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Matrix-assisted Laser Desorption/Ionization Mass Spectrometry Sample Preparation Techniques Designed for Various Peptide and Protein Analytes†

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This study encompasses a collection of experiences with regard to numerous matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) sample preparation techniques in terms of their suitability for different peptide and protein analytes. Variants of both established and new sample preparation techniques for the MALDI-MS analysis of peptides and proteins are described. The importance of matrix selection, matrix and analyte concentration, pH adjustment, crystallization conditions and the use of additives is evaluated. The tolerance of the different sample preparations towards salts, buffers, synthetic polymers, detergents, denaturants and other contaminants, and also the influence of the preparation methods on undesired amino acid side-chain oxidation, are investigated. Moreover, the performance of on-target tryptic digestion and on-target disulfide reduction is shown and a microscale purification procedure is described. According to this study, there is no universally applicable sample preparation for a broad variety of analytes. Rather, it is necessary to specifically adapt the sample preparation to the analyte properties. © **1997 by John Wiley & Sons, Ltd.**

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INTRODUCTION

Sample preparation is known to be the crucial procedure in the matrix-assisted laser desorption/ionization mass spectrometric (MALDI-MS) analysis of peptides and proteins. The sample preparation includes two steps. The first represents the isolation and the purification of a single component or a mixture, free of contaminants such as buffers, salts, detergents or denaturants. The second step comprises the sample processing on the MALDI target, i.e. choice of matrix, matrix and analyte concentration, pH adjustment, crystallization conditions, use of additives and on-target sample clean-up.

To date, a variety of MALDI sample preparation recipes has been reported, among which the most frequently used is the dried-droplet method.[1] In one approach, the tolerance of MALDI towards involatile contaminants has been improved by a slow matrix crystallization procedure.[2] Later, the preparation of a thin, homogeneous layer of sample/matrix co-crystals was shown to improve sensitivity and mass accuracy in the MALDI-MS analysis of peptides.[3,4] The choice of matrix and the use or presence of matrix additives, e.g. detergents, have been demonstrated to influence significantly the quality of MALDI mass spectra of peptides and proteins.[5–7] Cohen and Chait[8] and Jensen *et al.*[9] have systematically studied the influence of matrix–sample solvents, sample pH and crystal growth time on the quality of MALDI-MS analyses.

As a supplement to the investigations mentioned above, we describe here two new and variants of several established sample preparation techniques designed for peptide and protein analytes of different origin, nature and purity. In particular, the new procedures can tolerate the presence of salts, contaminants and additives and enable further sample processing on the target, e.g. disulfide bond reduction, proteolytic digestion and efficient washing steps. Furthermore, we report on a new

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cheap and fast microscale purification technique, which allows efficient concentration and clean-up of small analyte quantities prior to MALDI-MS analysis.

EXPERIMENTAL

Mass spectrometry

MALDI mass spectra were recorded on a Bruker Reflex mass spectrometer (single probe inlet) on a PerSeptive Voyager Elite mass spectrometer, both equipped with delayed ion extraction technology. All spectra shown were acquired in positive-ion linear or positive-ion reflector mode. Typically, 50–200 laser shots were added per spectrum.

Sample preparation for MALDI-MS

Matrices. The following matrices were used: α-cyano-4-hydroxycinnamic acid[10] (HCCA, Sigma); sinapic acid[11] (SA, Fluka); 2,5-dihydroxybenzoic acid[12] (DHB, Aldrich or Hewlett-Packard); a 9:1 mixture[13] of DHB and 2-hydroxy-5-methoxybenzoic acid (Aldrich); and 2,4,6-trihydroxyacetophenone (THAP, Aldrich).

Matrix solutions. The following matrix solutions were prepared:

HCCA: (I) 20 μg μl^{-1} in acetonitrile (ACN)–0.1% trifluoroacetic acid (TFA) (70:30, v/v); (II) 20 μg μl^{-1} in acetone–water (99:1, v/v).

SA: (I) 20 μg μl^{-1} in ACN–0.1% TFA (40:60, v/v); (II) 20 μg μl^{-1} in acetone–water (99:1, v/v).

DHB: (I) DHB (Aldrich): 20 μg μl^{-1} in ACN–0.1% TFA (20:80–0:100, v/v); (II) 9:1 mixture of DHB and 2-hydroxy-5-methoxybenzoic acid (Aldrich): 20 μg μl^{-1} in ACN–0.1% TFA (20:80–0:100, v/v); (III) DHB matrix solution from Hewlett-Packard, No. HP G2056A.

THAP: (I) 15 μg μl^{-1} in ACN–water (70:30, v/v); (II) 20 μg μl^{-1} in 100% MeOH.

On-target sample preparation. The sample preparation techniques used in this study can be classified as follows:

1. Dried-droplet method. A 0.5–2 μl volume of sample and 0.5–1 μl of matrix solution (HCCA (I), SA (I), DHB (I, II, III), THAP (I, II)) were mixed on the target and allowed to dry in the ambient air or, optionally, in a gentle stream of forced air or argon. In the case of on-target sample acidification, 0.5 μl of aqueous TFA was added according to Table 2. If the analyte solution contained buffers, urea, guanidinium hydrochloride or other involatile contaminants, and if HCCA, SA or THAP were used as the matrices, the sample was washed as follows: 1–3 times 5–10 μl of ice-cold 0.1% TFA were placed on the target and pipetted off after a few seconds.

2. Thin-layer methods. (*a*) *HCCA or THAP*: a thin layer of small, homogeneous matrix crystals was prepared on the target by placing (a) 0.5–1 μl of HCCA (II) or (b) 0.5–1 μl of THAP (II) on the target, and allowing the droplet to spread and dry. In the case of acidic analyte solutions (pH < 2), 0.5 μl thereof was placed on top of this matrix layer. Otherwise, 0.5 μl of aqueous TFA solution according to Table 2 was first deposited on the matrix layer followed by addition of 0.5 μl of analyte solution. After solvent evaporation, the sample was washed 1–3 times as follows: 5–10 μl of 0.1% TFA were added on the sample and removed after a few seconds using forced air.

