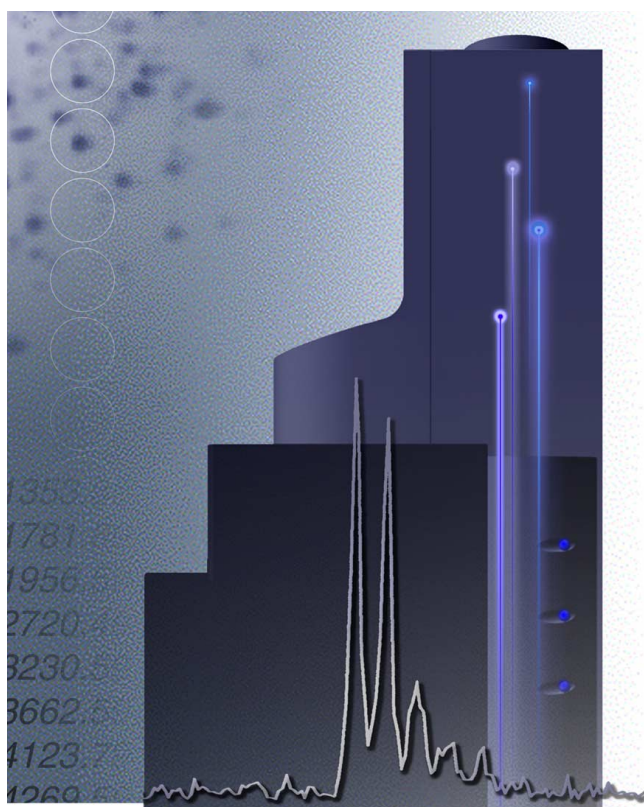


Ettan *MALDI-ToF*

Method Handbook



All goods and services are sold subject to the terms and conditions of sale of the company within the Amersham Biosciences group which supplies them. A copy of these terms and conditions is available on request.

Ettan is a trademark of Amersham Biotech Limited.

Amersham is a trademark of Nycomed Amersham plc.

ZipTip is a trademark of Millipore Inc.

©Amersham Biosciences AB 2000
-All rights reserved

Office Addresses

Amersham Biosciences AB
SE-751 84 Uppsala
Sweden

Amersham Biosciences UK Limited
Amersham Place Little Chalfont
Buckinghamshire
England HP7 9NA

Amersham Biosciences Inc.
800 Centennial Avenue
P.O. Box 1327 Piscataway N.J. 08855-1327
USA

Amersham Biosciences Europe GmbH
Munzinger Strasse 9
D-79111 Freiburg
Germany

Amersham Biosciences KK
Sanken Building
3-25-1 Hyakunincho, Shinjuku-ku
Tokyo
Japan

Contents

1 Introduction

1.1 Symbols and common abbreviations	8
--	---

2 An introduction to MALDI-ToF MS

2.1 Principles of MALDI-ToF mass spectrometry	10
2.2 Applications for MALDI-ToF mass spectrometry	12

3 Sample Preparation

3.1 Removal of contaminants	17
3.2 Sample Extraction from Electrophoresis Gels	19
3.3 Choosing the Matrix	20
3.4 Preparing Solutions for Crystallization	22
3.4.1 <i>Dissolving the protein or peptide</i>	23
3.4.2 <i>Dissolving the matrix</i>	23
3.5 Applying Sample to the Slide Surface	24
3.5.1 <i>Dried Droplet Method</i>	25
3.5.2 <i>Thin Layer Method</i>	26
3.5.3 <i>Slow Crystallization Method</i>	27
3.5.4 <i>Seeded Microcrystalline Film Method</i>	28
3.6 Preparation for Specific Applications	29
3.6.1 <i>Identification by peptide mass fingerprinting</i>	30
3.6.2 <i>Protein characterization I</i>	32
3.6.3 <i>Protein characterization II</i>	33
3.6.4 <i>Identification of protein phosphorylation sites</i>	36

1 Introduction

Ettan MALDI-ToF combines the protein knowledge of Amersham Biosciences that of experts in protein identification (Proteomics LLC) and mass spectrometry (Scientific Analysis Instruments Ltd.) to offer a MALDI-ToF mass spectrometer for fast, reliable protein identification. Ettan MALDI-ToF is particularly suited for high throughput work requiring rapid identification and high sensitivity detection, as in proteomic studies.

In order to obtain an accurate mass analysis and so achieve a reliable identification, the correct sample extraction and preparation procedures are as crucial as the interpretation and evaluation of mass spectra. The purpose of this handbook is to familiarize the reader with the key practical aspects that should be considered for successful protein identification.

Specific protocols are supplied for applications performed on Ettan MALDI-ToF systems, together with general background information that may be of use to those new to MALDI-ToF mass spectrometry.

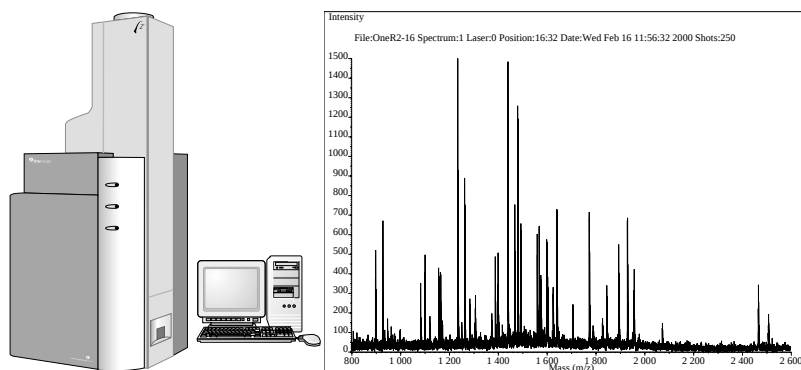


Fig 1-1. The Ettan MALDI-ToF system, Single high resolution result and ProFound identification report

1.1 Symbols and common abbreviations



general advice that can improve procedures or recommendations for action under specific situations



warning, when special care should be taken in a procedure

MS: mass spectrometry

MALDI-ToF: matrix-assisted laser desorption and ionization-time-of-flight

4-HCCA: α -cyano-4-hydroxycinnaminic acid

DHB: 2,5-dihydroxybenzoic acid, also known as gentisic acid

2 An introduction to MALDI-ToF MS

MALDI-ToF mass spectrometry has become one of the most important tools for the identification of proteins and peptides, and is particularly important in proteomic studies where it plays a key role in the identification of potential target proteins, as illustrated in Fig. 2-1. This chapter gives an overview of the key steps in mass analysis by MALDI-ToF MS.

