

Optimizing the MALDI-TOF-MS Observation of Peptides Containing Disulfide Bonds

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The observation of peaks corresponding to both disulfide-bonded and reduced peptides in matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra of disulfides could suggest that the samples are either mixtures prior to analysis or that the measurement process has converted single compounds into mixtures. This is an important distinction when characterizing potentially disulfide-bonded peptides obtained from proteolyzed proteins or from oxidized synthetic peptides. It is well documented that disulfides can undergo in-source decay (ISD) when using a 337-nm laser. However, the mixed matrix 2-(4-hydroxyphenylazo)benzoic acid: α -cyano-4-hydroxycinnamic acid (1:10) not only suppresses the ISD reduction of disulfides to thiols but allows the same low threshold laser power generally used with α -cyano-4-hydroxycinnamic acid to be applied.

KEY WORDS: Disulfides, peptides, MALDI-TOF-MS, ISD, mixed matrices.

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The analysis of potentially disulfide-bonded peptides by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) can be obscured by the experimental procedure. It is well documented that MALDI-TOF mass spectra often show the expected peaks corresponding to disulfides accompanied by their reduction products. Patterson and Katta¹ and Crimmins et al.² have offered convincing evidence that disulfides undergo in-source decay (ISD) during the MALDI experiment (337-nm laser) and that increasing the laser power increases the signal intensity for thiols while decreasing the intensity for the disulfide. However, the observation of peaks corresponding to both disulfide-bonded and reduced peptides in MALDI-TOF-MS could suggest that the samples are either mixtures prior to MALDI analysis or that the measurement process has converted single compounds into mixtures. This is an important distinction when characterizing potentially disulfide-bonded peptides obtained from proteolyzed proteins or from oxidized synthetic peptides.

While establishing the disulfide bond assignments in human thrombospondin type 1 domains,³ we searched for a way to use MALDI-TOF-MS to show that our isolated disulfides were not mixtures. Other laboratories have developed methods to characterize disulfide-bond-containing peptides: Jones et al.⁴ used post-source decay (PSD) to follow disulfide fragmentation; Katta et al.⁵ showed that ISD could be used for direct sequence analysis of disulfide-containing peptides; Mhatre et al.⁶ used on-plate reduction of protease digest mixtures to identify disulfide-containing peptides; and Schnaible et al.⁷ are automating a process that uses an algorithm to look for both disulfides and thiols in ISD spectra followed by fragmentation of the suspected disulfide.

While other laboratories have exploited the reactivity of disulfide bonds for analysis by ISD and PSD MS, our experiments involved changing the matrix and using mixed matrices. This report describes the conditions we found that control the redox state of sulfur-containing peptides during the MALDI experiment.

MATERIALS AND METHODS

The matrices α -cyano-4-hydroxycinnamic acid (CHCA), 2-(4-hydroxyphenylazo)benzoic acid (HABA), and 2,5-dihydroxybenzoic acid (DHB) were purchased from Sigma (St. Louis, MO). Peptides A and B were prepared from the third type 1 repeat of human thrombospondin-1 that had been expressed in the baculovirus system, purified, and shown to be fully disulfide bonded.³ The peptides were generated by thermolysin digestion at pH 6.5 and separated by high-performance liquid chromatography (HPLC) using a Vydac C18 column (Separations Group, Hesperia, CA). Peptide A contains one disulfide bond and peptide B contains two. MALDI targets were prepared by spotting 0.5 μ L of HPLC fraction followed by 0.5 μ L of matrix solution. Initially, three matrix solutions were used: CHCA (10 mg/mL in 70% acetonitrile/30% water), HABA (10 mg/mL in 70% acetonitrile/30% water); and DHB (20 mg/mL in 50% ethanol/50% water). While optimizing disulfide-bond analysis, two matrix mixtures were prepared: (1) 1 part HABA, 1 part CHCA; and (2) 1 part HABA, 10 parts CHCA.

Mass spectra were obtained using a Bruker REFLEX II (Billirica, MA) equipped with delayed extraction, a reflectron, and a 337-nm nitrogen laser. Unless noted, spectra were obtained using the reflectron mode and external calibration and are the average of at least 50 shots. Calibrants used included adrenocorticotrophic hormone fragment [18–39], angiotensin II, insulin, and matrix. Peptides A and B were reduced on-target with dithiothreitol* as follows: A 0.5- μ L aliquot of peptide, which had previously been dried and resuspended in water, was incubated with 0.5 μ L of dithiothreitol (20 mM in 8 mM $\text{NH}_3/\text{H}_2\text{O}$) for approximately 5 min. The reaction was quenched by the addition of 0.5 μ L of matrix solution. Dried spots containing either CHCA or HABA were washed when necessary to remove small water-soluble molecules by placing a drop on target with a pipet and then removing it with a pipet or tissue.