(*b*) *HCCA plus nitrocellulose (NC) as matrix additive*: two parts of HCCA and one part of NC (membrane from Bio-Rad) were dissolved in acetone–propan-2-ol (4:1) to final concentrations of 20 (HCCA) and 10 μg μl^{-1} (NC). A 0.5 μl volume of this solution was deposited on the target and allowed to spread and dry. The following steps were performed as described in (2a).

3. Thick-layer method with nitrocellulose as matrix additive (spin-dry technique[14,15]). Equal amounts of NC membrane and HCCA were first dissolved in acetone (40 μg μl^{-1} each), and this solution was subsequently diluted with propan-2-ol to 20 μg μl^{-1}. After addition of 1% (v/v) of 0.1% TFA, twice 10 μl of the resulting solution were applied to a rotating target so that the solution was immediately spin-dried and yielded a uniform NC–matrix layer. Then, 0.5 μl of 2% TFA and 0.5 μl of sample solution were placed on top of the NC–matrix layer and allowed to dry. If the sample was highly contaminated, it was washed 1–3 times with 10 μl of 0.1% TFA. After a few seconds of incubation, the TFA droplet was removed by spinning. Finally, another 0.5 μl of 2% TFA and 0.5 μl of HCCA (I) were deposited and dried. If indicated by poor spectrum quality, 1–2 further washing steps according to (1) were performed.

Alternatively, if using a multi-sample target (Voyager Elite mass spectrometer) difficult to spin, the NC–matrix layer was prepared on a small piece of Scotch tape (adhesive on both sides) glued on the rotator, and subsequently washed with 100 μl of 0.1% TFA. This tape was transferred to the multi-sample target and the following steps were carried out as described before.

4. Sandwich method. A thin layer of matrix crystals was prepared as in procedure (2a). Subsequently, droplets of (a) 1–2 μl of aqueous TFA solution (Table 2), (b) 0.5 μl of sample solution and (c) 0.5 μl of matrix solution (HCCA (I), SA (I)) were added and this mixture was allowed to dry. Optionally, the sample was washed 1–3 times with 5–10 μl of 0.1% TFA by placing the droplet on the target, incubating for a few seconds and removing it with a pipette.

Whenever large amounts of contaminants such as urea or guanidinium hydrochloride were present, 5–20 μl of 0.1% TFA were added ~1–2 min after addition of HCCA (I) or SA (I), i.e. shortly after crystallization had commenced. After another 1–2 min, the solvent was pipetted off, followed by 1–3 washing steps (see procedure (4)).

On-target dithiothreitol (DTT) reduction. After a first analysis of a sample prepared with the matrix SA according to the dried-droplet method, the reduction of

disulfide bonds was performed by placing a 5 μl droplet of DTT solution (150 mM ammonium hydrogencarbonate, pH 7.8) on top of the matrix–sample crystals. After a 10 min reaction time at ambient temperature, the moist sample was acidified with 5 μl of 2% TFA. The resulting droplet was pipetted off and the sample was washed 1–3 times with 5–10 μl of 0.1% TFA according to (1). Finally, 0.5 μl of matrix solution (HCCA (I), SA (I)) was added and allowed to dry.

On-target proteolytic digestion. A protein sample preparation according to the dried-droplet method with SA as the matrix can be followed by proteolytic digestion on-target. A lyophilized aliquot of 50–100 ng trypsin was dissolved in 2–5 μl of 50 mM ammonium hydrogencarbonate (pH 6–8) or 50 mM ammonium acetate (pH 6–8) (enzyme:substrate ratio (E:S) $\approx$ 1:10). This protease solution was placed on top of the sample–SA layer. After 30 min of reaction at 37 °C in a moist environment, the buffer droplet was either removed and used as the sample solution in a subsequent sandwich sample preparation or the moist sample was acidified, dried and washed as described for the on-target DTT reduction.

Alternatively, *n*-octyl glucopyranoside (OGP) was used for on-target digestions under denaturing conditions: subsequently, 1 μl of sample solution (freshly prepared in buffer or a dried-droplet sample preparation redissolved in 50 mM hydrogencarbonate), 0.5 μl of a 10 mM solution of OGP in water, 1 μl of 50 mM hydrogencarbonate buffer (pH 7.8) and 0.5 μl of trypsin solution (E:S $\approx$ 1:10) were added to the target. The reaction mixture was kept for 1 h at 37 °C in a moist environment. Thereafter, 1 μl of 2% TFA and 0.5 μl of HCCA (I) were added, the sample was allowed to dry and it was washed according to procedure (1).

Microscale sample purification. For each sample to be purified, a micro purification column was prepared as follows (Fig. 1). A GelLoader tip (Eppendorf) was carefully squeezed at the lower end of the extended outlet with a pair of flat pliers such that the inner diameter is reduced to <50 μm. This procedure is needed to avoid losses of stationary phase material during column packing and operation. Then, 50 μl of methanol were filled into the GelLoader tip using a 200 μl pipette tip. Subsequently, 2–3 μl of a suspension of Poros 50 R1 (PerSeptive, in the case of proteins) or Poros 50 R2 (PerSeptive, in the case of peptides) material in methanol were carefully pipetted into the methanol layer with a 1–10 μl tip so that no air bubbles were included. After the Poros material had settled (prepacked column), the GelLoader tip was mounted on a 1.25 ml Eppendorf Combitip (No. 0030 048.083) loaded with air and the methanol was quickly pressed through. During this procedure, the Poros material forms a small column with a volume of $\sim$1 μl in the GelLoader tip outlet above the squeezed section with the reduced diameter (see Fig. 1). After packing, the column was equilibrated as follows: 20 μl of 0.1% TFA were loaded using a 1–10 μl pipette tip and $\sim$80% thereof were passed over the stationary phase as described above. In this state, the columns can be stored at 4 °C for at least one day if they have been sealed.