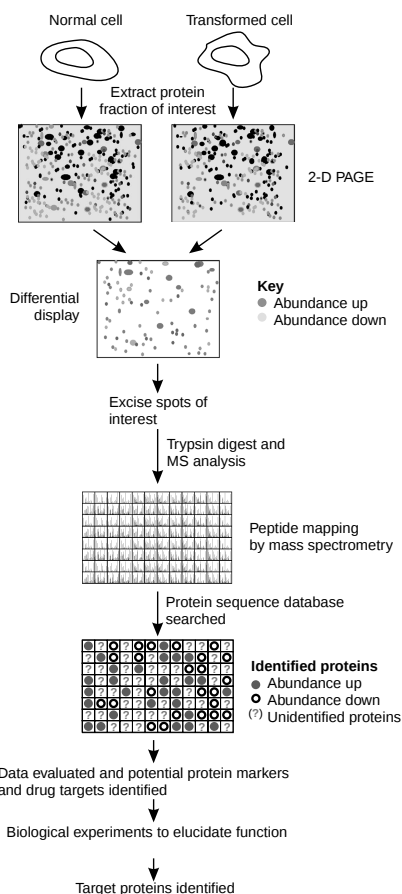


Fig 2-1. The role of mass spectrometry in proteomic studies

2.1 Principles of MALDI-ToF mass spectrometry

A mass analysis begins by careful sample preparation. Most frequently the source material originates from an SDS electrophoresis gel and samples must be extracted and cleaned before preparation for the MS analysis.

The cleaned protein or peptide is mixed with a *matrix*. The matrix ensures that the surroundings of a protein or peptide are such that, when subjected to the intense light pulse from a laser, the molecules become *ionized*. As the matrix *crystallizes* on the surface of the *sample slide* (Fig. 2-2), the proteins or peptides become incorporated into the crystals. The sample slide is used to carry the samples into the flight tube of the mass spectrometer.

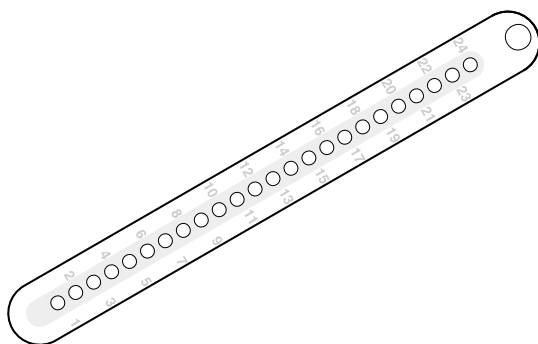


Fig 2-2. Sample slide from Ettan MALDI-ToF

Fig. 2-3 illustrates the process that follows as soon as the samples are loaded into the mass spectrometer. It is at this stage that *Matrix-Assisted Laser Desorption and Ionization* (MALDI) begins.

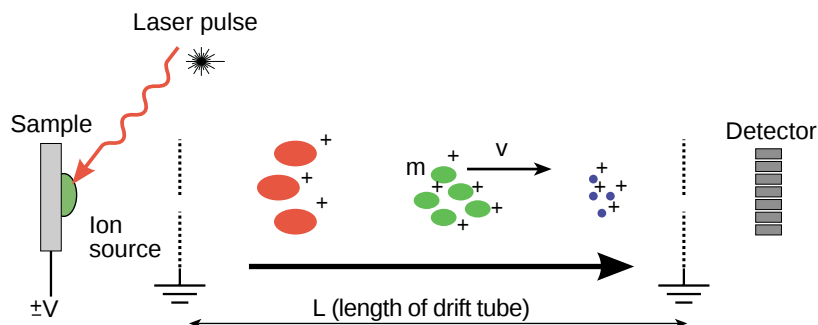


Fig 2-3. Matrix Assisted Laser Desorption, Ionization and Detection

The sample slide and laser are together referred to as the *ion source* used for sample irradiation.

Short-duration (nanosecond) pulses of laser light are directed at the crystals. The energy absorbed causes a portion of the crystal's surface to be *volatilized and ionized*, creating an ion cloud. The application of an electrostatic field prompts the ions to move through the vacuum of the flight tube towards the detector. If the ion cloud contains protein or peptide molecules, they will also move. The energy deposited by the laser can be adjusted to optimize conditions for this *ion ablation* step.

The gas-phase ions are accelerated to a fixed energy (i.e. a common kinetic energy) and, after passing through the flight tube, they impact onto an ion detector. By measuring the time between the application of the accelerating electrostatic field and the arrival of the ion at the detector (Δt_m), the mass of the ion can be determined by *Time of Flight* (ToF) mass analysis:

$$m = 2qV\Delta t_m^2/l^2$$

m = ion mass

q = ion charge

V = potential through which ion is accelerated

l = length of flight tube

The complete derivation of this equation is given in the *Ettan MALDI-ToF Instrument Handbook*.

In simple terms, when all ions are equally charged, a lighter mass will travel faster than a heavier mass. As each ion reaches the detector, a peak is generated, creating a mass spectrum i.e. a plot of signal intensity generated against time, as shown in Fig. 2-4. The mass-to-charge ratio (m/z) that corresponds to a particular peak is calculated using simple equations and the time-to-intensity data is converted into an m/z -to-intensity histogram.

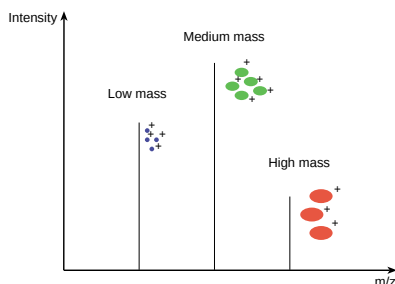


Fig 2-4. Example of a mass spectrum

Each peak in a mass spectrum is now assigned a specific mass. By recording time-of-flight mass spectra as intensity versus time-of-flight tables, the mass-to-charge (m/z) data is calculated as follows:

$$(m/z)^{1/2} = at + b$$

Although the constants a and b are related to the geometry of the ion source and mass analyser, they are most commonly determined by the simple and accurate method of selecting two peaks of known m and z and deriving the constants from their measured flight times. With the constants a and b established, the values can be applied to calibrate other spectra produced under the same experimental conditions. Further details on calibration methods are outlined in the *Ettan MALDI-ToF User Manual*.



It should be noted that a two point calibration is generally satisfactory when working with peptides. However, in some instruments, this calibration may not be completely linear so that higher terms will be required in the equation. Under these circumstances an accurate calibration, over a wider mass range, should be obtained using more than two points (multi-point calibration).

Depending upon the matrix solution, as well as other specific sample conditions, the MALDI-ToF spectrum may contain single- or double-charged species. Evaluation software is used to identify the peaks that have originated from a single mass with different charges.

Ettan MALDI-ToF uses the most powerful data analysis software available for the identification process. The list of masses (for example, a peptide mass fingerprint generated from mass analysis of a proteolytic digest) is compared with real or theoretically generated data stored in databases, until a match is found or a new protein discovered.

2.2 Applications for MALDI-ToF mass spectrometry

The diagram shown in Fig. 2-3 illustrates a MALDI-ToF mass spectrometer used in *linear detector mode*: ions, under the influence of an electric field, travel in a *straight line* towards the detector and lighter masses arrive before the heavier masses. In the simplest terms, the mass relates to the time of flight and a mass spectrum shows the separation between the masses according to their flight time to reach the detector.

For *mass analysis of full length proteins*, it is usually sufficient to use a MALDI-ToF instrument in linear detection mode.