RESULTS AND DISCUSSION

In the MALDI experiment, the disulfide is first mixed with a matrix and then dried. Once in a vacuum, the spot is hit with energy in the form of 337-nm light. Usually the matrix is present in great excess (>1000), and it readily absorbs 337-nm light energy. Two locations where the matrix and analyte are in close con-

*See Chait and Field⁸ and Caprioli and Fan⁹ for early examples of on-probe reactions.

tact so that reactions can occur are in the preparation of the spot before the laser fires and in the vacuum just after the laser fires and before the plume disperses. Figure 1 contains the structures of the peptides and matrices used in this report. Figure 2 contains the mass spectra of peptide A in three standard matrices. Peptide A contains one disulfide bond. With CHCA and DHB, the spectra show peaks where the two thiols resulting from reduction of the disulfide bond would be expected. Although the peaks are not large relative to the disulfide peak, they are present. However, with HABA and the same peptide solution used to obtain the CHCA and DHB spectra, only a peak for the disulfide was observed. Why did HABA produce a clean spectrum?

When aliquots of peptide B were subjected to on-target reduction with dithiothreitol^{8,9} mixed with the same three matrices, and subjected to MALDI-TOF-MS, complete reduction of the disulfide bonds was observed. The results are shown in Figure 3. Both CHCA and DHB clearly produced the expected thiols. While peaks corresponding to thiols could be observed using HABA, the spectra were weaker and generally had a more complicated background. Because a lower laser power can be used with CHCA relative to DHB and HABA, the matrix of choice for thiols is CHCA.

In order to understand and verify the protective effect of HABA for disulfides, we mixed HABA with other matrices. Figure 4 contains three of the best results for peptide A. In Figure 4a, an aliquot of peptide A was put on the target, immediately followed by an aliquot of HABA and then an aliquot of CHCA. Unlike our general practice of pulling the mixture into the pipet for a final mix, no extra mixing was needed. In Figure 4b, one part HABA was mixed with 10 parts CHCA before being mixed with the peptide solution. In Figure 4c, one part HABA and one part CHCA were mixed before being mixed with the peptide solution. No peaks that could be assigned to disulfide were present in any of the spectra shown in Figure 4. The best signal-to-noise ratio for peptide A was observed with the 1:10 HABA:CHCA mix. Figure 5 displays mass spectra obtained for peptide B. The data collected using just CHCA (Fig. 5a) show strong evidence of decomposition of the disulfide bonds, whereas the data collected using HABA (Fig. 5b) and 1:10 HABA:CHCA (Fig. 5c) show only peptide B.

Several notable points were revealed upon examination of the matrix regions for CHCA, HABA, the 1:10 HABA:CHCA mixture, and the 1:1 HABA:CHCA mixture, obtained at the same laser attenuation and number of laser pulses (Fig. 6). First, whenever HABA is present in combination with CHCA, the signals for

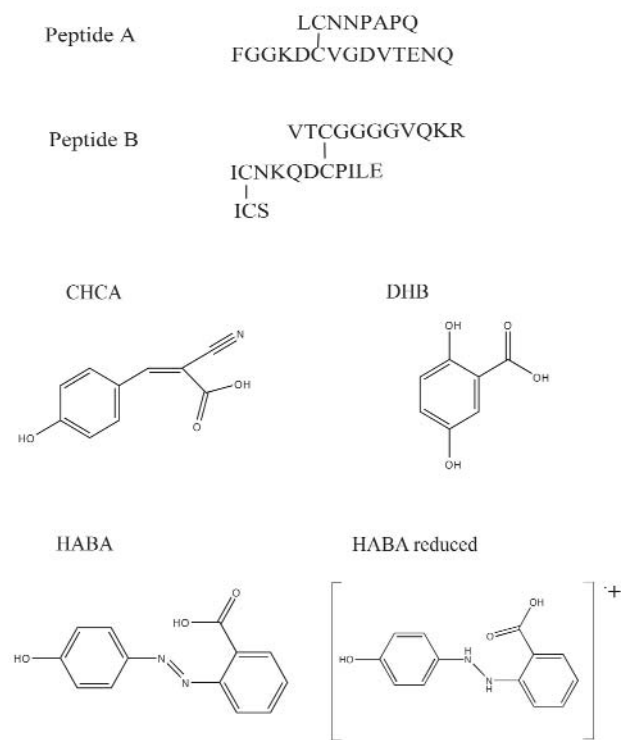


FIGURE 1

Structures of the peptides and matrices used in this study.