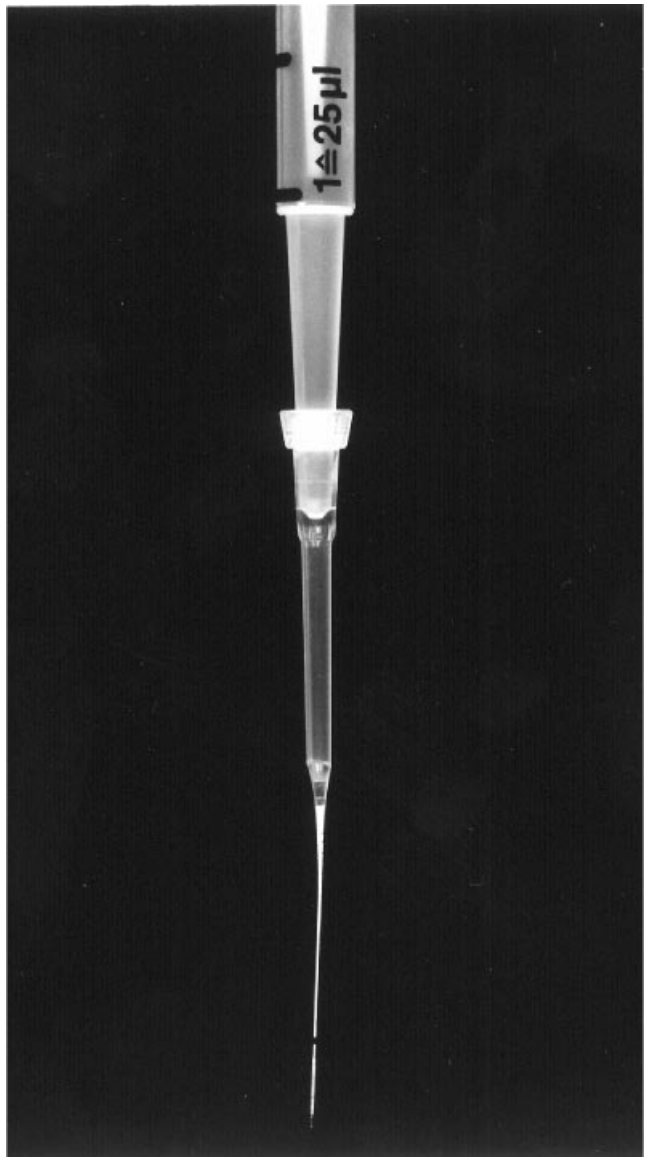

Figure 1. A self-assembled, disposable microscale reversed-phase column. Poros 50 R1 material is packed and equilibrated in the extended outlet of an Eppendorf GelLoader tip (white material on photograph; volume $\sim$1 μl). The GelLoader tip volume above the column material serves as the solvent reservoir and the Eppendorf Combitip, mounted on the GelLoader tip, is used to pass the solvent over the column.

Prior to the following clean-up procedure, it is necessary to acidify the sample solution with 0.1–2% TFA to pH <2 and to ensure that the amount of organic solvent (e.g. ACN, methanol) in the sample solution still allows efficient retention of the analyte molecules on the stationary phase. If the latter is difficult to predict, no organic solvent should be contained in the sample solution. In the case of sample volumes <40 μl, the sample was placed above the micro column using a second GelLoader tip and passed over as described above. In the case of volumes >40 μl, the sample solution was loaded into a second Eppendorf Combitip and the latter was used to pass the sample solution over the column. Subsequently, 100–200 μl of washing buffer ((100 − X)% 0.1% TFA–X% ACN (v/v); X = % ACN in the sample solution) were filled into the syringe and passed over the column. Finally, 2 μl of elution buffer ((100 − Y)% 0.1%

TFA–*Y*% ACN; *Y* = % ACN ensuring complete elution) were placed on top of the stationary phase using a third GelLoader tip and the sample was eluted directly on to the MALDI target for subsequent MALDI-MS sample preparation. When *Y*% was difficult to predict, 0.1% TFA–ACN (20:80, v/v) was used for the elution of both peptides and proteins.

RESULTS

Matrix selection

The choice of matrix must be adapted to the properties of the analyte. Our preferences of matrix selection dependent on the analyte and the matrix compatibility with the sample preparation techniques (1)–(4) (see Experimental) are summarized in Table 1.

HCCA is our first choice for peptide mapping analyses, and it is compatible with all sample preparation procedures described in this study. It is mostly used in the sandwich technique because this combination yields high peptide ion abundances (even for highly contaminated samples), good protein sequence coverage and little methionine or tryptophan side-chain oxidation. The HCCA–sandwich combination has, with respect to the latter, proven superior to the use of HCCA in the dried-droplet or thin-layer method. However, for peptide sample amounts <100 fmol, the thin-layer method, preferentially with NC as additive, exhibits a higher detection sensitivity.

Suppression of low-mass peptide ions with the matrix HCCA has been studied systematically by Cohen and Chait[8] as a function of sample–matrix solvents, sample pH and crystallization times. One result was that fast evaporation of the solvent in the dried-droplet method favours the detection of small peptides whereas larger ones are discriminated. Moreover, the choice of matrix can considerably influence the sequence coverage in peptide mapping, as demonstrated in Fig. 2, where the comparison of HCCA and THAP in a thin-layer sample preparation of a tryptic digest of reduced and alkylated cystatin C (isolated from human cerebrospinal fluid (hCSF)) is shown. With this method, THAP [Fig. 2(a)] yields more abundant peptide ions in the lower mass range (T1, T4, T5, T6) and more alkali adduct ions than HCCA [Fig. 2(b)]. According to our experience, peptide mapping results obtained with the matrix THAP can complement those obtained with HCCA in terms of protein sequence coverage. In this example, the detection of low-mass ions revealed a modified peptide T1 with the mass increment of 16 Da, found to be due to proline hydroxylation.[16]