However, for *mass analysis of a peptide mixture* (as required for protein identification by peptide mass fingerprinting), a MALDI-ToF instrument is used in *reflectron mode*. The masses in a peptide mixture are smaller and likely to be distributed over a narrow range of mass values. The ions generated must be made to travel longer distances in order to enhance the differences in flight times between these ions. The reflectron acts as an *ion mirror* which corrects for variations in energy of ions of equivalent mass/charge (m/z) ratio. The reflectron increases the flight path of the ions, by applying a stepwise increase in voltage across the ion flight path, as illustrated in Fig. 2-5. A longer flight path results in a greater separation between the different masses, seen as higher resolution in the mass spectrum. The overall result is a better time-focus at the detector and high resolution mass spectra with excellent mass accuracy.

Peptides can be studied further by creating conditions under which the peptides themselves are broken apart within the mass spectrometer and the resulting fragments are analysed. This analysis not only provides more information about the original protein, but, if initial identification has been unsuccessful, it can also be used for database comparisons.

To perform *fragmentation analysis*, a MALDI-ToF instrument is used in reflectron mode, but now the peptides are ionized at an increased laser power, thus giving them more internal energy. Bonds are broken apart, giving rise to a product ion and a neutral fragment. This phenomenon is known as *post source decay* (PSD).

The linear section of the Ettan MALDI-ToF is used to separate ions of different m/z ratio and bring them into time focus at the entrance to the reflectron. A timed ion gate selects a precursor mass for PSD analysis in the reflectron. In PSD experiments the energy of ionization is increased by the application of high laser power, this generates ions of higher energy and promotes fragmentation. When fragmentation occurs the fragment ions continue to travel with the same velocity as the precursor ion, but have lower energy. Thus, fragments from a precursor ion and the intact precursor ion, arrive at the timed ion gate simultaneously. The flight times of the fragment ions in the reflectron vary by virtue of their differing m/z ratio and a fragment ion spectrum is obtained. In this way the fragments of a single specific peptide can be detected and analysed.

Ettan MALDI-ToF contains a unique reflectron, based on patented z^2 technology, that applies an increasing voltage, non-linearly, to create a perfect quadratic field, as shown in Fig. 2-5.

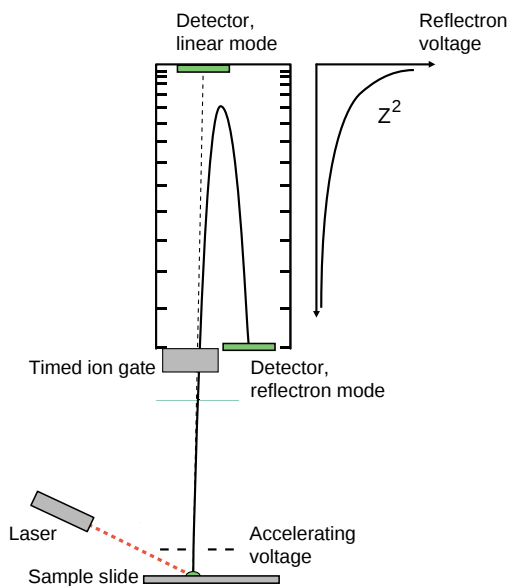


Fig 2-5. Quadratic-field reflectron of Ettan MALDI-ToF.

This unique quadratic-field reflectron provides time focusing of all ions irrespective of energy over the entire range of m/z . Hence a complete fragment ion mass spectrum is obtained under the same experimental conditions with each pulse of the laser, as shown in Fig. 2-6.

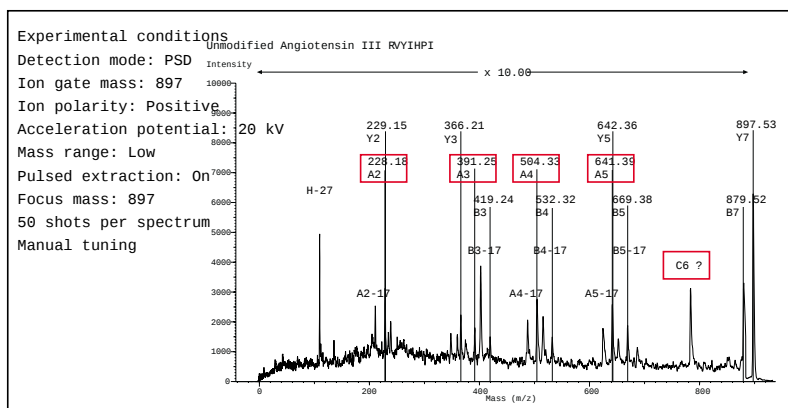


Fig 2-6. Example of a MS spectrum of ile^7 Angiotensin III, using PSD.

In all Ettan MALDI-ToF modes, a low mass rejection gate eliminates interference from low molecular weight compounds, such as those generated by components of the matrix, to further improve the detection and resolution of peaks in the mass spectrum. Ettan MALDI-ToF is designed to switch easily between linear and reflectron mode to give the performance required for a specific application.

3 Sample Preparation

3.1 Removal of contaminants

To achieve the accurate mass analysis needed for reliable identification, the correct sample preparation is crucial. The protein or peptide should be transferred intact into the flight tube of the mass spectrometer. The presence of contaminants from previous techniques, the solutions used to dissolve the sample and the type of matrix used to contain the sample on the slide surface may all affect this process. The MALDI-ToF technique is quite tolerant of certain contaminants (Table 3-1). However, to achieve a high quality mass analysis, it is advisable to remove as many contaminants as possible or, at least, reduce them to acceptable levels.



When working with low amounts of material (e.g. an extract from a weak 2D spot), the removal of salt will improve the signal to noise ratio.

<i>Potential contaminant</i>	<i>Tolerated levels</i>
Phosphate buffers	< 20 mM
Tris buffer	< 50 mM
Detergents	< 0.1%
Alkali metal salts	< 1 M
Guanidine	< 1 M
Ammonium bicarbonate	< 30 mM
Glycerol	To be avoided
SDS	To be avoided
Sodium azide	To be avoided

Table 3-1. Tolerance levels for contaminants in a MALDI-ToF analysis.

A range of solvents and chemicals are frequently used for protein and peptide dissolution. Although necessary, they provide a source of unwanted contaminants during sample preparation and mass analysis.



The solvent chosen to dissolve the sample must be compatible with the matrix.

Table 3-2 shows the most commonly used solvents and chemicals, together with the actions required to avoid any interference in a subsequent mass analysis. Methods for the application of sample to the slide surface are also referred to in this table and are described in detail further on in this chapter.

Potential contaminants	Comments
Sodium azide	MUST be removed. Suppresses ion formation in the mass spectrometer.
Formic acid: 2-propanol:water (1:2:3 v/v)	Recommended for hydrophobic peptides and proteins.
High concentration formic acid	Avoid long term exposure if mass accuracy is essential (forms formyl derivatives of N-terminal amino acids).
High concentration salts, buffers or urea	Remove if possible. May interfere with crystallization. If present use thin layer method.
Glycerol	MUST remove, concentrations > 1% interfere with crystallization.
Strong ionic detergents	MUST be removed. Prevent incorporation of samples into growing crystals.
Non-ionic detergents e.g. Triton X or N-octylglucoside	May interfere with crystallization, avoid if possible. Effects vary according to specific detergent, e.g. N-octylglucoside may have a beneficial effect at low concentrations. If present, use slow crystallization method (N.B. this requires some practice).