HABA ions dominate. Second, lower signal intensity is obtained for HABA relative to CHCA. To achieve the same signal intensity for HABA, lower laser attenuation (higher power) is required (data not shown). With the 1:10 and 1:1 HABA:CHCA mixtures, HABA ions dominate. Interestingly, with the 1:10 mix the HABA ions appear at the same laser attenuation used for CHCA alone. So while CHCA ions do not dominate, the presence of CHCA allows the MALDI experiment to be run at same high-laser attenuation (low power) settings used for CHCA. This advantage is not evident in the 1:1 mixture, in which the HABA ions still dominate but the laser power advantage of CHCA has disappeared.

Looking carefully at Figure 6, it can be seen that, in the presence of CHCA, the isotope pattern for the $[\text{HABA}+\text{H}]^+$ ion does not match the predicted pattern. The intensity of the peak at m/z 244 is too high relative to m/z 243, indicating that more than one species is responsible for the pattern. The elemental formula for $[\text{HABA}+\text{H}]^+$ is $[\text{C}_{13}\text{H}_{11}\text{N}_2\text{O}_3]^+$ and has a predicted monoisotopic mass of 243. Using an isotope pattern calculator—such as IsoPro 3.1,¹⁰ the University of Sheffield's ChemPuter website,¹¹ or one that is part of a mass spectrometer's software—the intensity for

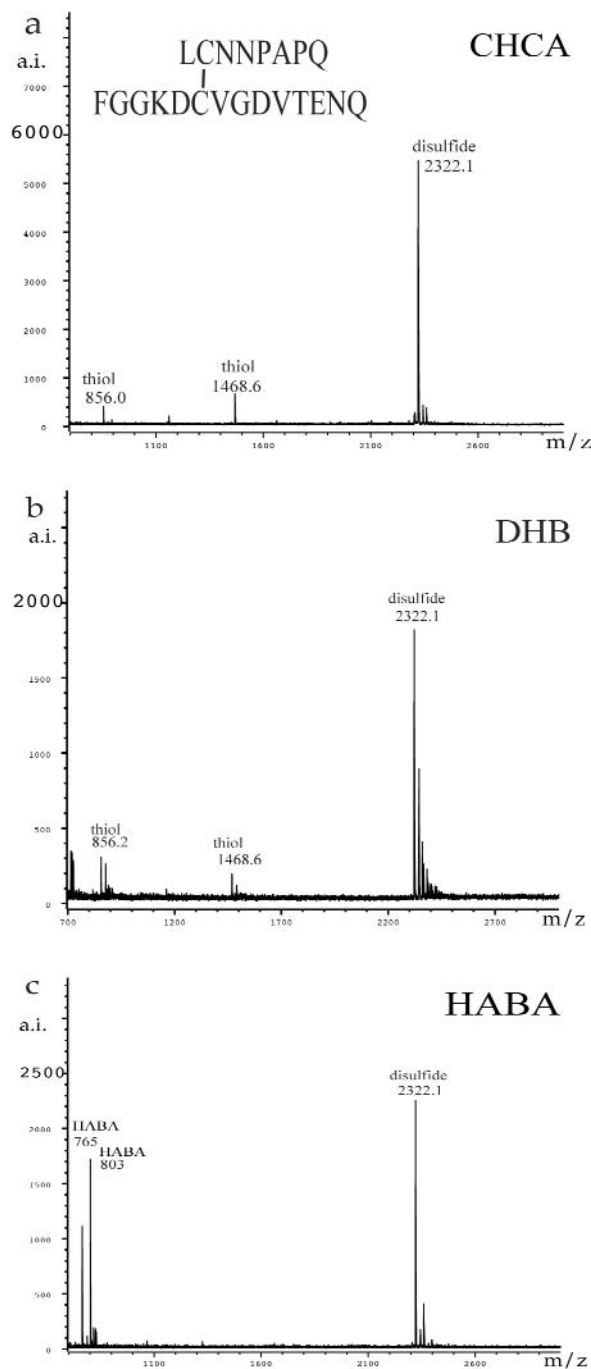


FIGURE 2

The MALDI-TOF mass spectra of peptide A obtained with (a) CHCA, (b) DHB, and (c) HABA.