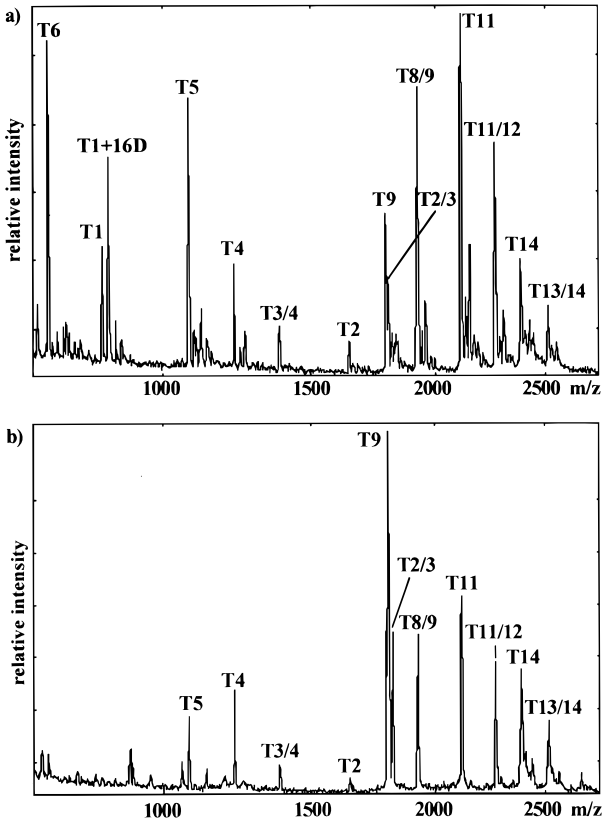

Figure 2. Linear mode MALDI mass spectra of a tryptic digest of reduced and alkylated cystatin C, obtained with (a) THAP and (b) HCCA as the matrix. Both samples were prepared according to the thin-layer method (~1–2 pmol on-target). The matrix THAP yields more low-mass peptide ions and more alkali adducts ions than HCCA.

Sinapic acid is our favourite matrix for protein analysis, whereas for glycopeptides and glycoproteins DHB (I–III) is preferred (see Table 1). As reported previously[17,18] and shown in Fig. 3, the quality of glycopeptide mass spectra obtained with DHB in the dried-droplet sample preparation is highly dependent on the morphology of the sample–matrix crystals. The spectra of a heterogeneously glycosylated peptide, carrying 4–7 GalNac residues (assigned by '4S' to '7S' indices), recorded with DHB (II) as the matrix, are shown. Spectrum (a) was obtained from the large rim crystals of the sample whereas spectrum (b) stems from the smaller crystals in the central area. The rim crystals almost exclusively yielded protonated molecules,

Table 1. Preference (1–3: high to low priority) of matrix selection for different analytes and their compatibility with the sample preparation techniques (1)–(4) (see Experimental)

Matrix	Peptide mapping	Small proteins	Large proteins	Glycopeptides	Glycoproteins	Compatibility
HCCA	1	3		2		Dried-droplet, thin/thick-layer, sandwich
SA		1	1	3	2	Dried-droplet, sandwich
DHB	2	2	1	1	1	Dried-droplet
THAP	2					Dried-droplet, thin-layer

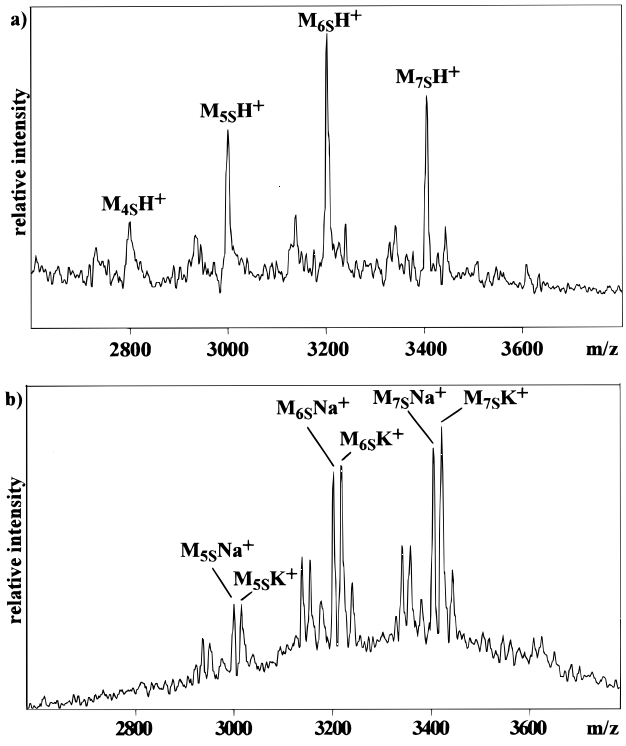

Figure 3. Linear mode delayed extraction (DE) MALDI mass spectra of a heterogeneously glycosylated peptide (~1 pmol on target), carrying 4–7 GalNac residues (assigned by '4S' to '7S' indices). The spectra were both recorded after dried-droplet sample preparations with the matrix DHB. Spectrum (a) was obtained by desorption from the large rim crystals, preferentially showing MH^+ ions, whereas the smaller crystals in the central sample area yielded spectrum (b), dominated by alkali metal adduct ions.

whereas the central sample area preferentially exhibited sodium and potassium adduct ions. Thus, the on-target crystallization process includes the separation of alkali metal salts.

However, HCCA with the sandwich method sometimes yields better results in glycopeptide analysis and is therefore always used as the matrix in a second sample preparation.

Influence of pH

While pH 3 has been reported as the upper limit for sensitive MALDI-MS peptide detection with HCCA as the matrix,[8] acidification to a pH below 2.0 is essential for samples containing considerable amounts of salts, buffers and detergents. Table 2 lists the TFA concentrations needed for on-target acidification to pH < 2 for a variety of buffers and buffer concentrations.[9]

Advantages of the sandwich sample preparation

The sandwich technique is compatible with the matrices HCCA and SA, and it often yields significantly better peptide mapping results than the dried-droplet method. The sandwich method has in particular proven superior in terms of tolerance towards high amounts of impurities (denaturants, detergents, salts, buffers), especially when the sample is not allowed to dry before washing. The spectrum in Fig. 4 shows an abundant series of singly and multiply protonated molecules of the His-tagged C-signalling A protein (CsgA, from *Myxococcus xanthus*). The analyte solution contained 10–20 pmol μl^{-1} of protein in 20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 6 mM imidazole and 8 M urea. A 0.5 µl volume of this solution was directly used for a sandwich sample preparation with HCCA and three washing steps (3 × 10 µl of 0.1% TFA) were performed 1 min after addition of HCCA (I). By contrast, with none of the other matrices and sample preparations described in this study were abundant protein molecular ions observed.

