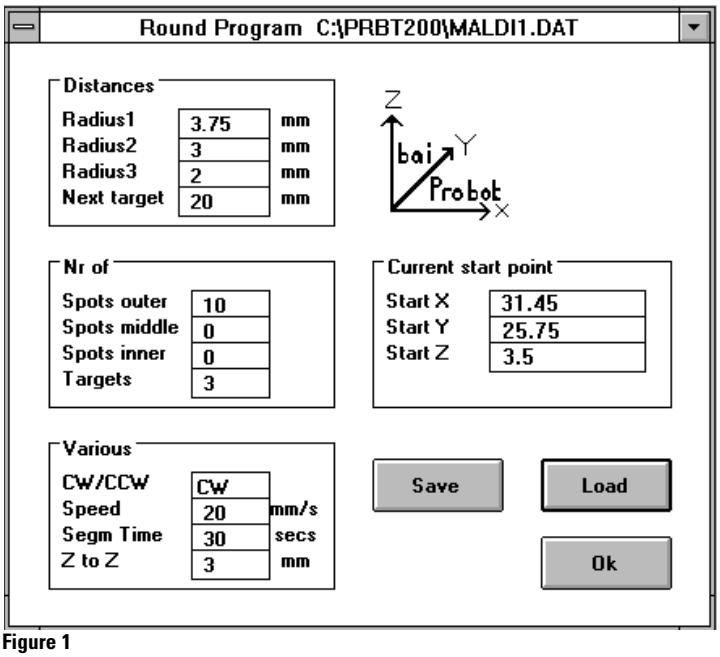


Figure 1
Programming of the ProBot system for microfraction collection onto MALDI targets

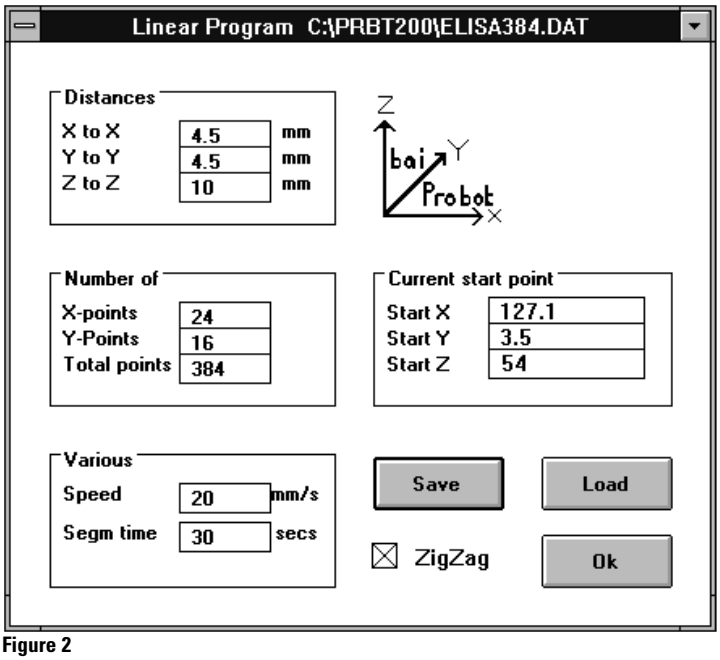


Figure 2
Programming of the ProBot system for microfraction collection into 384-well microtitre plates

Conditions for peptide mapping

Mobile phase:
Solvent A: H_20, 0.05% TFA
Solvent B: Acetonitrile, 0.045% TFA

Gradient composition:
1. short gradient (180 µm and 300 µm id column)

0 min	1% B
35 min	35% B
50 min	70% B
55 min	1% B

2. long gradient (1.0 mm id column)

0 min	1% B
120 min	45% B
160 min	90% B
165 min	1% B

Flow:
180 µm id column: 2 µl/min
(with flow splitter IC–100–VAR, LC Packings)
300 µm id column: 4 µl/min
(with flow splitter IC–100–VAR, LC Packings)
1.0 mm id column: 50 µl/min
(without flow splitter)

Injection:
Typical injection volumes for capillary LC separations are between 0.1–2.0 µl. To avoid a potentially large delay time caused by the injection loop volume when using such low flow rates, the injection valve is bypassed shortly after injection using a simple injector program:

Draw	1.0 µl from sample
Inject	
Wait	4.00 min
Valve	bypass
Wait	60 min
Valve	mainpass

For standard separations using 1.0 mm id columns the injection loop was not bypassed.