m/z 244 should be 15% of that for m/z 243 (Fig. 7). In the 1:10 mix and the 1:1 mix, the relative intensity is much greater than predicted (Fig. 7). If the observed excess intensity at m/z 244 corresponds to reduced

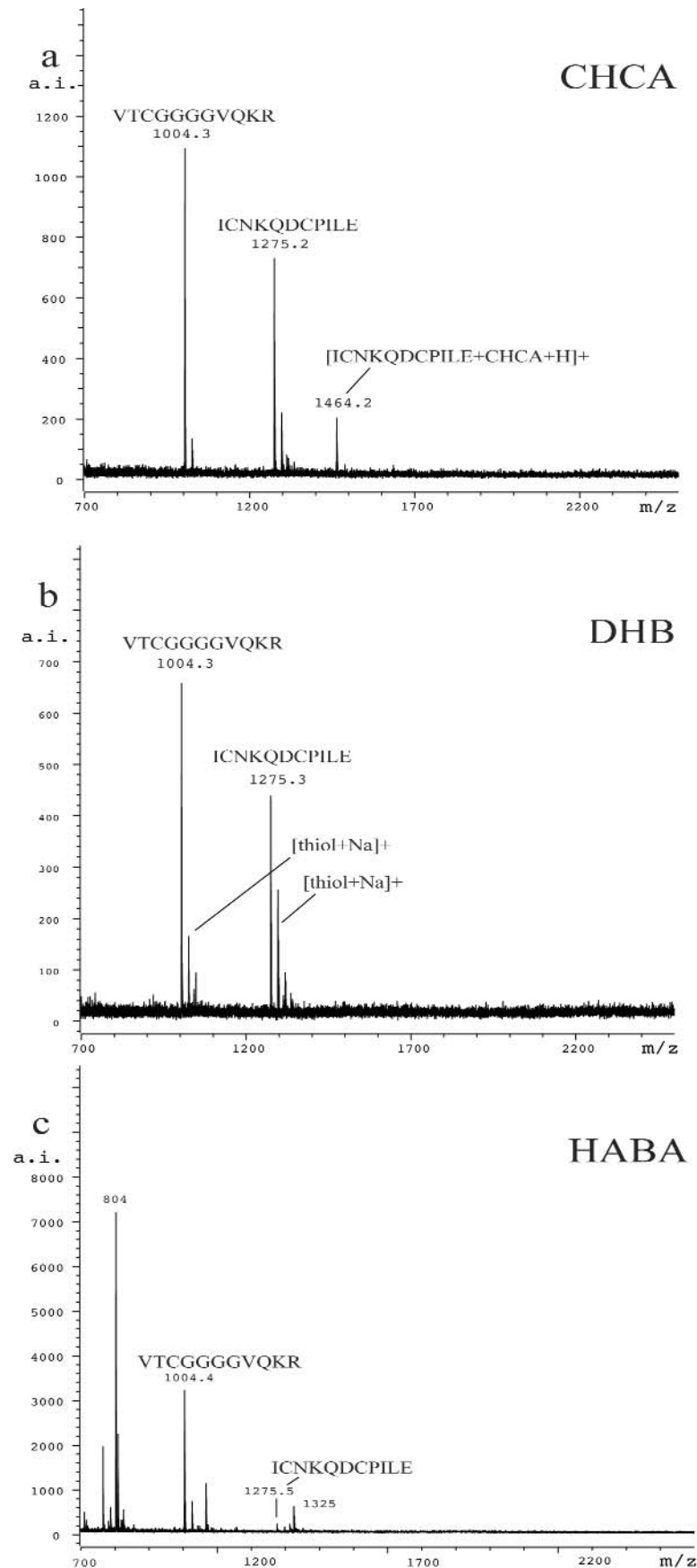


FIGURE 3

The MALDI-TOF mass spectra of peptide B obtained after on-target reduction was quenched with (a) CHCA, (b) DHB, and (c) HABA.

