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β-Carboline Alkaloids as Matrices for Matrix-assisted Ultraviolet Laser Desorption Time-of-flight Mass Spectrometry of Proteins and Sulfated Oligosaccharides: a Comparative Study Using Phenylcarbonyl Compounds, Carbazoles and Classical Matrices

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The successful use of six β-carbolines (i.e. *nor*-harmane (9H-pyrido[3,4-*b*]indole), harmane (1-methyl-9*H*-pyrido[3,4-*b*]indole), harmine (7-methoxy-1-methyl-9*H*-pyrido[3,4-*b*]indole), harmol (1-methyl-9*H*-pyrido[3,4-*b*]indol-7-ol), harmaline (3,4-dihydro-7-methoxy-1-methyl-9*H*-pyrido[3,4-*b*]indole) and harmalol (3,4-dihydro-1-methyl-9*H*-pyrido[3,4-*b*]indol-7-ol)) is reported as matrices in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF-MS) ($\lambda_{exc} = 337$ nm) for proteins (proteins checked: gramicidin S, bovine insulin, aprotinin, horse heart cytochrome *c*, ribonuclease A, lysozyme, myoglobin, trypsin, protease and bovine serum albumin) and sulfated oligosaccharides (λ-carrageenans of M_r 549, 712, 1570 and 1733), using stainless-steel probes and membranes (poly(vinylidene difluoride) (PVDF) polymers) to prepare the samples. The possible use of some phenylcarbonyl compounds (hydroxyphenyl ketones, amino- and hydroxybenzoic acids) and a few carbazole derivatives as matrices is also discussed briefly. In addition, the usefulness of the new matrices in MALDI/TOF-MS ($\lambda_{exc} = 337$ nm) of proteins and sulfated oligosaccharides is compared with those of classical MALDI matrices such as α-cyano-4-hydroxycinnamic acid, gentisic acid, sinapinic acid, 6-aza-2-thiothymine and 3-indole-*trans*-β-acrylic acid. Laser desorption/TOF-MS ($\lambda_{exc} = 337$ nm) of the new matrices is also described. © **1997 by John Wiley & Sons, Ltd.**

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INTRODUCTION

The development of the matrix-assisted laser desorption ionization (MALDI) technique by Karas and co-workers[1–4] has revolutionized the capability of time-of-flight mass spectrometry (TOF-MS) for the determination of the molecular masses of synthetic polymers[5] and biopolymers.[6] The key idea of MALDI is to ionize macromolecules for TOF-MS analysis by diluting them in a suitable matrix of small organic molecules which absorb laser photons. By UV photon absorption, the matrix molecules initially populate electronic excited states, inducing a strong perturbation of the condensed-phase matrix–analyte mixture. Although it is claimed that more attention must be paid to the photochemical properties of the compounds used as the matrix, the choice of and search for new matrix candidates are still a trial-and-error procedure.[7,8]

Considering the structure of the most commonly used matric materials[8,9] (pyridinecarboxylic acids,[8] pyrazinecarboxylic acids,[8] hydroxybenzoic acids,[8] cinnamic acids[5,8] and 6-aza-2-thiothymine[9]), in which simultaneously hydrogen donor and hydrogen acceptor groups are present, we decided to study the possible use as MALDI matrices ($\lambda_{exc} = 337$ nm) of two families of organic compounds (Table 1, phenylcarbonyl compounds and pyridoindoles) in which both structures are present. To begin with, 2-hydroxybenzoic acids, 2-hydroxyacetophenones, 2-hydroxybenzophenones and 9*H*-pyrido[3,4-*b*]indoles (β-carbolines) (Table 1) were selected for this study owing to their well known capability of proton transfer under UV irradiation yielding photoenols and zwitterions, respectively. As is known,

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photoenolization occurs by intramolecular proton transfer in 2-hydroxyacetophenones[10] and 2-hydroxybenzophenones.[10] Also, it was found previously that the acid–base properties of β-carbolines are dramatically modified after photon absorption.[11–16] e.g. defining ΔpK as $\Delta pK = pK^* - pK$, where pK was measured for the acid–base equilibrium in the ground state and pK^* for the same equilibrium in the electronic excited singlet state, the ΔpK values obtained in ethanol were 6.9 for nor-harmane, 6.2 for harmane, 6.4 for harmine and 13.0 for Harmaline. Further, the formation of the corresponding β-carboline zwitterion from β-carbolines in the electronic excited singlet and triplet state has been described previously.[11–16] Additionally, several carbazole derivatives, whose photochemical behavior has been studied in our laboratory,[17–21] have been checked as MALDI matrices.

