

OMS Letters

Dear Sir

α -Cyano-4-hydroxycinnamic Acid as a Matrix for Matrix-assisted Laser Desorption Mass Spectrometry

The development of the matrix-assisted laser desorption technique by Karas and Hillenkamp¹⁻⁴ has considerably improved the capability of time-of-flight mass spectrometry for the determination of the molecular masses of peptides and proteins. In the original laser desorption technique,¹ the sample of interest was mixed into a solution containing a large molar excess of nicotinic acid and this solution was dried on a metal substrate. The solid deposit was then placed in a time-of-flight mass spectrometer and analyzed by laser desorption using short duration (10 ns) pulses of laser irradiation with a wavelength (266 nm) at which the nicotinic acid matrix absorbs strongly. A laser pulse with sufficient energy causes the mixture of sample and matrix to undergo a conversion into the gas phase and also produces a significant amount of ionization of the sample.

Although nicotinic acid is an effective laser desorption matrix for many peptides and proteins, it has certain undesirable properties. These include: (a) a propensity for forming unwanted adducts between the protein and decarboxylated photoproducts produced from the nicotinic acid matrix;⁵ and (b) a tendency to discriminate against certain protein components in mixtures. A search for matrix materials with improved properties led to the discovery that the cinnamic acid derivatives sinapinic acid, ferulic acid, and caffeic acid produced mass spectra of proteins exhibiting considerably reduced adduction compared with nicotinic acid.⁶ In addition, these cinnamic acid derivatives showed a reduced tendency to discriminate between the components of complex mixtures of peptides and proteins, and were also highly tolerant of large molar excesses of non-proteinaceous impurities such as salts and lipids.⁷ The cinnamic acid derivatives also absorb strongly at longer wavelengths than nicotinic acid, allowing the use of 355 nm radiation from the frequency tripled output of Nd(YAG) lasers,⁸ 308 nm radiation from excimer lasers,⁹ and 337 nm radiation from nitrogen lasers.¹⁰⁻¹³

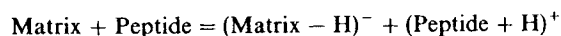
Sinapinic acid, while constituting an improvement over nicotinic acid as a laser desorption matrix, still has two undesirable properties: (a) the formation of an adduct of a photo-dehydration product of sinapinic acid with proteins,⁶ having a mass 206 u higher than the protonated protein; and (b) the production of an intense background of ions in the m/z range below 1000, limiting the utility of the technique for the measurement of low-mass peptides. In a continuing search for improved laser desorption matrices for peptides and proteins, we have discovered that α -cyano-4-hydroxycinnamic acid (4HCCA) has certain interesting properties as a laser desorption matrix for peptides and proteins. We describe these properties here.

Samples were prepared for mass spectrometry in the following manner. The peptide or protein was dissolved in a 1:2 (v/v) solution of acetonitrile 0.1% trifluoroacetic acid to give an analyte concentration in the range 10^{-7} – 2×10^{-5} M. 1 μ l of this solution was added to 1–10 μ l of a 5 g/l concentration solution of α -cyano-4-hydroxycinnamic acid (Aldrich Chemical Co., Milwaukee, WI) in the same solvent system used for the peptide or protein. 0.5 μ l of the resulting mixture (containing ≤ 1 pmol of peptide or protein) was applied to the probe of a laser desorption time-of-flight mass spectrometer and dried by a stream of air at ambient temperature. The probe was then inserted into the mass spectrometer where the

sample was thoroughly dried in vacuum. All sample handling was carried out under reduced lighting and matrix solutions were freshly made every day. Substance P, the angiotensins, bovine α -casein, bovine transferrin, bovine carbonic anhydrase II, equine cytochrome c , and human immunoglobulin G were obtained from the Sigma Chemical Co. (St. Louis, MO). Synthetic human β -endorphin and the dynorphins were obtained from Peninsula Laboratories Inc., (Belmont, CA), vasotocin was provided by R. Acher (University of Paris), the selenyl-methionine-containing peptides and the human chorionic gonadotropin peptides were provided by S. Birken (Columbia University, New York), and the HIV-I peptides were provided by S.B.H. Kent (Scripps Research Institute, La Jolla).