Table 3-2. Potential contaminants



Low signals (calibration peptides cannot be seen) and poor resolution (noisy background spectrum) indicate that the sample should be cleaned. Run the first analysis on only half of the sample. This will allow a second analysis on a cleaned up sample if required.



Reverse phase chromatography (RPC) is frequently used to remove salts and other low molecular weight substances. Peptides are bound to an RPC column containing 10-20 μm C18 medium. This process desalts and concentrates the sample, since the peptides bind in the presence of 0.1% TFA and are eluted in a small volume (1-4 μl) of 50% acetonitrile, 0.1% TFA. Columns are often handmade, by packing the RPC medium into microtips, or obtained as commercial products, such as ZipTip™.

3.2 Sample Extraction from Electrophoresis Gels

Samples are often extracted from an electrophoresis gel before being prepared for mass analysis. For example, in proteomic studies, potential target proteins are selected during expression analysis, a process that compares protein profiles between normal and altered tissue. Two dimensional electrophoresis separations generate patterns of protein spots that can be visualized and compared by image analysis.

The proteins of interest are then cut from the gel. The gel pieces are destained, subjected to an in-gel digest and the peptides extracted and cleaned, as illustrated in Fig. 3-1.

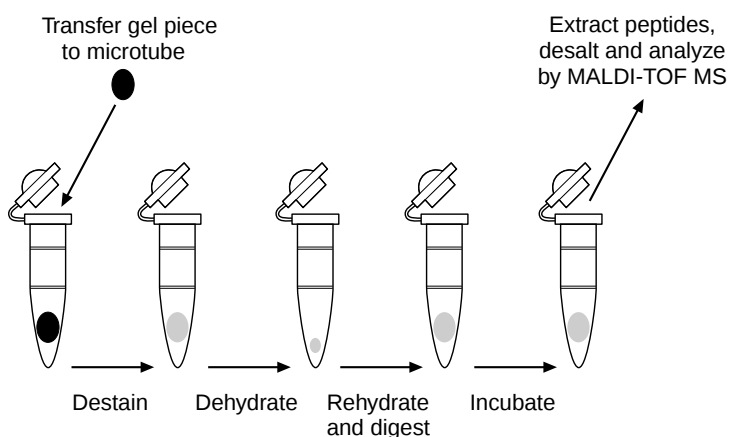


Fig 3-1. Preparation of protein spots for MS identification



Coomassie Blue is recommended for visualization of protein spots. The dye binds stoichiometrically to proteins, a significant advantage when determining *relative* amounts of protein. Staining is simple and the Coomassie Blue does not usually interfere with subsequent mass analysis. Coomassie Blue R-350 is the most sensitive form of the dye, available from Amersham Biosciences (code no: 17-0518-01).



The highest quality HPLC grade water should be used for every step after the sample has been excised from the gel.



Although silver staining is 100 times more sensitive than Coomassie Blue, it may interfere with mass analysis. However, a recent publication outlines a method that avoids this problem by using a modified silver staining protocol from which glutaraldehyde is eliminated (*Gharahdaghi et al., Electrophoresis, 20, p601-605, 1999*).



Using the modified protocol, while improving mass analysis results, may reduce the sensitivity of the silver staining.



Detergents and excess salts are usually eliminated during normal staining procedures.



Strong ionic detergents, such as sodium dodecyl sulphate (SDS), must be completely removed as they will interfere with crystallization and so disrupt the mass analysis.

3.3 Choosing the Matrix

The surroundings of a protein or peptide should ensure that, when subjected to the intense light pulse of a laser, the molecule is transferred, in an ionized state, into the flight tube. To achieve this effect the sample is incorporated into crystals of a second material- the *matrix*. The sample-containing crystal absorbs the light pulse and the absorbed energy causes desorption of material from the crystal's surface, creating an ion cloud. If the desorbed material contains protein or peptide molecules, they will also enter the flight tube.



It should be noted that, in earlier MALDI-ToF instruments, matrix compounds were seen to produce *adduct peaks*. These are satellite signals resulting from the chemical breakdown of the matrix into more reactive species that bind to the protein or peptide. They have slightly higher mass than the analyte molecule peaks. However, this phenomenon occurred when very high laser energies were required to compensate for lack of sensitivity. Adduct peaks are rarely seen in today's, more sensitive, MALDI-ToF instruments.



If salts are present in the sample then satellite peaks, containing Na^+ or K^+ that replace protons as the charge donor, may be seen. Similar satellite peaks will also occur when using poor quality matrix substances.

Table 3-3 shows the most commonly used matrix compounds and indicates the ionization states.

Matrix	Molecular mass	Ionization	
		Peptides	Proteins
α -cyano-4-hydroxycinnamic acid (4-HCCA)	189 Da	+++	+
Sinapinic acid (sinapic acid: <i>trans</i> -3,5-dimethoxy-4-hydroxycinnamic acid)	203 Da	+	+++
2,5-dihydroxybenzoic acid (gentisic acid or DHB)	154 Da	+	+

Table 3-3. Properties of commonly used matrices



An increased ionization state increases the signal intensity and gives a higher charge state to the most intense peak. However, it should be noted that charge states depend not only on the matrix used, but also on the concentration and nature of the acid present in the matrix solvent, and on the nature and respective concentrations of peptides and proteins in the sample.



Selection of the most suitable matrix for a particular sample may be a case of trial and error. However, experience has shown that 4-HCCA is a versatile and easy to use matrix, particularly for peptides and smaller proteins.

Table 3-4 gives guidelines as to the advantages and disadvantages of the matrix compounds for protein and peptide mass analysis.

Matrix	Comments
4-HCCA	First choice for peptides and smaller proteins $\leq 5\,000$ Da. Intense signals for proteins and peptides. No adduct peaks. Protein ions may undergo metastable decay at very low laser energies and low protein concentration.
Sinapinic acid	First choice for proteins. Peptides $< 3\,000$ Da may give weak signals.
DHB	Alternative matrix for peptides. Suitable when looking at metastable ions, e.g. when performing PSD analysis on peptides. May not always work with proteins.

Table 3-4. Guidelines for matrix selection

3.4 Preparing Solutions for Crystallization

Both the sample and the matrix should be dissolved separately before mixing and beginning the crystallization process. Fig. 3-2 to Fig. 3-4 shows the crystallization states of three of the most commonly used matrices. These examples show pure crystals. Their appearance may change according to the type of sample being analysed.



Fig 3-2. Crystals of α -cyano-4-hydroxycinnaminic acid

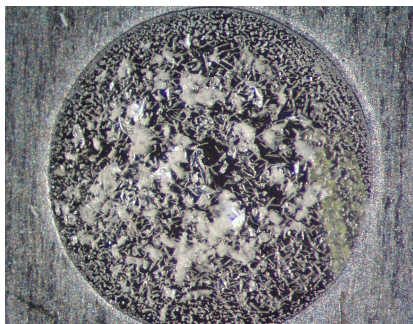


Fig 3-3. Crystals of sinapinic acid

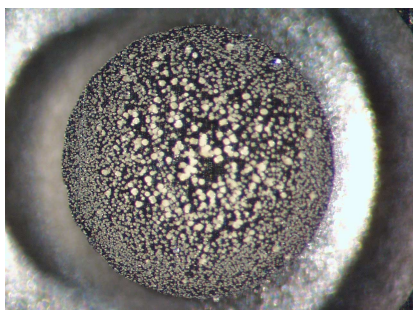


Fig 3-4. Crystals of 2,5-dihydroxybenzoic acid

3.4.1 Dissolving the protein or peptide

Please refer to Sections 3.1 and 3.2 .