In this paper, we report the successful use of six β-carbolines (i.e. *nor*-harmane (9*H*-pyrido[3,4-*b*]indole), harmane (1-methyl-9*H*-pyrido[3,4-*b*]indole), harmine (7-methoxy-1-methyl-9*H*-pyrido[3,4-*b*]indole), harmol (1-methyl-9*H*-pyrido[3,4-*b*]indol-7-ol), harmaline (3,4-dihydro-7-methoxy-1-methyl-9*H*-pyrido[3,4-*b*]indole) and harmalol (3,4-dihydro-1-methyl-9*H*-pyrido[3,4-*b*]indol-7-ol); see Tables 1–3) as matrices in MALDI/TOF-MS ($\lambda_{exc} = 337$ nm) of proteins and sulfated oligosaccharides, using stainless-steel probes and membranes (poly(vinylidene difluoride) (PVDF) polymers) to prepare the samples (see Experimental). The possible use of phenylcarbonyl compounds (hydroxyphenyl ketones and hydroxybenzoic acids) and a few carbazole derivatives as matrices is also discussed briefly (Tables 1–3). In addition, the usefulness of the new matrices in MALDI/TOF-MS ($\lambda_{exc} = 337$ nm) of proteins and sulfated oligosaccharides was compared with those of classical MALDI matrices such as α-cyano-4-hydroxycinnamic acid (CHC), gentisic acid, sinapinic acid, 6-aza-2-thiothymine (6-ATT) and 3-indole-*trans*-β-acrylic acid (indoleacrylic acid)[5] (Tables 2 and 3). Laser desorption (LD)/TOF-MS ($\lambda_{exc} = 337$ nm) of the new matrices is also described (Table 4).

EXPERIMENTAL

Materials

Matrix chemicals. The β-carbolines listed above were purchased from Aldrich, Sigma and Wako as free bases. Carbazole and 2-hydroxycarbazole were purchased from Wako and 1-nitrocarbazole was prepared from carbazole as described elsewhere.[17,18] The classical matrix compounds α-cyano-4-hydroxycinnamic acid (CHC), 2,5-dihydroxybenzoic acid (gentisic acid), 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), 6-aza-2-thiothymine (6-ATT) and 3-indole-*trans*-β-acrylic acid (indoleacrylic acid) were obtained from Sigma, Aldrich, Acros Organics and Wako. Phenylcarbonyl compounds such as 2,4-dihydroxyacetophenone, 2,5-dihydroxyacetophenone, 2,6-dihydroxyacetophenone, 3,5-dihydroxyacetophenone, 2,4,6-trihydroxyacetophenone, 2,6-dihydroxybenzoic acid, 2-hydroxybenzoic acid (salicylic acid), 2-aminobenzoic acid (anthranilic acid), 2-hydroxybenzophenone and 2,4-dihydroxybenzophenone were purchased from Aldrich, Sigma, Wako and TCI Tokyo Kasei.

Analyte chemicals. Proteins, i.e., gramicidin S (G0900, M_r 1141.47), bovine insulin (I5500, M_r 5733.5), aprotinin (A1153, M_r 6512), horse heart cytochrome *c* (C3256, M_r 12 384), ribonuclease A (R5500, M_r 13 700), lysozyme (L6876, M_r 14 307), myoglobin (M0630, M_r 16 950), trypsin (T8003, M_r 23 290), protease (subtilisin Carlsberg P5380, M_r 27 288.4) and bovine serum albumin (A7511, M_r 66 525) were obtained from Sigma.