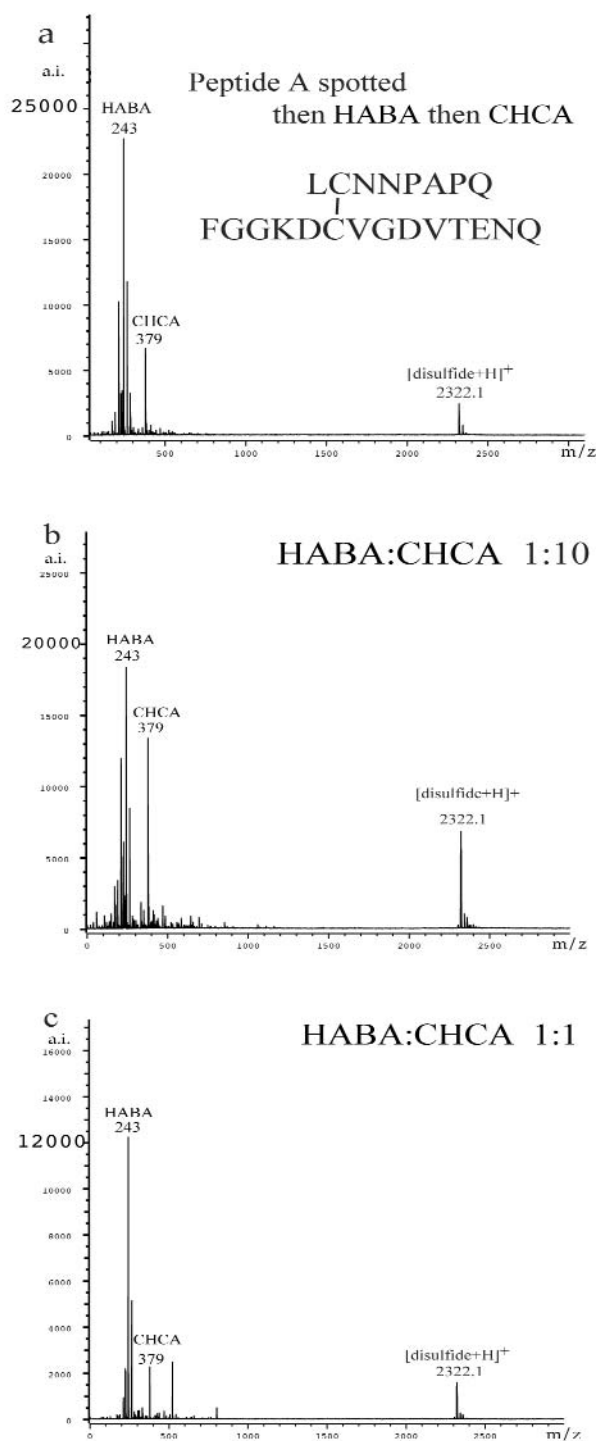


FIGURE 4

The MALDI-TOF mass spectra of peptide A obtained with (a) HABA then CHCA before drying, (b) 1:10 HABA:CHCA, and (c) 1:1 HABA:CHCA. No peaks assignable to thiols were observed.

HABA (radical cation with m/z 244), then the protective effect HABA has for disulfides can be explained by the reduction of HABA in the MALDI experiment prior to the disulfides. Looking at the isotopic clusters assignable to $[\text{CHCA}+\text{H}]^+$ at m/z 190, and the cluster assignable to $[\text{CHCA}+\text{Na}]^+$ at m/z 212, the patterns in the 1:10 mix are near normal (Fig. 7). However, when only CHCA is present, the amount of m/z 191 is too large, again indicating that more than one species is producing the cluster of peaks (data not shown). Increasing the laser power (lowering the attenuation) just further distorts the expected isotope pattern for m/z 243 (Fig. 7d). In mixtures of HABA and CHCA, HABA appears to be protecting CHCA from reduction by reacting first.

The observation of reduction in mass spectrometric desorption processes is not new. For example, several groups^{12–15} have reported reduction using fast atom bombardment (FAB). With FAB, a mixture of liquid matrix and analyte are splattered (desorbed) into the vacuum by beams of cesium ions or xenon atoms. The process momentarily adds energy to the mix, resulting in possible reactions. In 1987, Williams et al.¹² showed that reduction was a common process in FAB. Since FAB does not use 337-nm light, their observations suggest that the close proximity of excited matrix and analyte molecules is all that is needed for reduction to occur. Kyranos and Vouros¹⁴ reported that FAB reduction processes depended on the relative redox potentials of the analyte and the matrix. They investigated a series of dyes in several FAB matrices (glycerol, sulfolane, thioglycerol, hydroxyethyl disulfide, *m*-nitrobenzyl alcohol). Methyl red, which has an azo group, has also been studied using FAB by Florencio and Heerma.¹⁵