3.4.2 Dissolving the matrix

Typically a water: organic solvent solution is used to dissolve the matrix compounds.



Use reagent quality acetonitrile, methanol or propanol and the highest quality HPLC grade water.

Table 3-5 shows suggested water:solvent ratios for solubilization of the matrix compounds.

Matrix	Water:Solvent
4-HCCA	2:1 to 1:1
Sinapinic acid	2:1 to 1:1
DHB	9:1 to 3:1

Table 3-5. Recommended water:solvent ratios for matrix solubilization



Conditions may need to be adjusted according to the type of sample application method (see next section) or if precipitation occurs. Although the final crystallization mixture may contain acids, salts, lipids, detergents and organic solvents, it is essential that neither the matrix, nor the protein or peptide, precipitate when the two solutions mix.

There are several points to note for successful crystallization



The protein must be truly dissolved in the solvent (a slurry is not acceptable).



Avoid inadvertent changes in solvent composition that may cause precipitation. Always keep tubes closed when not in use.



Use fresh matrix solutions whenever possible. Solutions will decompose under normal laboratory conditions.



Keep the concentration of non-protein materials to a minimum. It may be possible to wash the crystals to remove non-protein materials.



Maintain the pH of the crystal-growing solution below pH 4 by adding 0.1 – 1.0% TFA. This avoids ionization of the matrices. The optimal amount of TFA depends upon the specific matrix and analyte.



Aim for 5 – 10 pmoles protein or 10 fmoles of peptide on the sample slide.



MOST IMPORTANT! There must be enough protein or peptide dissolved in the crystal-growing solution to give a signal of sufficient intensity, although reducing protein concentration can sometimes increase signal intensity.



Analyse as soon as possible after crystallization.



Do not leave matrix crystals in the protein-matrix solution, this causes loss of signal. Remove by centrifugation if necessary.



Take care during sample handling; protein adsorption to surfaces such as pipettes or Eppendorf tubes can significantly reduce the true sample concentration, particularly if the original concentration is low.

3.5 Applying Sample to the Slide Surface

After mixing with the selected matrix solution the samples are applied onto the surface of the *sample slide* - the stage used to carry them into the flight tube of the mass spectrometer.



The dried droplet method is a suitable sample application method for the majority of applications. For completeness, three alternative methods are also described.



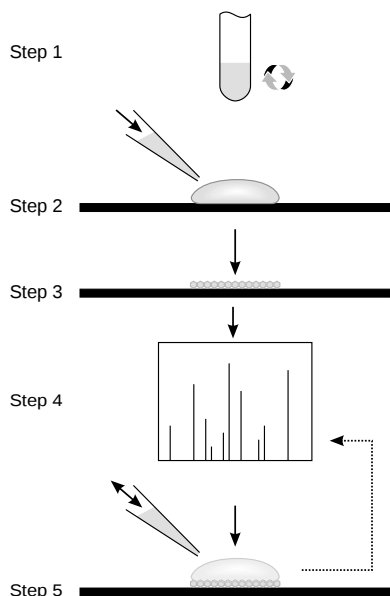
Thin layer and microfilm methods produce more noise than the dried droplet method and a lower signal intensity for peptides > 3 000 Da. Consequently, the sensitivity may not be sufficient when working in reflectron mode. However, in linear mode the advantage of signal uniformity over the entire spot may outweigh any disadvantage of low sensitivity, particularly when requiring high throughput and when acquisition is automated

3.5.1 Dried Droplet Method

The dried droplet method is the first choice for most applications.



Do not use non-volatile solvents (e.g. glycerol, polyethylene glycol, DMSO, Triton X) with this method.



- 1 Mix peptides or proteins with saturated matrix solution
⇒ final concentrations: peptide approximately 40 nM and protein approximately 10 μ M.
- 2 Apply 0.5 μ l of solution to the sample slide.
- 3 Leave to dry at ambient temperature.
- 4 Insert into the mass spectrometer. Dry sample thoroughly (1-2 minutes). Acquire the mass spectrum.
- 5 ⇒ If heavy salt or buffer contamination is suspected, remove from the vacuum and wash with cold (4 °C) 0.1% aqueous TFA solution. Wait 2 - 10 seconds and remove washing solution by vacuum suction.



Do not heat the droplet to speed up the drying process (this will alter matrix crystallization).



Homogeneity of the film can be improved by adjusting the TFA concentration.

3.5.2 Thin Layer Method

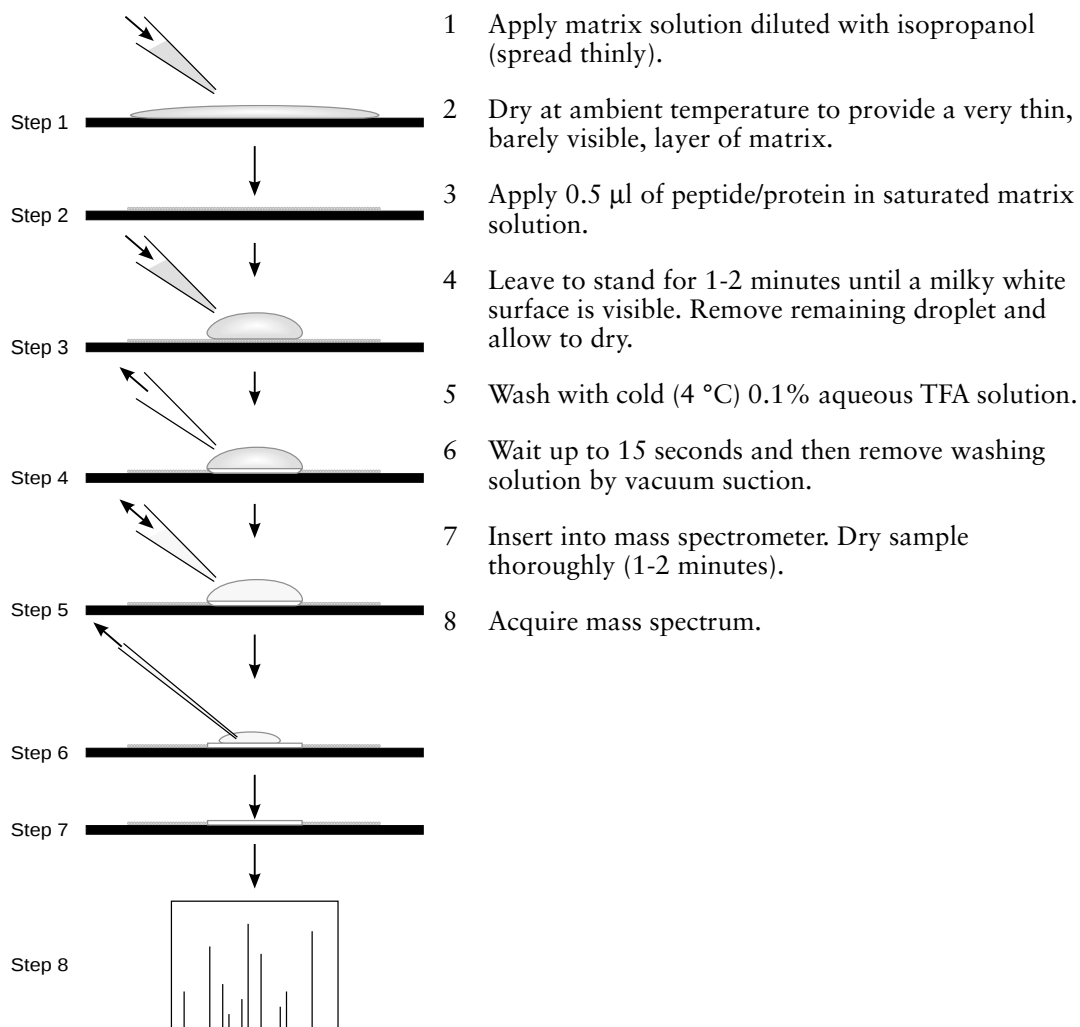
The thin layer method produces a uniform layer of small crystals that are mechanically well adhered to the surface of the sample slide allowing contaminants to be washed away.