The thin-layer sample preparation often results in considerable oxidation of methionine and tryptophan side-chains. The sandwich method is an alternative in order to circumvent this amino acid modification during sample preparation. Figure 5(a) shows the MALDI mass spectrum of glucagon (porcine pancreas, purchased from Sigma, No. G9279; contains Met^{27}, Trp^{25}) obtained from a thin-layer sample preparation and the spectrum in (b) was recorded after application of the sandwich method. Whereas in spectrum (a) molecular ions of singly and multiply oxidized glucagon can be seen ($\Delta m/z$ 16, 32 and 48, respectively, relative to MH^+ of native glucagon), spectrum (b) almost exclusively shows the molecular ion of unmodified glucagon. The triply oxidized glucagon derivative observed in spectrum (a) probably contains a methionine sulfoxide (+16 Da) and a doubly oxidized tryptophan side-chain (+32 Da).

On-target reactions

After having recorded a spectrum of a peptide or protein, it is possible to perform further reactions with the same sample on the same target. On-target protein chemistry has been developed for plasma desorption MS[19] and can also be applied to MALDI-MS.

Table 2. TFA concentrations (%) needed for on-target acidification to pH < 2 with 0.5–1 µl of TFA solution[a]

Buffer	5 mM	25 mM	50 mM	75 mM	100 mM	500 mM
NH_4HCO_3	0	0.5	0.5	1.0	1.0	5.0
NaH_2PO_4	0.5	0.5	0.5	1.0	1.0	5.0
Na_2HPO_4	0.5	1.0	1.0	2.0	2.0	10.0
Tris–HCl	0.5	0.5	1.0	1.0	1.0	5.0

[a] The adjustment is given for different buffers and buffer concentrations.

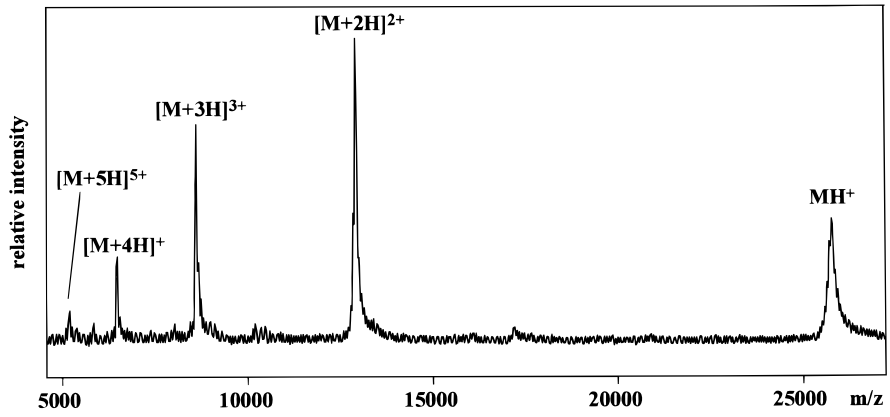

Figure 4. Linear mode DE-MALDI mass spectrum of the protein CSgA showing an abundant series of singly and multiply protonated molecules. The analyte solution contained 10–20 pmol μl^{-1} of protein, 8 M urea and various buffers and salts. A 0.5 µl volume of this solution was subjected to a sandwich sample preparation including extensive washing according to procedure (4).

On-target DTT reduction. On-target DTT reduction of peptides and peptide mixtures was successfully applied to disulfide bond determination of the major cat allergen fel D 1.[20] Sometimes, particularly if the on-target reduction was applied to complex peptide mixtures, not all of the expected reduced cysteinyl peptides were observed.

On-target proteolytic digestion. As an alternative to conventional digestion in solution, for instance in the case of very low sample amounts or the need of a fast protein identification, the proteolytic digestion can be performed on-target. If a protein is particularly resistant to proteolysis, even in 8 M urea or 6 M guanidinium hydrochloride, proteolytic degradation can sometimes be achieved by addition of OGP to the (on-target) digestion mixture. OGP is a non-ionic detergent known to aid in protein solubilization and also to often improve the quality of MALDI mass spectra of proteins or peptide mixtures.[8] The latter finding is in contrast to ionic detergents, e.g. sodium dodecyl sulfate (SDS), that drastically deteriorate the quality of MALDI mass spectra.

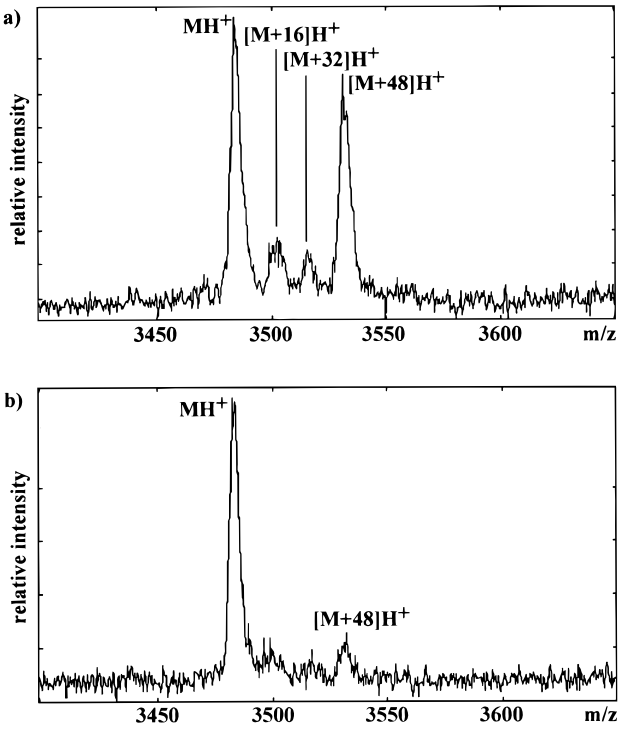

Figure 5. Linear mode MALDI mass spectrum of 1.5 pmol of porcine glucagon obtained with the matrix HCCA, (a) from a thin-layer and (b) from a sandwich sample preparation. Whereas in spectrum (a) molecular ions of singly and multiply oxidized glucagon were detected ($\Delta m/z$ 16, 32 and 48 Da, respectively), spectrum (b) almost exclusively shows the molecular ion of unmodified glucagon.