Columns:
A 300 µm or 180 µm id × 250 mm Vydac C-18 (Fusica, LC Packings) column is used for online fraction collection onto the MALDI targets or onto sequencing membranes. A 1.0 × 250 mm Vydac C-18 (Agilent 7991803-581) is used for fraction collection into the microtitre plate.

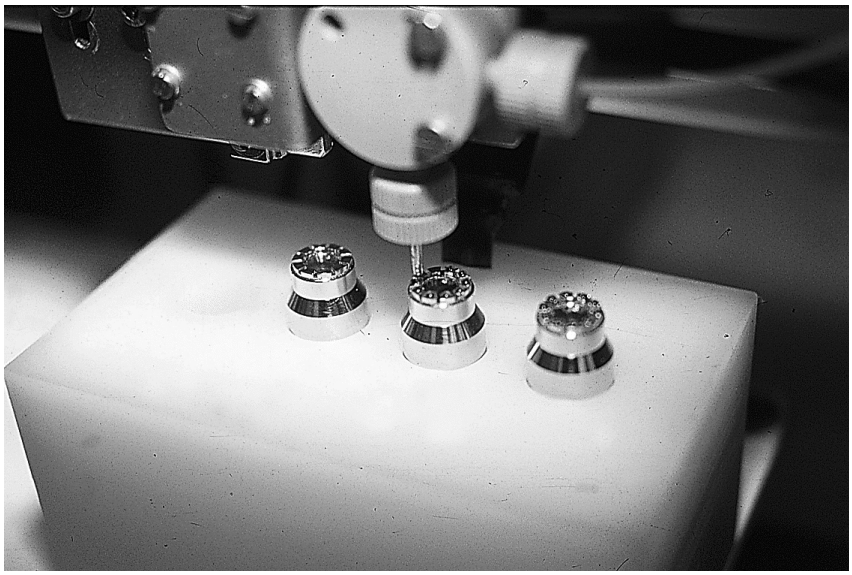


Figure 3
Automated microfraction collection of 1 µl peptide fractions from a peptide map onto MALDI targets

Preparation of targets for MALDI-TOF/MS analysis

α-cyano-4-hydroxycyanamic acid (Agilent order number G2054-85010) should be crystallized onto the targets prior to microfraction collection using the Agilent sample prep accessory (Agilent order number G2024A). After fraction collection samples are vacuum-dried and analyzed using MALDI-TOF/MS.

Results

Flow rates for separations on capillary columns of 180 or 300 µm inner diameter are typically 2–4 µl/min. Since the widths of eluting peaks are mostly between 0.3–0.5 min, the volume of fractions is between 600 nl and 2 µl.

Regular fraction collection of these very small volumes in small vials followed by pipetting steps would normally lead to significant sample loss, therefore automated online microfraction collection is essential.

Using the BAI microfraction collection system “ProBot”, reliable fully-automated fraction collection of 1 µl volumes directly onto the MALDI targets is achievable, see figure 3.

After collection, fractions are dried using the Agilent sample prep accessory and analyzed using MALDI-TOF mass spectrometry. A peptide map of 700 fmol myoglobin tryptic digest and some representative MALDI spectra are shown in figure 4.