Sulfated oligosaccharides of known molecular mass (λ-carrageenans, isolated from a red seaweed, *Gingartina skottisbergii*) were supplied by Dr A. S. Cerezo (Organic Chemistry Department, College of Natural and Exact Sciences, University of Buenos Aires).[22,23]

Probe support materials. Shimadzu P/N 670-19109-01 stainless-steel sample slides and Immobilon poly(vinylidene difluoride) (PVDF) membranes with 0.45 μm pores (IPVH000 10, Millipore, Bedford, MA, USA) were used.

Organic solvents. Methanol and ethanol (Nacalai Tesque, HPLC grade), acetonitrile (Sigma–Aldrich, HPLC grade), dimethyl sulfoxide (DMSO) (Wako–Dosindo) and trifluoroacetic acid (TFA) (Merck) were used as received. The purity of the solvents was monitored by electronic spectroscopy. Water of very low conductivity (Milli-Q grade, 56–59 nS cm^{-1}, with PURIC-S; Orugano, Tokyo, Japan) was used.

Mass spectrometry

Instrumentation. All measurements were performed on a Kompact MALDI III laser desorption time-of-flight mass spectrometer (Kratos Analytical, obtained from Shimadzu, Kyoto, Japan), equipped with a pulsed nitrogen laser ($\lambda = 337$ nm; pulse width = 3 ns). The analyzer was used at accelerating voltage of 20 kV. The laser can be fired at any spot or fired continuously along a selected length of the sample holder. The sample was irradiated just above the threshold for obtaining ions. Thus, the irradiance used for producing a mass spectrum was analyte dependent. Usually 50 spectra were accumulated. All mass spectra were taken (i) in the positive- and negative-ion modes and (ii) in the linear and reflectron modes.

Sample preparation. A matrix stock solution of 10^{-2} M was prepared by dissolving 10^{-5} mol of the matrix compound in 1 ml of the selected solvent (water, methanol, ethanol, DMSO, water containing 0.1% TFA, ethanol–water containing 0.1% TFA (1:1, v/v) and water–acetonitrile (7:3, v/v)). The stock solution was stored in the dark at low temperature to prevent photochemical decomposition. An analyte solution of 10^{-5} M was freshly prepared dissolving (i) the proteins in aqueous 0.1% TFA solution, (ii) the sulfated oligosaccharides in water and (iii) the low molecular mass nitrogen organic compounds in methanol, ethanol, DMSO,

acetonitrile and in the corresponding aqueous 0.1% TFA mixture.

Four sample preparation methods were tried. In the first (method A), to make an analyte–matrix deposit, typically 0.5 μl of the analyte solution was placed on the sample probe tip (2 mm diameter; stainless steel) and the solvent was removed by room-temperature evaporation by forced air (analyte film). Subsequently, 0.5 μl of the matrix solution was placed on the same probe tip covering the analyte film, and again the solvent was removed by forced air (matrix film). The analyte to matrix ratios used were 1:1, 1:2, 1:3, 2:1 and 3:1 (v/v).

In the second procedure (method B), to make a matrix–analyte deposit, we followed method A but first placed the matrix (matrix film) on the stainless-steel probe tip and then the analyte (analyte film).

In method C (dried-droplet deposit method), typically 5 μl of the matrix stock solution was placed in an Eppendorf tube and 0.5 μl each of the analyte solutions (10^{-5} M) to be analyzed was added. The solution was briefly mixed by vortex stirring. A small aliquot (0.5 μl) of the matrix–analyte solution was then placed on the 2 mm diameter, flat metal probe tip and dried at room temperature (see method A).

In the fourth procedure (method D), PVDF membrane slices (70 × 5 mm) were secured to the sample slide with tape and β-carbolines, CHC, gentisic acid, sinapinic acid and 6-ATT were used as matrix. The matrices were applied on the PVDF membranes in methanol solutions (0.1 M) at an estimated matrix to analyte ratio of 1000:1 to 10 000:1. The application of the analyte (protein; sulfated oligosaccharide) at each spot was accomplished by successively applying 1 μl of methanol to wet the membrane, 0.5 μl of water and 0.5 μl of the analyte solution. The membrane was kept wet throughout this stage and then allowed to dry partially before applying the matrix (0.5 μl). Once the membrane had dried, two more applications of matrix were made. The sample slide with attached membrane was then inserted into the mass spectrometer.