More recently, Zenobi's group¹⁶ has investigated the reduction of Cu(II) to Cu(I) seen in MALDI spectra when only Cu(II) has been placed on the target. They state that two processes are generally available to accomplish the task. The first is gas-phase charge exchange with matrix molecules such as nicotinic acid, dithranol, and 2,5-dihydroxybenzoic acid. The second is reduction with free electrons generated when the laser hits thin layers of matrix on a metal target. Knochenmuss¹⁷ has built a quantitative model of the MALDI process incorporating thermodynamically favored reactions in the plume just after the laser shot hits the matrix–analyte spot and incorporating free electron capture. His model supports the idea that chemicals added to the analyte–matrix mix can dictate what the detector will register. Thus it makes sense that adding a protecting agent (HABA in this report) to the analyte–matrix mixture leads to observation of nonreduced disulfides.

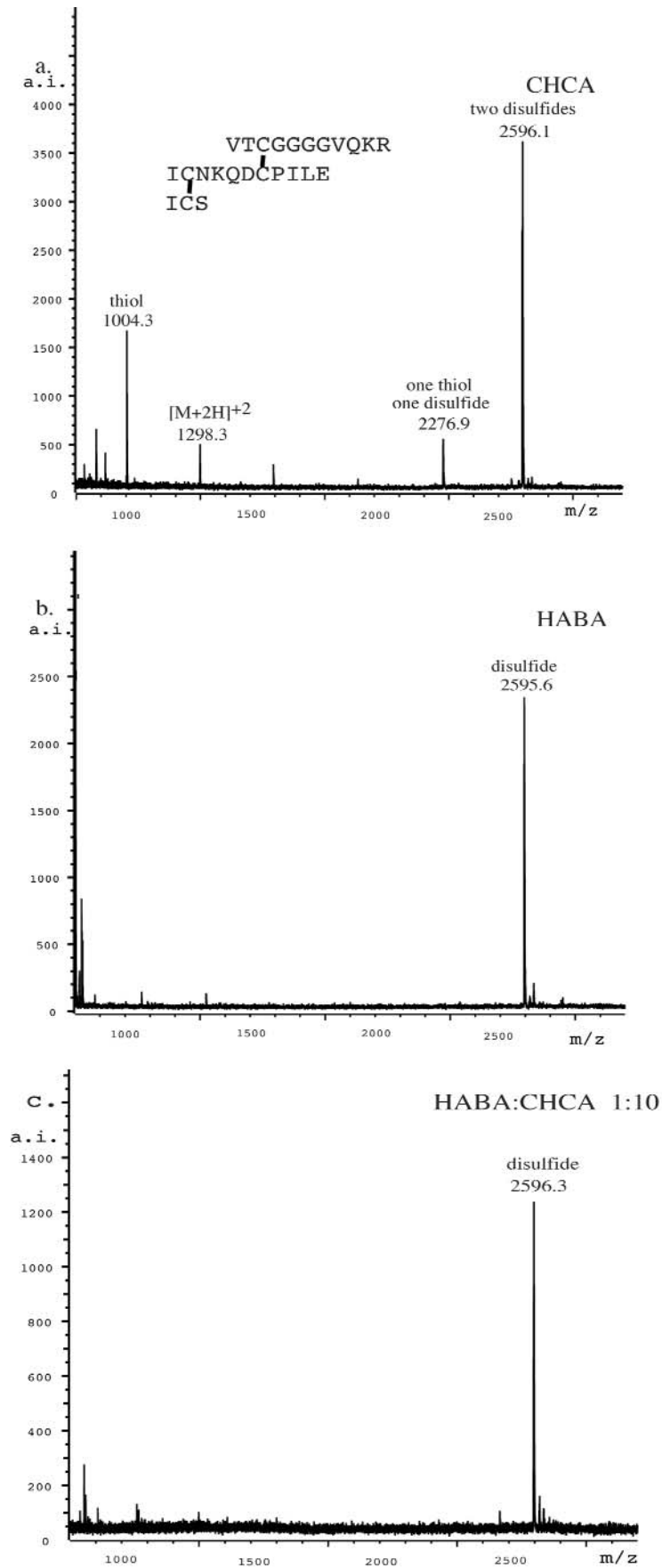


FIGURE 5

The MALDI-TOF mass spectra of peptide B obtained with (a) CHCA, (b) HABA, and (c) 1:10 HABA:CHCA.