Figure 6(a) and (b) show the MALDI mass spectra of two variants of HPLC-purified cystatin B with a mass difference of ~330 Da. The spectra in (c) and (d) represent the MALDI-MS peptide maps obtained by tryptic on-target digestions of (a) and (b), respectively. The peptide map in (c) yielded a sequence coverage of ~95%, considering the fact that T1 was found modified. The molecular ion at m/z 2710.2 represents the doubly oxidized (at Met^1 and Met^2) and cysteinylated (at Cys^3) peptide T1 (these modifications were further confirmed[16]). Spectrum (d) reveals the 330 Da mass difference between the two cystatin B isoforms [spectra (a) and (b)] as being due to further modification of T1, i.e. glycosylation in addition to oxidation and cysteinylation. The T1 peak pattern suggests a heterogeneously glycosylated peptide, probably carrying HexNAc and HexNAc plus a pentose on one of the residues Ser^7, Thr^9, Thr^{13} or Thr^{16}.

Nitrocellulose as matrix additive

The preparation of a homogeneous matrix–NC layer is important. In addition, particularly if the modified NC preparation on Scotch tape is used, the calibration is crucial. Preferably, the sample is spiked with an internal standard. Alternatively, an external calibration, obtained from a protein or peptide standard also prepared with the thick-layer method, is applied.

The addition of NC to the matrix results in significantly improved spectra from samples contaminated with salts and synthetic polymers (e.g. polyethylene glycol (PEG)), a finding illustrated in Fig. 7: Spectrum