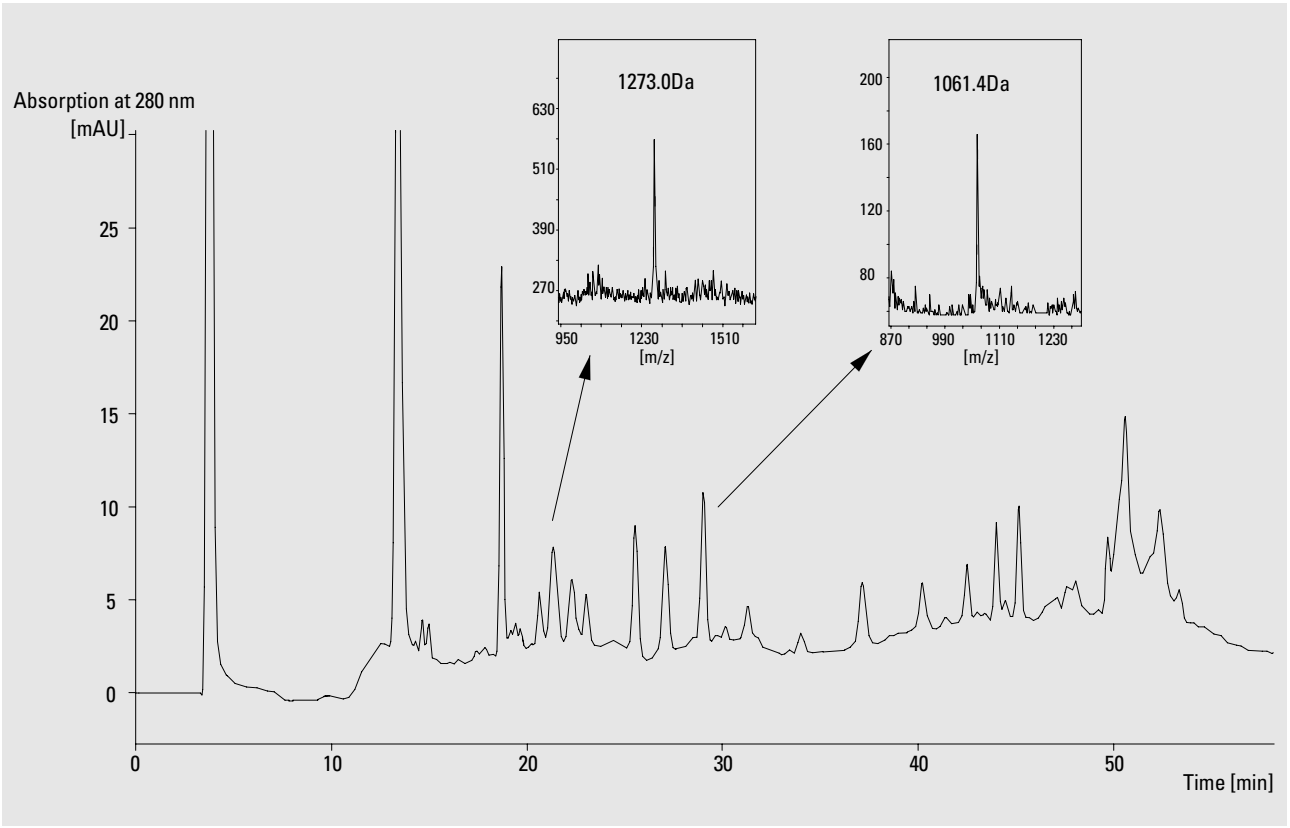


Figure 4
Separation of 700 fmol myoglobin trypic digest followed by automated microfraction collection onto MALDI targets and MALDI-TOF/MS analysis

