application note

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Effective desalting and concentration of in-gel digest samples with Vivapure® C18 Micro spin columns prior to MALDI-TOF analysis.

A. Kocourek[1], M. Naldrett[2] and R. Zeidler[1].

1. Vivascience AG, Hanover, Germany

2. John Innes Centre Proteomics Facility, Norwich, UK

Introduction

The identification of proteins plays an important role in today's pharmaceutical and proteomics research. Commonly used methods for separating proteins from complex samples are 1D or 2D gels. Relevant protein spots are cut out of the gel and after in-gel digestion the peptides are spotted onto a target for matrix-assisted laser desorption/ionisation - time of flight mass spectrometry (MALDI-TOF-MS). Subsequently, the protein can be identified by comparison of the resulting signals with different databases. However, it is well known that the success of protein identification can be enhanced by desalting and concentration of the peptide samples. Traditionally, the peptide solution is purified with self-prepared or commercially available pipette tips containing C-18 material prior to MALDI analysis.

Normally, many repetitive pipette steps are necessary and the sample volume is limited to 10-20 µl. Alternatively, the newly developed Vivapure® C18 Micro spin columns containing a C-18 membrane can be utilized for sample preparation. They are designed to be used in a standard laboratory micro centrifuge and can be loaded with up to 200 µl of sample solution. During centrifugation the solution flows through the approximately 3 mm^2 membrane area. The membrane is an innovative combination of Sartorius membrane adsorber technology with C-18 chemistry. The bound peptides can be desalted easily by subsequent centrifugation steps. For elution, a 200 µl PCR tube is attached to the base of the device. Peptides are recovered with 2-4 µl 50% acetonitrile/ 0.1% trifluoroacetic acid (TFA) containing matrix and are directly spotted onto a target for MALDI analysis.

This technique offers a very fast, parallel and effective method to desalt and concentrate up to 200 µl of highly dilute peptide solutions from any source e.g. 2D PAGE, chromatographic methods or directly from biological samples. Here, we describe the preparation of very low concentration (<1 fmol/µl) peptide samples generated from bovine serum albumin (BSA) digestion with the Vivapure® C18 Micro spin columns. The samples were spotted with the dried droplet method and the data were acquired automatically.

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Methods and Materials

In-gel digestion of the samples

1. Place the gel plugs (no bigger than 2 mm^3) containing 100 fmol BSA into a PCR tube.
2. Wash the gel plug in 50 µl solution B for 20 min (to equilibrate to ~pH 8 and to remove excess stain and sodiumdodecylsulfate), then remove the liquid. Solution B is freshly prepared 400 mM ammonium bicarbonate and 100% acetonitrile, in a ratio of 1:1.
3. Shrink the gel piece(s) with 50 µl 100% acetonitrile for 10 min, then remove the acetontrile.
4. Repeat step 2.
5. Repeat step 3.
6. Add dithiothreitol (DTT) solution (40 µl of 10 mM DTT in 25 mM ammonium bicarbonate) and incubate for 30 min (or longer) at 60°C.
7. Cool to room temperature and remove the DTT.
8. Add iodoacetamide solution (30 µl of 100 mM iodoacetamide in 50 mM ammonium bicarbonate) and incubate for 40 min at room temperature.
   Remove the liquid.
9. Wash the gel pieces with 50 µl of 25 mM ammonium bicarbonate for 10 min.
10. Dehydrate with 50 µl of acetonitrile for 15 min and remove the liquid.
11. Rehydrate with 50 µl 25 mM ammonium bicarbonate for 15 min and remove the liquid.
12. Dehydrate with 50 µl acetonitrile for 10 min and remove the liquid.
13. Dehydrate with 50 µl acetonitrile for a further 5 min and remove the liquid.
14. Rehydrate the gel pieces for 10 min with 10 µl of 25 mM ammonium bicarbonate solution containing 75-100 ng of trypsin solution
15. Without removing surplus trypsin solution add 15 µl of 25 mM ammonium bicarbonate. Incubate for 3 hours at 37°C.
16. Add 7 µl 10% formic acid.
17. These samples were frozen in liquid nitrogen and stored at –80°C until required.
18. For the purpose of this experiment samples were diluted to 100 µl with 0.1% TFA just prior to use to give a nominal concentration of 1 fmol/µl. It should be noted that such low concentration samples are extremely unstable and following dilution should be used within 5 min.