Mass spectra were obtained on a laser desorption time-of-flight mass spectrometer constructed at The Rockefeller University and described previously.^{5,14} Pulses of 355 nm wavelength light, of 10 ns duration, from a Nd(YAG) laser were directed at the solid sample-matrix mixture and the resulting ions accelerated to an energy of 30 keV/charge and detected at the end of a 2 metre long flight-tube by a hybrid microchannel plate/gridded discrete dynode electron multiplier detector.¹⁵ The spectra were recorded in a LeCroy Model 8828D transient digitizer (200 Msamples/second). 100–200 single shot spectra, taken at a rate of 2.5/second, were added together to give improved statistics.

Figure 1 shows the time-of-flight mass spectrum of the 11-residue peptide substance P (molecular mass = 1347.7 u) taken by adding together 100 single shot spectra at a laser fluence just barely above the threshold for ion production. The dominant features of the spectrum are the intense peak corresponding to protonated substance P at $m/z = 1348.7$ and an oxidized impurity of substance P at $m/z = 1364.9$. Also observed are several weak peaks arising from the 4HCCA matrix, including the protonated monomer at $m/z = 190$ and the protonated dimer at $m/z = 379$. The low relative intensity of matrix-related ions compared with peptide ions raises interesting questions as to the mechanism of protonation of the peptide. One possible mechanism involves the disproportionation reaction



A second possible mechanism involves production of protonated matrix followed by exhaustive reaction of these protonated matrix molecules with the peptide or neutralization by some other unknown process.

In practice, we frequently operate at laser fluences up to 50% higher than the threshold for ion production to obtain more intense mass spectra of peptides. At these higher laser fluences, a larger relative intensity of matrix-related ions is observed. However, no intense background is observed above $m/z = 442$. Some of the spectra of peptides exhibited relatively intense peaks arising from copper adduction to the protonated molecule. The copper was present as a low-level impurity (~ 1 ppm) in the 4HCCA sample. This interesting phenomenon will be discussed in detail in a separate publication.

In addition to substance P, we have successfully used 4HCCA as a matrix to measure 160 other peptides including five different dynorphins ranging in length from 6–17 residues; angiotensins I, II, and III; vasotocin; fragments of bovine α -casein generated by, respectively, pepsin, trypsin, and cyanogen bromide cleavage; human β -endorphin; short synthetic peptides containing selenyl-methionine; constituent synthetic peptides of HIV-I protease ranging in length from 10 to 99 residues; and 10 glycopeptide fragments of human chorionic

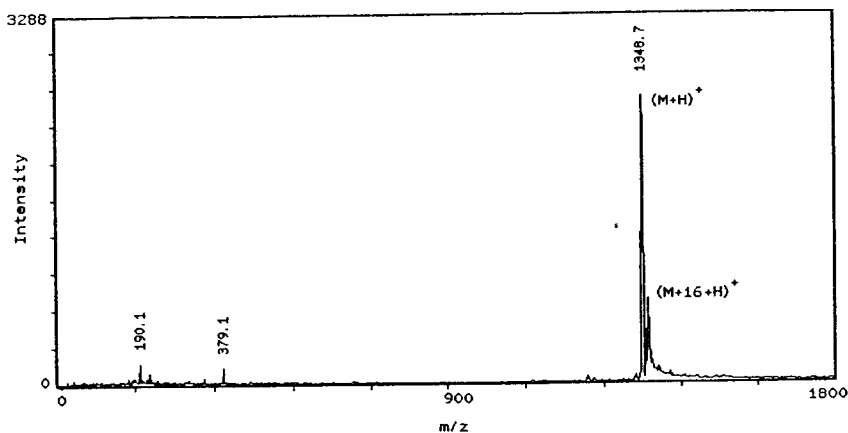


Figure 1. Matrix-assisted laser desorption mass spectrum of substance P obtained with a matrix of α -cyano-4-hydroxycinnamic acid.

gonadotropin. These results, which will be described in detail elsewhere, indicate that 4HCCA has general utility for the measurement of peptides and glycopeptides having more than five residues. The accuracy of the mass determination for these peptides was 1–2 parts in 10^4 .