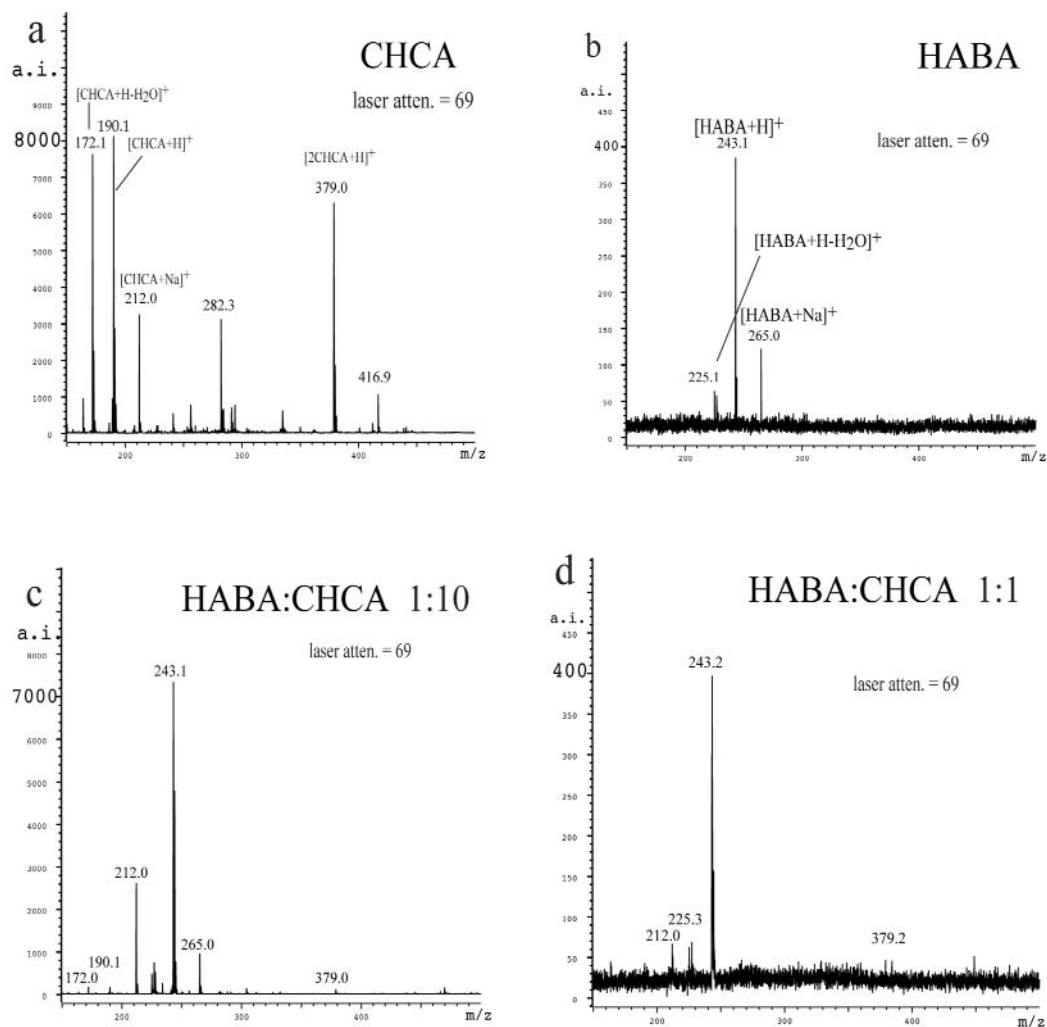


FIGURE 6

A comparison of the matrix regions of (a) CHCA, (b) 1:10 HABA:CHCA, (c) 1:1 HABA:CHCA, and (d) HABA.