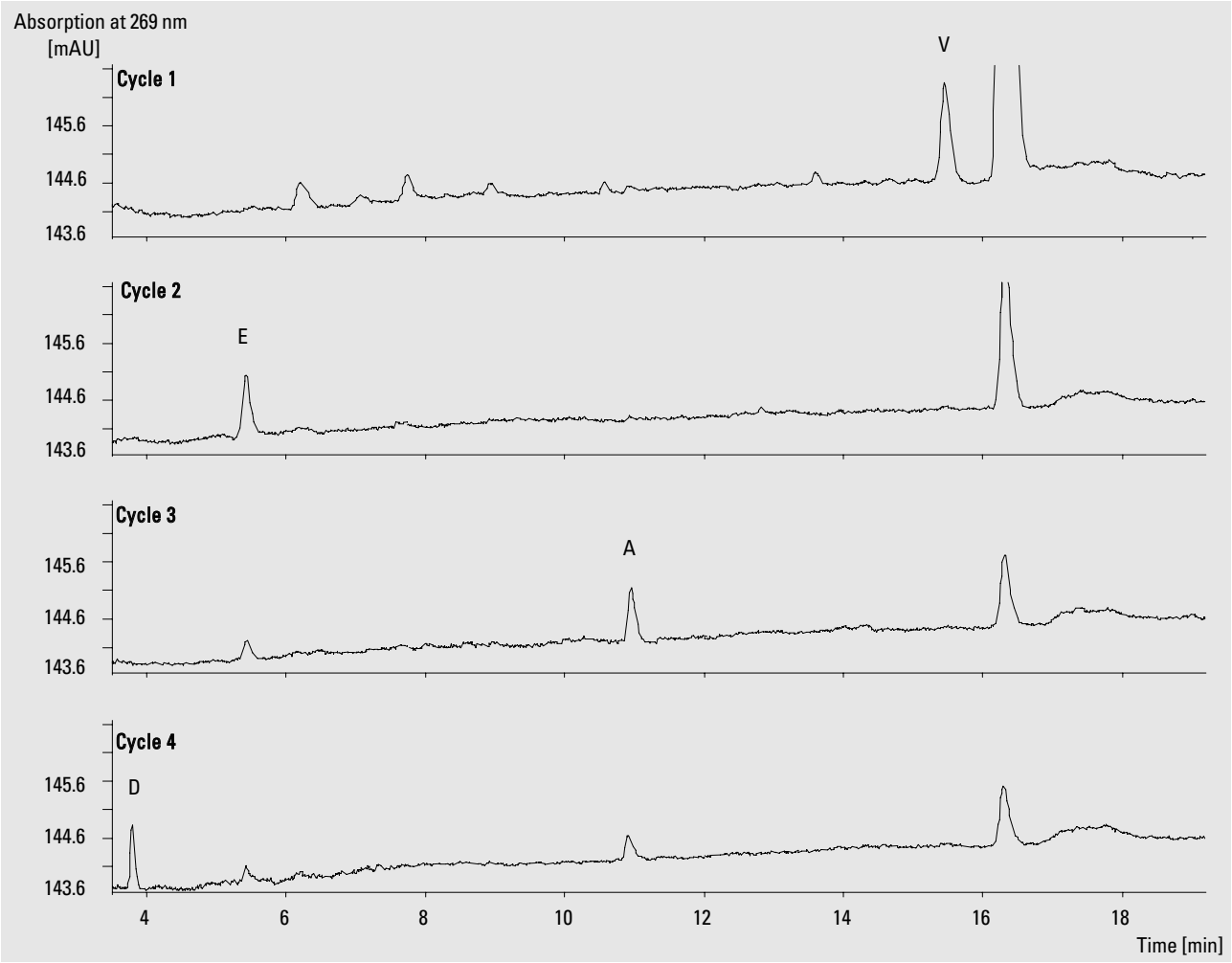


Figure 5
Protein sequence analysis of a peptide fraction of a myoglobin tryptic digest collected onto a sequencing membrane. Cycles 1–4 are shown

Using a similar approach, peptide fractions of 1–4 µl volumes can also easily be collected onto a sequencing membrane. After collection of the fractions—which are easily detected by visual inspection of the membrane surface for marks of the collection capillary—the spot of interest is excised with a scalpel and placed into an empty sequencing cartridge. Protein sequence analysis of a collected fraction from a 8 pmol myoglobinpeptide map demonstrating Edman degradation cycles 1–4 is shown in figure 5.

The "ProBot" microfraction collector can also be used for automated fraction collection of larger fraction volumes which are typically obtained from separations using 1.0 mm id columns at 50 µl/min flow rate. In tests, a very complex peptide map, a tryptic digest of the 125 kDa plant photoreceptor protein phytochrome, was separated on a 1.0 × 250 mm id Vydac C-18 column. Fractions of 30 seconds corresponding to 25 µl volume were collected automatically into a 384-well microtitre plate. After collection aliquots of several fractions were analyzed by MALDI-TOF mass spectrometry, see figure 6. Further confirmation of the tryptic fragments were obtained by protein sequencing of the residual fraction.