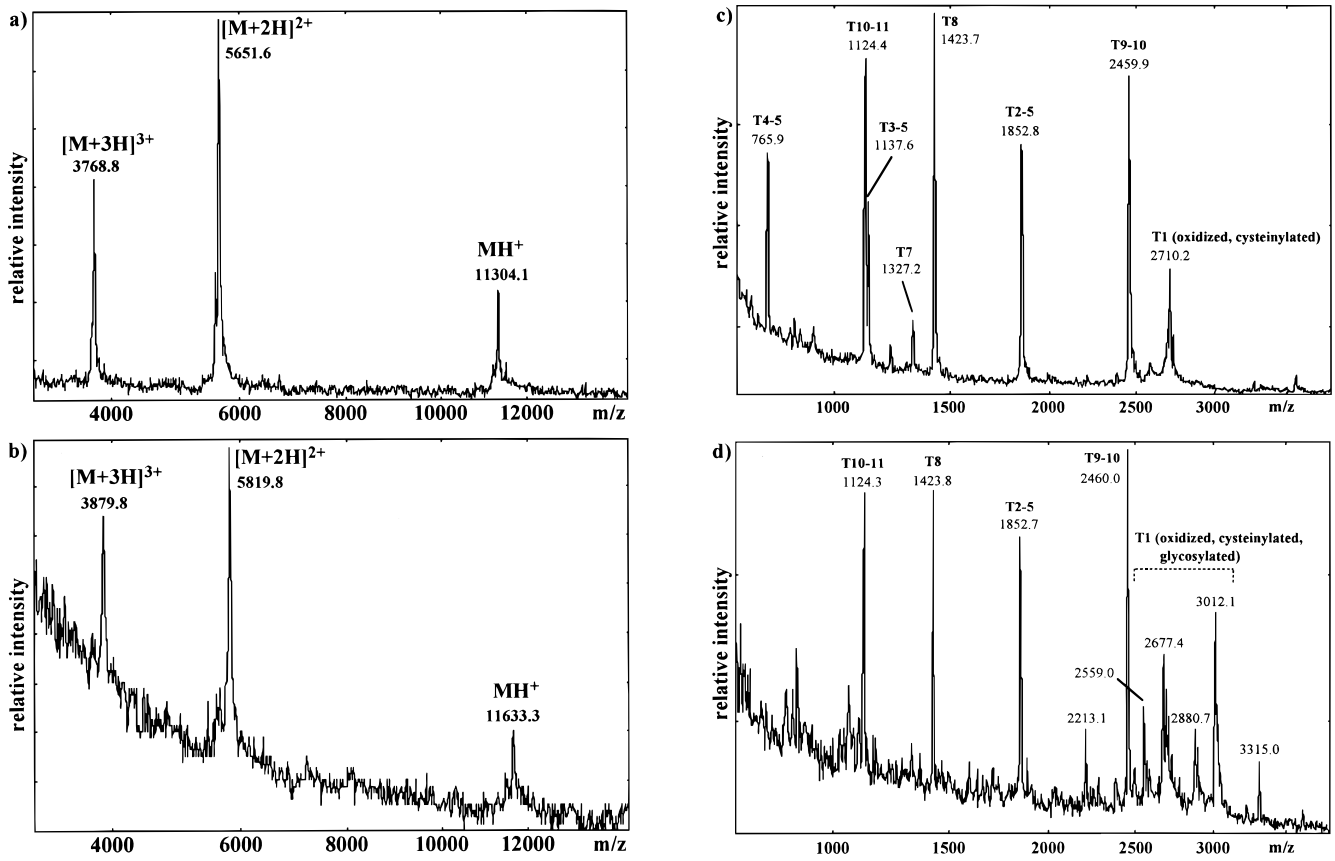

Figure 6. (a) and (b): linear mode MALDI mass spectra of two variants of HPLC-purified cystatin B (~1–2 pmol of each variant on-target) with a mass difference of ~330 Da; (c) and (d) show linear mode MALDI mass spectra obtained after tryptic on-target digestions of (a) and (b), respectively. The peptide map in (d) reveals the 330 Da mass difference being due to a modification of the tryptic peptide T1. In spectrum (c), the major peak corresponding to T1 (*m/z* 2710.2) represents the doubly oxidized (at Met^1 and Met^2) cysteinylated species. In spectrum (d), the predominant T1 signal (*m/z* 3012.1) is the cysteinylated and probably glycosylated (HexNAc, +203 Da; pentose, +132 Da), but not oxidized species. The extent of methionine oxidation depends on sample preparation conditions and varies between different spectra. The O-glycans probably account for the ~330 Da mass difference observed in spectra (a) and (b) of the intact cystatin B variants.

(a) shows a peptide map obtained from a sandwich sample preparation with HCCA as the matrix. The peptides were extracted from an in-gel tryptic digest of a yeast protein isolated by 2D SDS polyacrylamide gel electrophoresis (PAGE). The low-mass polymer signals with a spacing of *m/z* 44, corresponding to PEG molecular ions separated by ethylene glycol monomer units, are predominant and only few and low abundant peptide molecular ions were detected. The spectrum quality is further reduced by excessive alkali adduct formation. In contrast, when using NC in a thick-layer sample preparation [Fig. 7 (b)], the polymer signals are nearly suppressed and no peptide alkali adducts but instead intense MH^+ signals of the expected tryptic peptides were detected. Apparently, the PEG contamination and the alkali ions were efficiently retained by the NC.

Figure 8 shows the spectra of a tryptic β-lactoglobulin digest obtained with (a) the thin-layer HCCA–NC sample preparation, (b) the thick-layer HCCA method and (c) the sandwich sample preparation. Aliquots of 100 fmol of the digest in 50 mM hydrogencarbonate were directly applied to the target. From (a) to (c), a decreasing signal-to-noise ratio is observed, in particular, when following on the ion abundances of the peptides 77–85, 77–86 and 31–56.

Microscale sample purification

If contaminants prevented the detection of peptide or protein molecular ions even after application of all sample preparation variants and on-target purifications mentioned above, or if an analyte concentration step was needed prior to sample preparation, a micropurification procedure, based on self-assembled, disposable micro LC columns, was performed (see Fig. 1). Poros material (PerSeptive) with reversed-phase (RP) properties was packed and equilibrated in GelLoader tips in order to obtain RP columns with volumes of 1–2 µl. Features of this technique are efficient purification and concentration of pmol to low fmol amounts of peptides and proteins and circumvention of memory effects due to the use of one column for each sample.

The performance of this micropurification is illustrated by the DE-MALDI mass spectrum of bovine tau proteins (purchased from Sigma) shown in Fig. 9: four groups of molecular ions, representing different sequence variants of tau, were detected and the partial resolution of the two most abundant molecular ion series revealing spacings of $m/z \approx 80$ suggests the presence of multiply phosphorylated forms of these two species. Without prior micropurification, no spectra

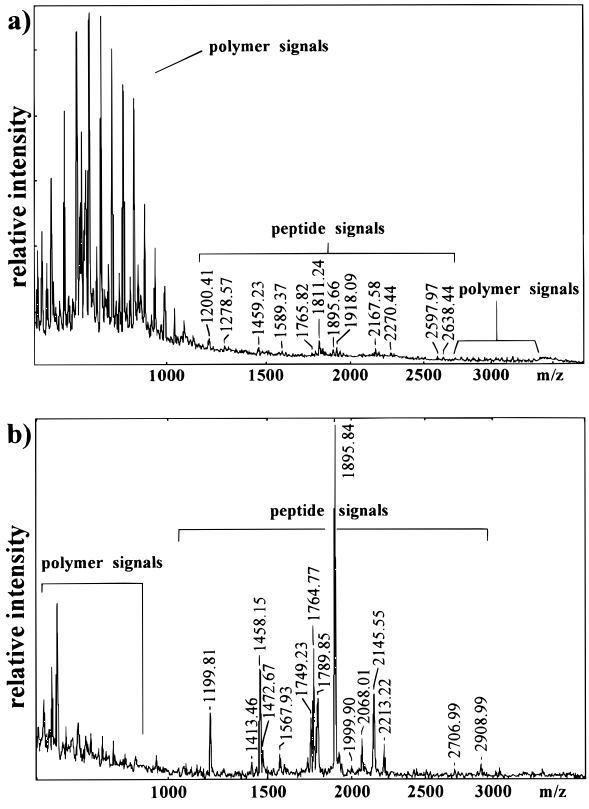

Figure 7. (a) MALDI-MS peptide map of an in-gel tryptic digest of a yeast protein isolated by 2D SDS-PAGE (sandwich sample preparation, matrix HCCA, ~1 pmol on-target, acquired in linear mode). A PEG molecular ion series (contamination) is predominant and only low-abundant peptide molecular ions are detected. (b) The same amount of the same sample analyzed with the thick-layer sample preparation (matrix HCCA–NC, ~1 pmol on-target, linear mode): intense MH^+ signals of the expected tryptic peptides are observed and the PEG signals are nearly suppressed.