Spectrum calibration. Spectra were calibrated by use of (i) a matrix ion and a standard protein applied to the sample (internal calibrant regent, e.g. gramicidin S for oligosaccharides) and (ii) Cs, Na and standard chemicals (external calibrant regent) using the Kratos Kompact calibration program.

UV/visible and fluorescence spectroscopy

In order to establish which β-carboline species (neutral, protonated (cationic), anionic and/or zwitterionic) were present in the matrix–analyte deposit obtained using the different sample preparation methods (A–D), we measured the UV/visible and fluorescence emission and excitation spectra of the solid deposits and compared them with those previously obtained from the corresponding solution.

Instrumentation. Absorption spectra were recorded with stoppered quartz cells (Fine quartz cell, Japan) of length 1 cm in a Beckman DU-50 spectrophotometer using the corresponding solvent as reference (298 K).

The fluorescence measurements were performed on a Jasco FP-777 spectrofluorimeter whose output is automatically corrected for instrumental response by means of a Rhodamine B quantum counter and equipped with a Hamamatsu R928 photomultiplier tube. The excitation spectra were measured on the same spectrometer. The fluorescence emission and excitation spectra of the solvent blanks and the solid matrices used (steel probe tip; PVDF membranes) were measured in each case to check that they showed negligible emission over the wavelength range monitored for emission (λ_{exc} = 300–420 nm) and excitation experiments. The measurements at room temperature (298 K) were recorded with stoppered quartz cells (Jasco, P005K-MFP-1004, 10 mm) using the 90° mode. Using the Front Surface Accessory (FSA, Jasco P/N 105018), measurements of the intensity of the fluorescence from solid film layers of matrix and/or analytes placed on the stainless-steel probe tip and on the PVDF membranes were made.

RESULTS AND DISCUSSION

Search for useful matrix compounds

Phenylcarbonyl compounds were classified into three groups according to differences in the residue attached to the carbonyl group, i.e. acetophenones (R = CH_3), benzoic acids (R = OH) and benzophenones (R = phenol residue) (Table 1). When proteins were used as analytes, acetophenones and benzoic acids acted as effective matrices, but benzophenones did not (Table 1). Changes in the locations of hydroxy groups in acetophenones did not affect clearly their usefulness as matrices for protein analyses (Table 1 and 2). In benzoic acids, addition of a hydroxy group or replacement of a hydroxy group with an amino group affected their usefulness as matrices for protein analyses (Tables 1 and 2). However, none of phenyl ketones could be used as matrices in TOF-MS for sulfated oligosaccharide analyses (Table 1).

Modification of carbazole structure was effected by adding substituents to the carbazole, i.e. hydroxy, nitro, bromide, chloride, methyl, phenyl, vinyl, acetyl and benzoyl groups (Table 1). Only carbazole, 2-hydroxycarbazole and 1-nitrocarbazole could be used as matrices for protein analyses, and only 1-nitrocarbazole could be used as matrix for sulfated oligosaccharide analyses (Table 1).

Although β-carbolines have a similar structure to that of carbazoles, all the β-carbolines used in the present study, i.e. *nor*-harmane, harmane, harmine, harmol, harmaline and harmalol, acted as useful matrices for both protein and oligosaccharide analyses (Table 1). The presence of the basic nitrogen pyridinic moiety in the β-carboline structure would account for this fact. 7-Azaindole[7] was checked in order to compare it with pyridoindoles, but it was not useful as a matrix for either protein or sulfated oligosaccharide analyses (Table 1).