CONCLUSIONS

The mixed matrix HABA:CHCA (1:10) is an excellent choice for MALDI analysis of disulfide-bonded peptides. Not only is the ISD reduction of disulfides to thiols suppressed but the same low-threshold laser power generally used with CHCA is still feasible. Energy transfer from CHCA to HABA is implied by the observation that HABA ions dominate over CHCA in mixed matrices, even when CHCA is present in excess and when the lower laser powers applicable for CHCA are used. CHCA is the matrix of choice for cysteine-containing peptides, as its use leads to clean spectra at low-threshold laser powers. Cysteine-containing peptides are easily generated from solutions of the corresponding disulfides on-target. HABA appears

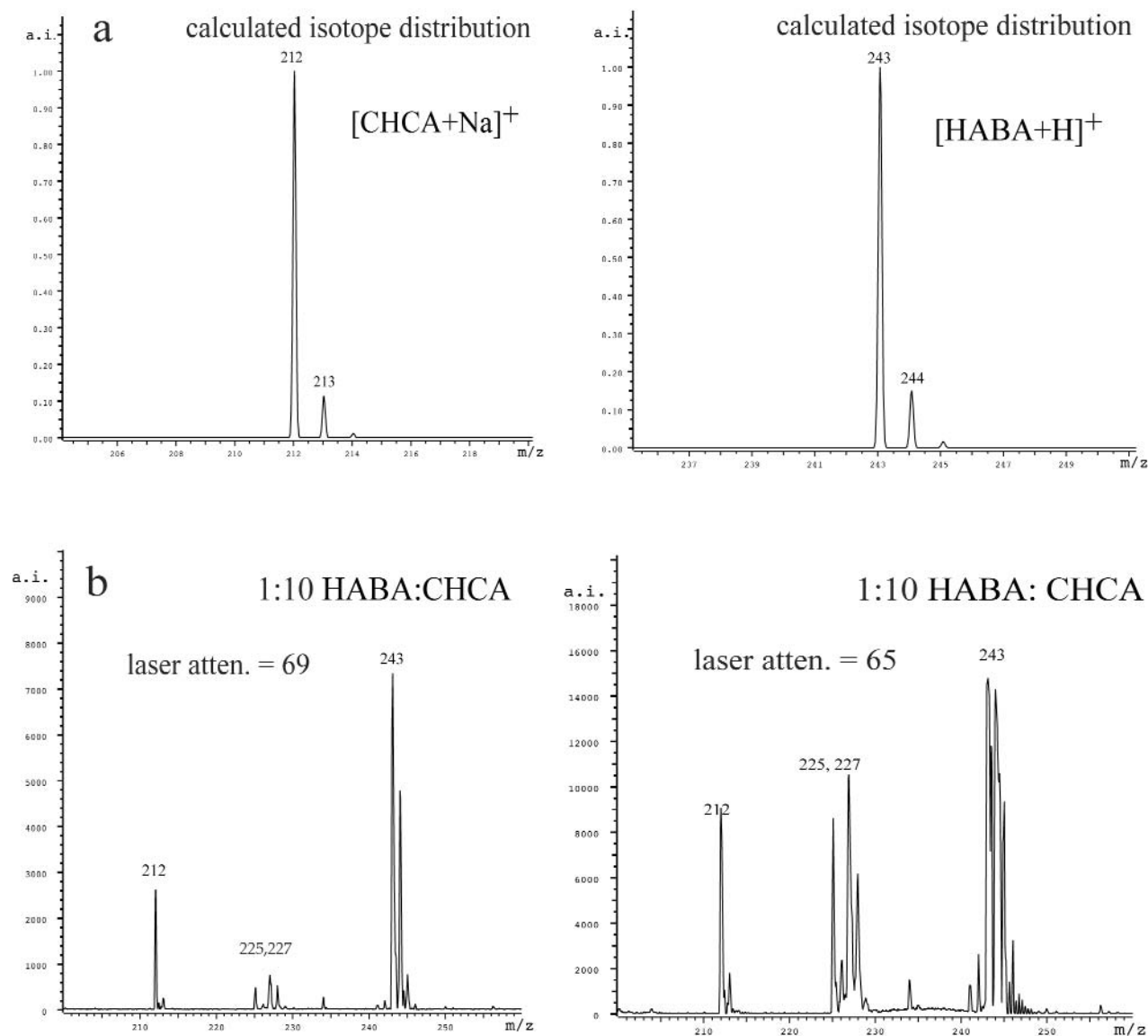
to be protecting disulfides from reduction by being reduced preferentially. The idea of using a mixed matrix and/or adding a protective reagent to a matrix for MALDI-TOF-MS may be of general utility for other redox active compounds.

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**FIGURE 7**

a: The calculated isotope patterns for [CHCA+Na]⁺ and [HABA+H]⁺. **b:** The observed matrix region for 1:10 HABA:CHCA at two laser attenuations.

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