Useful when samples contain non-peptide contaminants, such as buffering substances, 4M urea or salts.



Suitable for use with 4-HCCA and sinapinic acid



3.5.3 Slow Crystallization Method

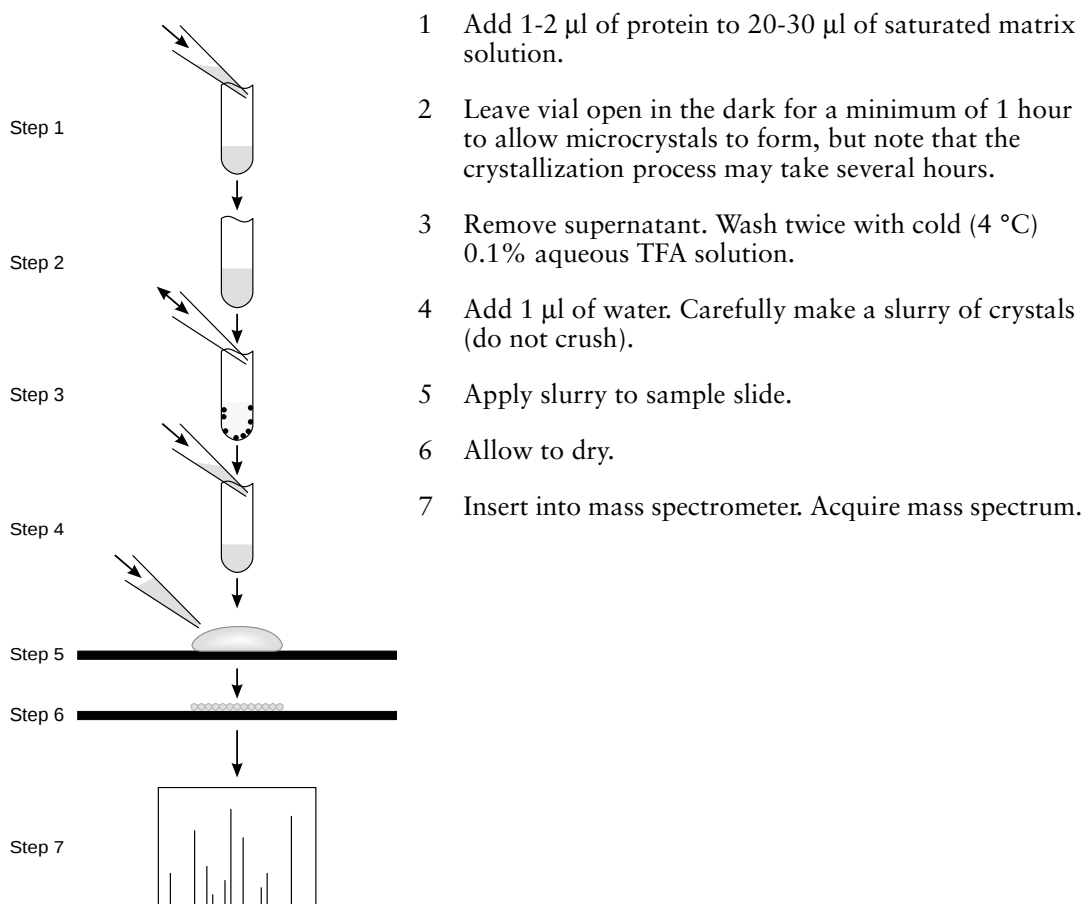
The slow crystallization method may require some practice to achieve a successful result.



Useful when samples contain involatile solvents (e.g. 30% glycerol), high salt concentrations (e.g. 4M NaCl), low protein concentrations ($<1\mu\text{M}$), or if the dried droplet approach has failed.



Suitable for use with 4-HCCA and sinapinic acid



3.5.4 Seeded Microcrystalline Film Method

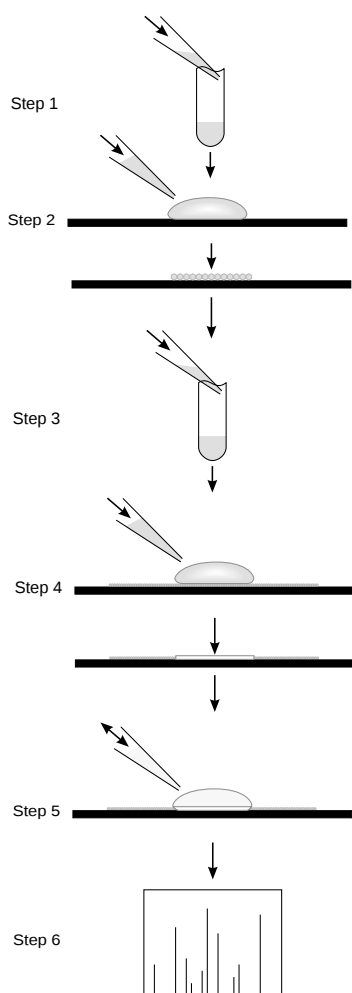
The seeded microcrystalline film method produces very uniform samples and the crystals are well adhered to the surface of the sample slide. It is a direct replacement for the dried droplet technique.



May improve the mass resolution and mass accuracy obtained in reflectron mass spectrometers.



Suitable for use with 4-HCCA and sinapinic acid



- 1 Add matrix to acetone containing 1-2% water until the solution is nearly saturated.
- 2 Place a droplet (e.g. 0.5 μl) onto the sample slide and leave to spread out and dry, forming a uniform deposit.
- 3 Dissolve the sample of interest in an aqueous solution containing 20-30% organic solvent.
- 4 Place approximately 0.5 μl onto the sample slide and leave to dry at room temperature.
- 5 Wash the dried deposit with pure water or 0.1% TFA for 2 – 10 sec. and remove excess water.
- 6 Insert into mass spectrometer. Acquire mass spectrum.