Table 1. Compounds whose usefulness as matrices for MALDI TOF-MS was checked (λ_{exc} = 337 nm)

Matrix	Useful matrix for proteins	Useful matrix for sulfated oligosaccharides	Compound structures
Phenylcarbonyl compounds:			
Benzoic acids (R = OH):			
2-Hydroxybenzoic acid (salicylic acid)	Yes	No	
2,6-Dihydroxybenzoic acid (2,6-DHOBA)	Yes	No	
2-Aminobenzoic acid (anthranilic acid)	Yes	No	
Acetophenones (R = CH_3):			
2,4-Dihydroxyacetophenone (2,4-DHOA)	Yes	No	
2,5-Dihydroxyacetophenone (2,5-DHOA)	Yes	No	
2,6-Dihydroxyacetophenone (2,6-DHOA)	Yes	No	
3,5-Dihydroxyacetophenone (3,5-DHOA)	Yes	No	
2,4,6-Trihydroxyacetophenone (2,4,6-THOA)	Yes	No	
Benzophenones (R = phenol residue):			
2-Hydroxybenzophenone	No	No	
2,4-Dihydroxybenzophenone	No	No	
Pyridoindoles:			
β-Carbolines:			
9*H*-Pyrido[3,4-*b*]indole (*nor*-harmane)	Yes	Yes	
1-Methyl-9*H*-pyrido[3,4-*b*]indole (harmane)	Yes	Yes	
7-Methoxy-1-methyl-9*H*-pyrido[3,4-*b*]indole (harmine)	Yes	Yes	
1-Methyl-9*H*-pyrido[3,4-*b*]indol-7-ol (harmol)	Yes	Yes	
3,4-Dihydro-7-methoxy-1-methyl-9*H*-pyrido[3,4-*b*]indole (harmaline)	Yes	Yes	
3,4-Dihydro-1-methyl-9*H*-pyrido[3,4-*b*]indol-7-ol (harmalol)	Yes	Yes	
7-Azaindole	No	No	
Benzoindoles:			
Carbazoles:			
Carbazole	Yes	No	
2-Hydroxycarbazole	Yes	No	
1-Nitrocarbazole	Yes	Yes	
3-Bromocarbazole	No	No	
3,6-Dibromocarbazole	No	No	
3-Chlorocarbazole	No	No	
3,6-Dichlorocarbazole	No	No	
1,6-Dichlorocarbazole	No	No	
1,3,6-Trichlorocarbazole	No	No	
1,3,6,8-Tetrachlorocarbazole	No	No	
9-Methylcarbazole	No	No	
9-Phenylcarbazole	No	No	
9-Vinylcarbazole	No	No	
3-Chloro-9-acetylcarbazole	No	No	
3,6-Dichloro-9-acetylcarbazole	No	No	
3-Chloro-9-benzoylcarbazole	No	No	
3,6-Dichloro-9-benzoylcarbazole	No	No	
Classical matrices:			
α-Cyano-4-hydroxycinnamic acid (CHC)	Yes	Yes	
3,5-Dimethoxy-4-hydroxycinnamic acid (sinapinic acid)	Yes	Yes	
2,5-Dihydroxybenzoic acid (gentisic acid)	Yes	Yes	
6-Aza-2-thiothymine (6-ATT)	Yes	Yes	
3-Indole-*trans*-β-acrylic acid (indoleacrylic acid)	Yes	No	

The classical matrices examined acted as useful matrices for both protein and oligosaccharide analyses, except indoleacrylic acid for sulfated oligosaccharide analyses (Table 1).

Systematic check of mass dependence in protein analysis

In order to perform MALDI/TOF-MS protein analyses, we compared the results obtained for each compound using a new matrix, following the different sample preparation methods described in Experimental, with those obtained with classical matrices (Tables 1 and 2) under the same experimental conditions.