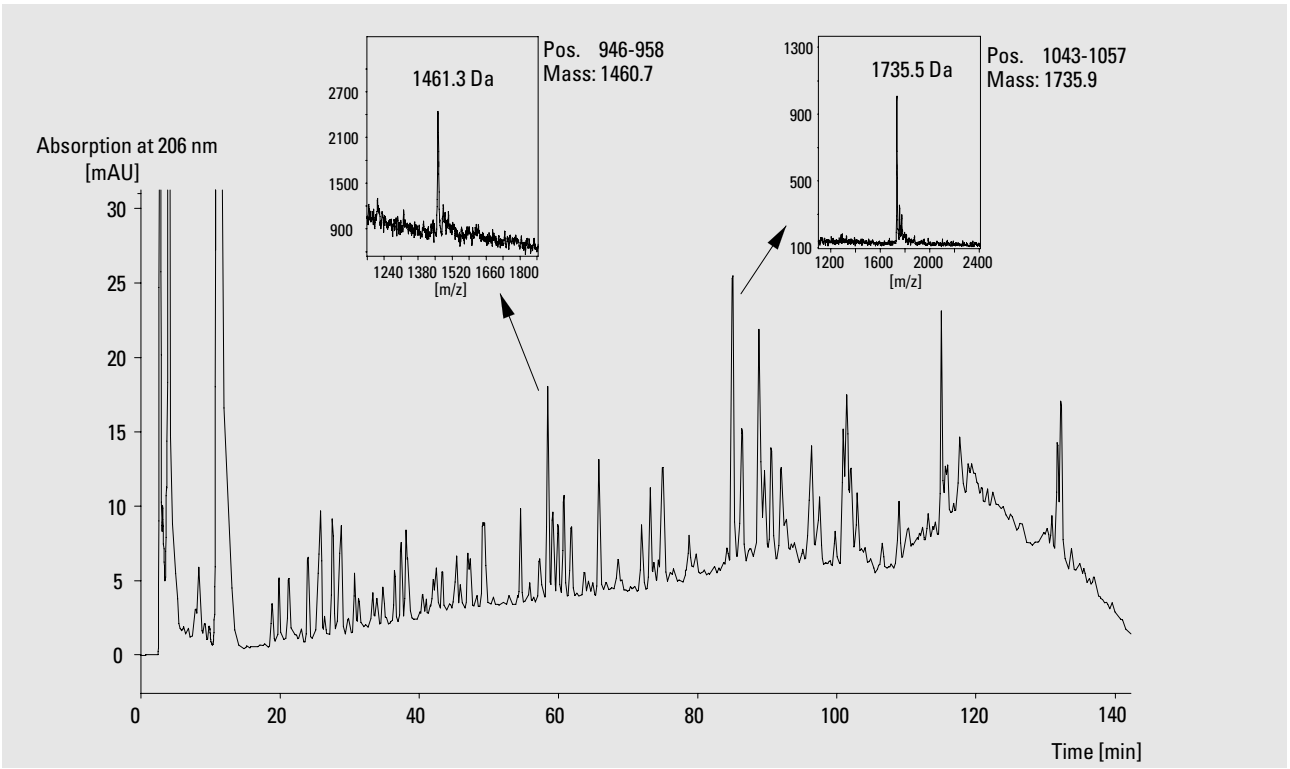


Figure 6
Peptide mapping of a trypic digest of phytochrome. MALDI-TOF/MS analysis of 2 representative automatically collected fractions

Conclusions

With the use of the BAI "ProBot" microfraction collection system, fully-automated microfraction collection of nanoliter to microliter volumes can be achieved reliably onto MALDI targets, sequencing membranes or into wells of microtitre plates. The system is easy to program and there are no limits on the number of fractions that can be collected. With its excellent flexibility it represents an ideal device for unlimited microfraction collection from capillary LC separations. Microsample handling is simplified and sample loss significantly reduced allowing offline MS analysis of peptides down to the femtomol range and protein sequence analysis at the lowest detection limit of the sequencing system.

References

1
"Capillary liquid chromatography with the HP 1100 Series modules and systems for HPLC", *Agilent Technologies Technical Note*, **1996**, publication number 5965-1351E

2
Serwe, M., Glatz, B., "Peptide separations by microbore reversed-phase HPLC", *Agilent Technologies Application Note*, **1996**, publication number 5964-9935E

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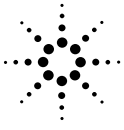

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