3.6 Preparation for Specific Applications

Sample preparation methods may need to be slightly adjusted in order to achieve the most satisfactory result for an application. The general recipes described here can be used as a basis to develop optimized sample preparation methods protocols for some of the most common applications of mass analysis by MALDI-ToF mass spectrometry.



Proteins that are already in solution should be prepared following the recommendations given earlier in this chapter.



Always use HPLC quality solvents, reagent-grade chemicals and the highest quality HPLC grade water.



Always use siliconized microtubes for sample processing to avoid loss of material by non-specific adsorption.



Use sequencing-grade trypsin that is prepared to be resistant to autodigestion, for example, trypsin that has undergone lysine alkylation.



Trypsin should not be protected by polyside derivatisation of lysines as this can dramatically decrease efficiency when handling glycoproteins.

3.6.1 Identification by peptide mass fingerprinting

Starting material is often a protein gel band isolated by SDS-PAGE. The protein is digested in-gel with trypsin and the proteolytic peptides ('the digest') are extracted from the gel and analysed. The protein is identified by comparing the digest peaks with a computer-generated database of tryptic peptides from known proteins.

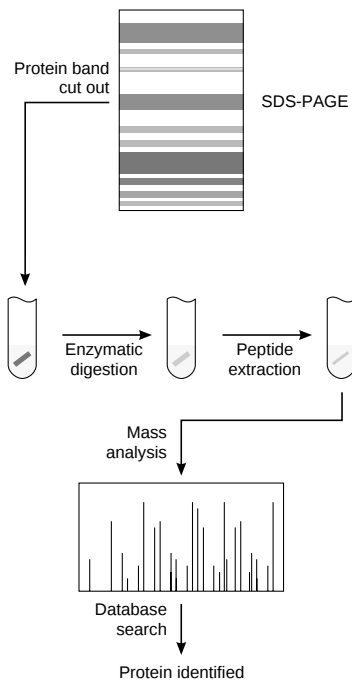


Fig 3-5. Identification by peptide mass fingerprinting

Table 3-6 gives examples of the chemicals and reagents that can be used as a basis for development of an optimized protocol.

Destaining solution for Coomassie Blue stained gels	50 mM NH_4CO_3 :acetonitrile (50:50) or 50 mM NH_4CO_3 :methanol (50:50)
Trypsin digestion (0.2 – 0.5 μg trypsin / digestion)	Trypsin in 50 mM NH_4CO_3 , 5 mM CaCl_2 , pH 8.0. (CaCl_2 is not required if trypsin has undergone lysine alkylation)
Extraction solvent	0.1 – 0.5% aqueous TFA:acetonitrile (30:70 or 40:60)
Peptide mass calibrants (mix 50:50 with matrix solution)	hACTH* fragment 18-39 (2464.191 amu) hACTH fragment 7-38 (3656.923 amu) [ile7]-Angiotensin III (896.5232 amu) Dissolve lyophilized material in water (200 pmol/ μl), dilute 10-fold with 0.5% TFA, 50% acetonitrile. Store at – 20 °C. *human adrenocorticotrophic hormone
Peptide matrix solution	4-HCCA in 0.5% TFA:acetonitrile (50:50) or 0.1 M DHB in water:acetonitrile (50:50)

Table 3-6. Examples of chemicals and reagents used for peptide mass fingerprinting.



Use gloves to prevent keratin contamination.



Excised gels and extracted, dried down sample pellets can be stored at – 20 °C or used immediately.



Always excise a small strip of gel from a background area to use as a control sample during analysis.



Use the dried droplet method to apply samples to the sample slide and leave the crystals to air-dry for 5 minutes before beginning an analysis.

Suggested Evaluation Procedure:

- 1 Filter spectra
- 2 Perform an external calibration of each protein tryptic digest mass spectrum, and background control spectrum.
- 3 Perform an internal recalibration of each protein tryptic digest mass spectrum using trypsin autolysis peaks.
- 4 Extract peak masses.
- 5 Remove background peak masses.

6 Begin database search

Results will provide a list of proteins that are identified at the selected significance level.

3.6.2 Protein characterization I

Proteins of known sequence are characterized, i.e. the most common modifications identified and located after proteolysis in solution. The starting material is the purified protein, lyophilized or in solution. The protein is digested with trypsin, and proteolytic peptides are analysed by mass spectrometry. Modifications are identified by comparing the proteolytic peptide masses with peptide masses calculated from the sequence of the unmodified versus modified protein.

Table 3-7 gives examples of the chemicals and reagents that can be used as a basis for development of an optimized protocol.

Trypsin digestion (approximately 0.2 µg trypsin / digestion)	Trypsin in 50 mM NH ₄ CO ₃ , 5 mM CaCl ₂ , pH 8.0 (CaCl ₂ is not required if trypsin has undergone lysine alkylation)
Protein mass calibrants (dissolve directly into protein matrix solution)	Apomyoglobin 20 ng, carbonic anhydrase 70 ng, enolase 280 ng
Protein matrix solution	0.04 M sinapinic acid, 67% of a 0.1% aqueous TFA solution, 33% acetonitrile
Peptide mass calibrant solution (mix 50:50 with peptide matrix solution)	hACTH* fragment 18-39 (2464.191 amu) hACTH fragment 7-38 (3656.923 amu) [ile ⁷¹]-Angiotensin III (896.5232 amu) Dissolve lyophilized material in water (200 pmol/µl), dilute 10-fold with 0.5% TFA, 50% acetonitrile. Store at – 20 °C. *human adrenocorticotrophic hormone
Peptide matrix solution	4-HCCA in 0.5% TFA:acetonitrile (50:50) or 0.1 M DHB in water:acetonitrile (50:50)

Table 3-7. Examples of chemicals and reagents.



Use the dried droplet method to apply samples to the sample slide and leave the crystals to air-dry for 5 minutes before beginning an analysis.

Suggested Evaluation Procedure

- 1 Filter spectra.
- 2 Perform external calibration of each protein mass spectrum.
- 3 Perform internal recalibration of each protein mass spectrum if peaks for internal calibration are present.
- 4 Extract peak masses from protein mass spectrum.
- 5 Compare measured mass with protein sequence.
- 6 Perform external calibration of each tryptic peptide mass spectrum.
- 7 Perform internal recalibration of tryptic peptide mass spectrum using trypsin autolysis peaks.
- 8 Extract peak masses from tryptic peptide mass spectrum.
- 9 Remove background peak masses in tryptic peptide mass spectrum (using a list of trypsin autolysis peptide masses).
- 10 Compare measured tryptic peptide masses with unmodified protein sequence
- 11 Compare measured tryptic peptide masses with protein sequence allowing for common modifications

Results will provide a comparison of measured protein mass with protein sequence, a list of contaminant masses, coverage of protein sequence and indicate possible modifications.