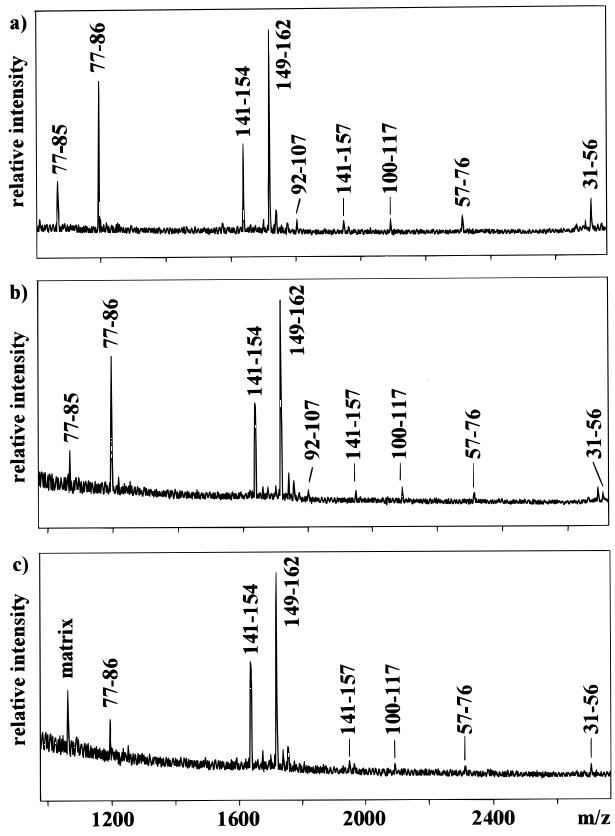

Figure 8. Reflector mode DE-MALDI mass spectra of a tryptic β-lactoglobulin digest obtained with (a) the thin-layer HCCA–NC sample preparation, (b) the thick-layer HCCA–NC method and (c) the sandwich technique. In each case, 100 fmol aliquots of the digest in 50 mM hydrogencarbonate (pH 7.8) were directly applied to the target. From (a) to (c), the absolute signal intensity and the signal-to-noise ratio deteriorate.

could be obtained with any of the previously described sample preparation methods.

DISCUSSION

There is no universal sample preparation method yielding good results for a broad variety of peptide and protein analytes. According to our experience, rather a number of alternatives should be available. These include several matrix compounds combined with various sample preparation techniques. However, based on specific information on the analyte properties, it is possible to select a promising first attempt (Table 1), but often subsequent variations are needed to optimize the results.

The adjustment of the sample–matrix mixture to $pH < 2$, depending on the buffer contents, is essential and allows direct analysis of salt and buffer containing samples. The sandwich method represents, in terms of the preparation procedure, a combination of the thin-layer and the dried-droplet method. The sandwich method is superior to the thin-layer method in terms of less methionine and tryptophan side-chain oxidation. The sandwich method is furthermore highly compatible with on-target sample clean-up, whereas the thin-layer method is susceptible to significant losses of low-mass and/or hydrophilic peptides, if the sample is extensively washed.

We have found HCCA to be universally applicable to all sample preparations described in this study, whereas, for instance, SA cannot be used in the thin-layer preparation, and DHB is exclusively compatible with the dried-droplet method. However, the combinations of matrix compounds, sample preparation procedures, and the use of additives described in this study do not claim completeness.

The addition of nitrocellulose to the matrix solution suppresses alkali adduct formation and predominant molecular ion series of synthetic polymers, e.g. PEG, and thus significantly increases the detection sensitivity, especially for peptides and proteins extracted from gels. When comparing the two NC sample preparations, the thick-layer variant turns out to have a larger capacity of adsorbing contaminants whereas the thin-layer method reveals a higher ultimate sensitivity.

The question of which method provides the highest detection sensitivity for a given analyte is predominantly dependent on the amount and composition of contaminants in the sample. In our experience, in the case of samples containing moderate amounts of buffers (e.g. 50 mM hydrogencarbonate; see Fig. 9), little detergent, denaturant or synthetic polymer, the thin-layer

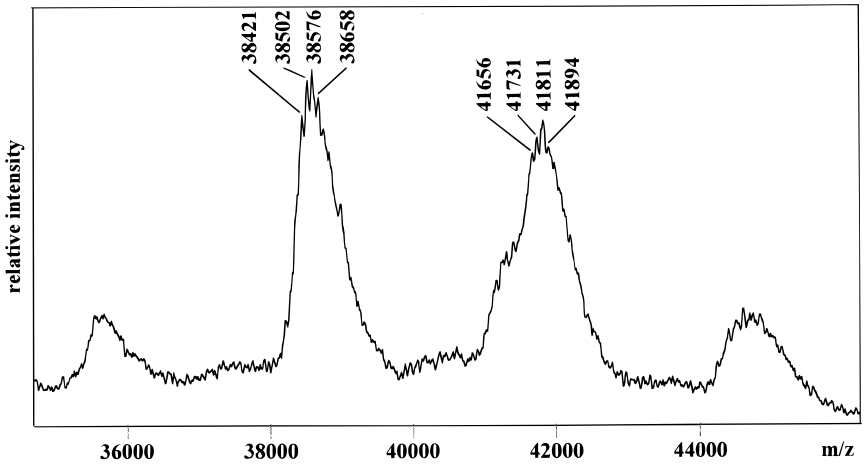

Figure 9. Linear mode DE-MALDI mass spectrum of bovine tau proteins (5 pmol subjected to micro purification, protein eluted with 2 μl on to the target; dried-droplet sample preparation with SA as the matrix. Four groups of molecular ions, representing different sequence variants of tau, were detected and the partial resolution of the most abundant molecular ions with spacings of $m/z \approx 80$ suggests, in addition, the presence of differently phosphorylated species.

HCCA–NC method is more sensitive for peptide detection than the thick-layer technique, and the latter is more sensitive than the sandwich method. However, if large amounts of contaminants, e.g. urea (see Fig. 4), guanidinium hydrochloride or PEG (see Fig. 8) are present, the sandwich or the thick-layer method, respectively, are advantageous because the contaminants can be washed off on-target (sandwich) or are efficiently adsorbed by the matrix (thick-layer).

Moreover, we have shown that chemical reactions and proteolytic digestions can be carried out on-target, e.g. a protein can be analysed in a dried-droplet sample preparation with SA as the matrix and subsequently (reduced and) digested on-target followed by subjecting the resulting reaction mixture to a sandwich sample preparation with the matrix HCCA.

Finally, a microscale sample purification is sometimes recommended whenever on-target sample purification was not successful and/or an analyte concentration step is needed prior to sample preparation.

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