Sample desalting and concentration with Vivapure® C18 Micro spin devices

General notes:

1. Always use the spin device in the same orientation, e.g. always align the hinge of the cap towards the centre of the rotor.
2. All peptide samples should be spotted directly onto the membrane and not against the plastic housing to avoid losses.

Sample clean-up: Binding peptides

1. Wash the membrane with 200 µl 0.1% TFA in acetonitrile by centrifugation for 20 s at 400 x g. Repeat. Discard the flow through.
2. Equilibrate the membrane with 200 µl 0.1% TFA in purest water (arium water system) by centrifugation for 30 s at 400 x g. Repeat. Discard the flow through.
3. Load the peptides in 10-200 µl of 0.1% TFA or 0.1% formic acid (higher amounts of acid should not be a problem). Centrifuge for 40 s at 400 x g. Discard the flow through.

Note: Check the orientation of the device; see general notes!

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4. Wash with 200 µl of 0.1% TFA by centrifugation for 40 s at 400 x g. Repeat this wash step. Discard the flow through.
5. Centrifuge at 13,000 x g for 30 s to reduce the aqueous content. Discard the flow through.
6. Attach a PCR tube to the bottom of the spin column and insert both into the 2 ml tube. Elution (for peptide samples from in-gel digest).

Note: Work quickly when using low elution volumes to avoid evaporation of solvent.

1. Load 3 µl of a solution of a-cyano-4-hydroxycinnamic acid (HCCA, 10 mg/ml) in 50% acetonitrile and 0.1% TFA onto the membrane.
2. Centrifuge for 30 s at 400 x g then 30 s at 13,000 x g.
3. Spot a portion or all of the eluate directly onto a target to give dried droplet preparations.

MALDI-TOF measurements

Samples were run on a Bruker Reflex III MALDI-ToF mass spectrometer in reflectron mode. Resulting spectra were calibrated using three BSA peptides as internal standards. Signals were annotated automatically.

Mascot search

The Mascot search was done with the following internet link from Matrix Science Ltd (London, UK): *www.matrixscience.com* using the following parameters

Search Parameters	
Type of search	Peptide Mass Fingerprint
Database	MSDB
Enzyme	Trypsin
Fixed modifications	Carbamidomethyl (C)
Variable modifications	Oxidation (M)
Mass values	Monoisotopic
Protein Mass	Unrestricted
Peptide Mass Tolerance	± 50 ppm
Peptide Charge State	1+
Max Missed Cleavages	1

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Preparation without Vivapure® C18 Micro spin column:

The BSA digest sample with a nominal concentration of 1 fmol/µl was mixed (1:1) with matrix in 50% acetonitrile / 0.1% TFA and 0.5 µl spotted onto a MALDI target nine times. Only one of nine spots of the sample gave signals that could be analysed. The spectrum is shown in the following figure.

The peak list was used for a Mascot search and the results are summarized in the following table:

Mascot score	111
Searched peptides	7
Matched peptides	7
Sequence coverage	14%

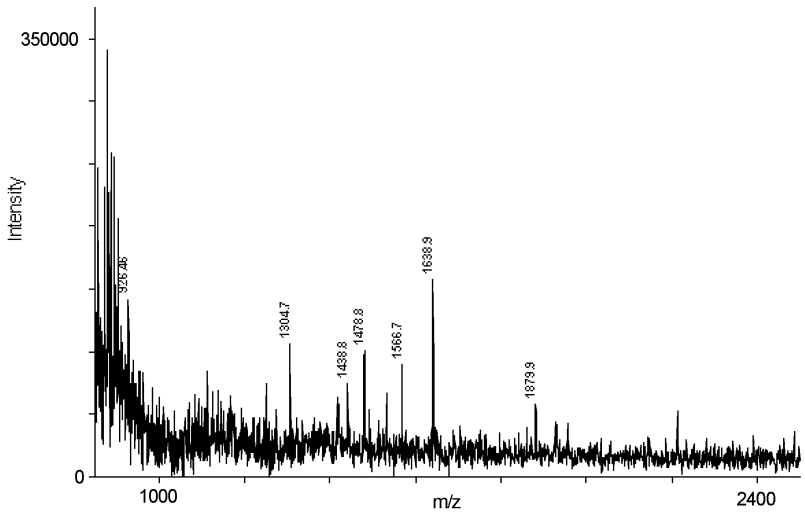

Fig. 1: MALDI spectrum of 0.5 µl of a < 1 fmol/µl BSA digest. The annotated signals belong to BSA peptides.

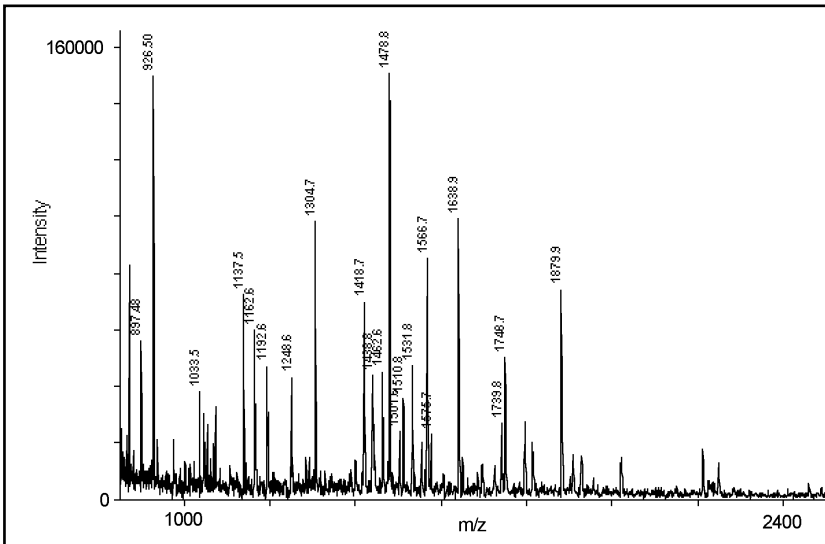

Fig. 2: MALDI spectrum of 0.5 µl of the eluate after preparation with the Vivapure® C18 device. The annotated signals belong to BSA peptides.

Preparation using Vivapure® C18 Micro spin column:

100 µl of the BSA digest were used for preparation with the Vivapure® C18 Micro spin columns. The bound peptides were eluted with 3 µl of a solution of a-cyano-4-hydroxycinnamic acid (HCCA, 10 mg/ml) in 50% acetonitrile and 0.1% TFA. 0.5 µl of the eluate were spotted six times onto a target to give dried droplet preparations. All spots gave very consistent results in contrast to the preparation without using the Vivapure® C18 spin device device. One of the resulting spectra is exemplarily shown below.

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The peaklist was used for a Mascot search and the result is summarized in the following table:

	Original Sample	Vivapure® C18
Mascot score	111	251 ± 35
Searched peptides	7	26 ± 1
Matched peptides	7	20 ± 4
Sequence coverage	14%	34 ± 3%

Conclusions

Highly diluted protein digest solutions are extremely unstable and difficult to handle and losses occur fast. The Vivapure® C18 Micro spin devices offered a chance to quickly desalt and concentrate a sample (<15 min) from a high volume of liquid (100 µl). Hence, many more signals for peptides of interest were detected in the MALDI spectrum. Subsequently the protein identification was much easier and more convincing as indicated from the higher Mascot score and sequence coverage. Whereas only one out of nine spectra from the original dilute sample could be analysed, the preparation with the spin devices gave very consistent results.