3.6.3 Protein characterization II

Proteins of known sequence are characterized, i.e. the most common modifications identified and located after proteolysis in solution. The starting material is the purified protein, lyophilized or in solution. The protein is digested in parallel with trypsin, chymotrypsin and endoproteinase Asp-N. Proteolytic peptides are analysed. Modifications are identified by comparing the proteolytic peptide masses with peptide masses calculated from the sequence of the unmodified versus modified protein. Table 3-8 gives examples of the chemicals and reagents that can be used as a basis for development of an optimized protocol.

Trypsin or chymotrypsin digestions (approximately 0.2 µg trypsin and 0.2 µg chymotrypsin per digestion)	*Trypsin or chymotrypsin in 50 mM NH_4CO_3 , 5 mM CaCl_2 , pH 8.0 (CaCl_2 is not required if trypsin has undergone lysine alkylation)
Endoproteinase Asp-N digestion (approximately 0.01 µg per digestion)	*Endoproteinase Asp-N in 50 mM $(\text{NH}_4)_2\text{PO}_3$, pH 7.0
Protein mass calibrants (dissolve directly into protein matrix solution)	Apomyoglobin 20 ng, carbonic anhydrase 70 ng, enolase 280 ng
Protein matrix solution	0.04 M sinapinic acid, 67% of a 0.1% aqueous TFA solution, 33% acetonitrile
Peptide mass calibrant solution (mix 50:50 with peptide matrix solution)	hACTH* fragment 18-39 (2464.191 amu) hACTH fragment 7-38 (3656.923 amu) [ile7]-Angiotensin III (896.5232 amu) Dissolve lyophilized material in water (200 pmol/µl), dilute 10-fold with 0.5% TFA, 50% acetonitrile. Store at – 20 °C. *human adrenocorticotrophic hormone
Peptide matrix solution	4-HCCA in 0.5% TFA:acetonitrile (50:50) or 0.1 M DHB in water:acetonitrile (50:50)

Note: * that the protein samples are dissolved in the buffers and then mixed with the appropriate lyophilized enzyme.

Table 3-8. Examples of reagents and chemicals.



Use sequencing grade chymotrypsin and endoproteinase Asp-N



Use the dried droplet method to apply samples to the sample slide and leave the crystals to air-dry for 5 minutes before beginning an analysis.

Suggested Evaluation Procedure

- 1 Filter spectra.
- 2 Perform external calibration of each protein mass spectrum.
- 3 Perform internal recalibration of each protein mass spectrum if peaks for internal calibration are present.

-
- 4 Extract peak masses from protein mass spectrum.
 - 5 Compare measured mass with protein sequence.
 - 6 Perform external calibration of each tryptic peptide mass spectrum.
 - 7 Perform internal recalibration of tryptic peptide mass spectrum using trypsin autolysis peaks.
 - 8 Extract peak masses from the tryptic peptide mass spectrum.
 - 9 Remove background peak masses in tryptic peptide mass spectrum (using a list of trypsin autolysis peptide masses).
 - 10 Compare measured tryptic peptide masses with unmodified protein sequence.
 - 11 Compare measured tryptic peptide masses with protein sequence allowing for common modifications.
 - 12 Steps 6 to 11 are repeated for chymotrypsin and endoproteinase Asp-N, respectively.

Results will provide a comparison of measured protein mass with protein sequence, a list of contaminant masses, coverage of protein sequence and indicate possible modifications.

3.6.4 Identification of protein phosphorylation sites

Protein phosphorylation sites are identified by in-gel trypsin digestion and comparison of phosphorylated/dephosphorylated peptide maps. The proteolytic peptides are analysed by Ettan MALDI-ToF and the phosphorylation sites are identified by comparing the peptide masses with masses calculated from the protein sequence. Table 3-9 gives examples of the chemicals and reagents that can be used as a basis for development of an optimized protocol.

Destaining solution for Coomassie Blue stained gels	50 mM NH_4CO_3 :acetonitrile (50:50) or 50 mM NH_4CO_3 :methanol (50:50)
Trypsin/bacterial alkaline phosphatase digestion (approximately 0.2 μg trypsin / digestion, 0.01 μg phosphatase/dephosphorylation)	Trypsin/phosphatase in 50 mM NH_4CO_3 , 5 mM CaCl_2 , pH 8.0 (CaCl_2 is not required if trypsin has undergone lysine alkylation)
Extraction solvent	0.1% aqueous TFA:acetonitrile (30:70 or 40:60)
Peptide mass calibrants (mix 50:50 with matrix solution)	hACTH* fragment 18-39 (2464.191 amu) hACTH fragment 7-38 (3656.923 amu) [Ile ⁷¹]-Angiotensin III (896.5232 amu) Dissolve lyophilized material in water (200 pmol/ μl), dilute 10-fold with 0.5% TFA, 50% acetonitrile. Store at -20°C . *human adrenocorticotrophic hormone
Peptide matrix solution	4-HCCA in 0.5% TFA:acetonitrile (50:50) or 0.1 M DHB in water:acetonitrile (50:50)

Table 3-9. Examples of chemicals and reagents



Use gloves to prevent keratin contamination.



Excised gels and extracted, dried down sample pellets can be stored at -20°C or used immediately.



Always excise a small strip of gel from a background area to use as a control sample during analysis.



Use the dried drop method to apply samples to the sample slide and leave the crystals to air-dry for 5 minutes before beginning an analysis.

Suggested Evaluation Procedure

- 1 Filter spectra.
- 2 Perform an external calibration of each protein tryptic digest mass spectrum (phosphorylated/ dephosphorylated), and of the background control spectrum.
- 3 Perform an internal recalibration of each protein tryptic digest mass spectrum (phosphorylated/ dephosphorylated) using trypsin autolysis peaks.
- 4 Extract peak masses.
- 5 Remove background peak masses.
- 6 Compare measured mass of the phosphorylated and non-phosphorylated tryptic peptide with protein sequence.

Results will provide a list of contaminant masses, coverage of the protein sequence and localization of phosphorylation sites.

Index

Numerics

4-HCCA8, 21

A

acetonitrile 23

adduct peaks 20

C

calibrants 31

calibration 12

chymotrypsin 33

contaminants 17

coomassie Blue 19

crystals 22

D

DHB8, 21

dried droplet method 25

E

electrophoresis gel 19

endoproteinase 33

Ettan MALDI-ToF 7

F

fragmentation analysis 13

full length proteins 12

G

gentisic acid 21

glycerol 27

I

ion ablation 11

ion source 10

K

keratin 31

L

linear detector mode 12

low mass rejection gate 15

M	
m/z	11
MALDI-ToF	8
matrix	10, 20
methanol	23
P	
peptide mass fingerprinting	30
phosphatase	36
phosphorylation sites	36
post source decay	13
ProFound	7
propanol	23
protein characterization	32, 33
proteomic	9
R	
reflectron mode	13
reverse phase chromatography	18
S	
sample extraction	19
sample slide	10, 24
SDS-PAGE	30
seeded microcrystalline film method	28
silver staining	19
sinapinic acid	21
slow crystallization method	27
sodium dodecyl sulphate	20
T	
thin layer method	26
time of flight	11
trypsin	29, 30, 31, 33
U	
urea	26
Z	
ZipTip	18