The intensities of the protonated peptide signals obtained with 4HCCA are substantially higher than those obtained from other cinnamic acid derivatives that we have tested (e.g. sinapinic acid (SA) and ferulic acid),⁶ indicating that the peptides are being ionized more efficiently from 4HCCA. This hypothesis is confirmed by a comparison of the laser desorption spectra of proteins obtained from 4HCCA and SA. Figure 2 shows a comparison of the spectra of bovine transferrin (molecular mass = 78 000 u) obtained from 4HCCA and SA. The degree of protonation is considerably higher in the

former matrix, with +2, +3, and +4 being the most intense charge states in 4HCCA compared with +1 from SA. The highest charge state observed from 4HCCA is +9 compared with +4 from SA. Similar enhancements of the degree of protonation in 4HCCA were obtained for equine cytochrome c, bovine carbonic anhydrase II, and human immunoglobulin G. Although the reason for the increased ionization efficiency has not yet been elucidated, we note that replacement of the hydrogen on the carbon atom adjacent to the carboxyl group by the nitrile group considerably increases the acidity of the proton on the carboxylate group. We further note that 4HCCA is not unique in its enhanced capability for ionizing proteins. Thus, for example, the ionization efficiency of proteins in caffeic acid (3,4-dihydroxycinnamic acid) is also significantly higher than for proteins in sinapinic acid (3,5-

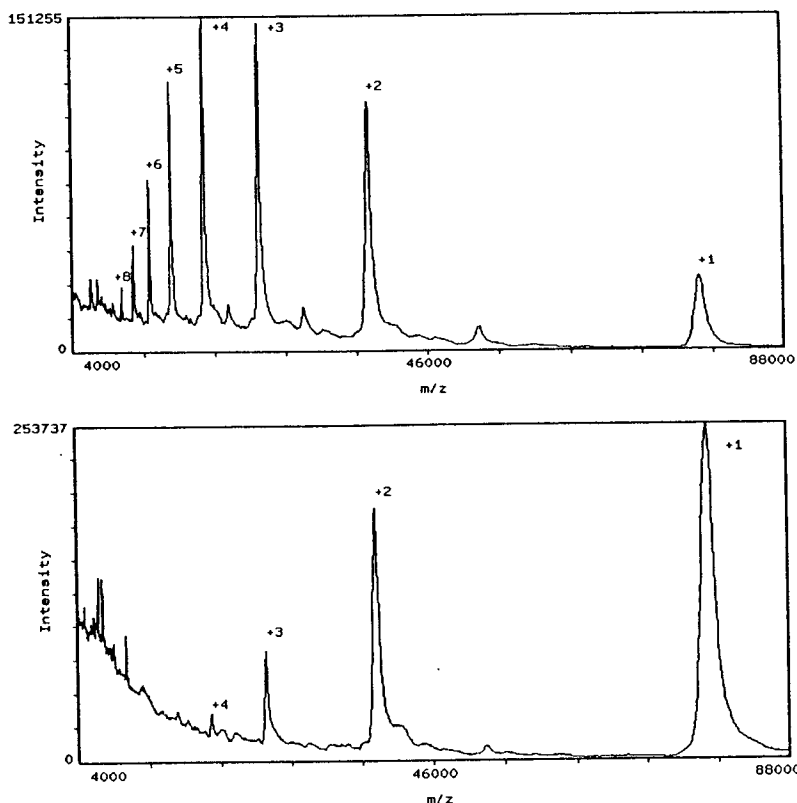


Figure 2. Comparison of the matrix-assisted laser desorption mass spectra of bovine transferrin obtained from 4HCCA (top panel) and SA (bottom panel). In both cases 1 pmol of the protein was applied to the probe of the mass spectrometer. The less intense peaks between the main series arise from multiple protonation of transferrin dimers.

dimethoxy-4-hydroxycinnamic acid) and ferulic acid (3-methoxy-4-hydroxycinnamic acid).

α -Cyano-4-hydroxycinnamic acid has been shown to be a highly effective and efficient matrix for the matrix-assisted laser desorption/ionization of peptides and glycopeptides in the molecular mass range 500–5000. In this range, the mass spectra are dominated by intense peaks corresponding to singly protonated peptide molecule ions. The relative absence of matrix-related ions obtained at fluences just above the threshold for ion production raises interesting questions as to the origin of the proton that attaches to the peptide.

The laser desorption mass spectra of proteins obtained from α -cyano-4-hydroxycinnamic acid show an increased tendency for multiple protonation compared with spectra obtained from other commonly applied laser desorption matrices. This finding confirms that structural details of the matrix molecule strongly influence the degree and extent of ionization of proteins in matrix-assisted laser desorption. Control over the degree and extent of ionization are likely to have a considerable impact on the practical utility of matrix-assisted laser desorption for the analysis of peptides and proteins.

Acknowledgements. This work was supported by Grants RR00862, Division of Research Resources and GM38274, National Institutes of General Medical Sciences.

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