As shown in Table 2, the only useful carbazoles examined are carbazole, 2-hydroxycarbazole and 1-nitrocarbazole, which acted as good matrices for proteins of low molecular mass (e.g., up to ribonuclease A for carbazole and aprotinin for 2-hydroxycarbazole and 1-nitrocarbazole). In our experience, the ions which are observed correspond to the intact protein molecule as cation (protonated species) in the positive mode and as anion (deprotonated species) in the negative mode (Table 2, +/−). Despite their limited usefulness, we examined the carbazole behavior as a matrix in order to

Table 2. Dependence of matrices for MALDI on the molecular mass protein analytes[a]

Matrix	Protein and molecular mass: Gramicidin S[f] 1141.47	Insulin-B[g] 5733.5	Aprotinin[h] 6512	Cytochrome C[h] 12384	Ribonuclease A[h] 13700	Lysozyme[h] 14307 (5288)[k]	Myoglobin[h] 16950 (5965)[k]	Trypsin[i] 23290	Subtilisin C[h] (protease) 27288.4	Albumin-B[j] 66525
Phenylcarbonyl compounds:										
Benzoic acids:										
Salicylic acid[b]	+	+/−	0	0	0	0	0	0	0	0
2,6-DHOBA[b]	+	+/−	+/−	0	+/−	+	+/−	+/−	0	0
Anthranilic acid[b]	+	+/−	+/−	0	+/−	+/−(s)	+/−	0	0	0
Acetophenones:										
2,4-DHOA[b]	+/−	+/−	+/−	+	+/−	+/−	+/−	+	0	0
2,5-DHOA[b]	+	+/−	+/−	+/−	+	+(s)	+/−	+	0	0
2,6-DHOA[b]	+	+/−	+(s)	0	0	0	0	0	0	0
3,5-DHOA[b]	+	0	+/−(s)	0	0	0	0	+	0	0
2,4,6-THOA[b]	+	+/−	+/−	+/−(s)	+/−	+	+/−	+/−	0	0
Pyridoindoles:										
β-Carbolines:										
nor-Harmane[b]	+	+/−	+/−	+(s)	+/−	+/−	+/−	0	0	0
Harmane[b]	+/−	+/−	+/−	+/−(s)	+/−	+/−	+/−	0	0	0
Harmine[b]	+	+/−	+/−	+/−(s)	+/−	+/−	+/−(s)	0	0	0
Harmol[b]	+/−	+/−	+/−	0	+/−	+/−	+/−(s)	0	0	0
Harmaline[b]	+/−	+/−	+/−	+/−(s)	+/−	+/−	+/−(s)	0	0	0
Harmalol[b]	+/−	+/−	+/−	0	+/−(s)	0	0	0	0	0
Benzoindoles:										
Carbazoles:										
Carbazole[c]	+/−	+/−	+/−	0	+/−(s)	0	0	0	0	0
2-Hydroxycarbazole[c]	+/−	+/−	+/−	0	0	0	0	0	0	0
1-Nitrocarbazole[c]	+/−	+/−	+/−	0	0	0	0	0	0	0
Classical matrices:										
CHC[d]	+	+/−	+/−	+/−	+/−	+/−	+/−	+/−	+/−	+/−
Sinapinic acid[d]	+/−	+/−	+/−	+/−	+/−	+/−	+/−	+/−	+/−	+/−
Gentisic acid[d]	+	+/−	+/−(s)	+/−(s)	+/−	+/−(s)	+/−(s)	0	0	0
6-ATT[d]	+	0	+/−	+/−(s)	+/−(s)	+/−	+/−(s)	+/−	0	0
Indoleacrylic acid[e]	+	+/−	+/−	+/−	+/−	+/−	+/−	+/−	0	0

[a] Shown are no spectrum signal (0), spectrum obtainable in the positive mode (+), in the negative mode (−), both modes (+/−) and spectrum with satellite signals (s). Data were obtained in the linear mode of the TOF-MS, and samples were placed on a stainless-steel probe tip as films according to method A (see Experimental). Solvent for matrices, ethanol–water containing 0.1% TFA (1 : 1, v/v). Solvent for proteins, water–0.1% TFA. $\lambda_{exc} = 337$ nm.
[b] 2.5 mg ml^{-1}.
[c] 2.5 mg ml^{-1} of DMSO.
[d] 10 mg ml^{-1}.
[e] 1.25 mg ml^{-1}.
[f] 1.1 mg ml^{-1}.
[g] 0.5 mg ml^{-1}.
[h] 0.3 mg ml^{-1}.
[i] 0.23 mg ml^{-1}.
[j] 1.8 mg ml^{-1}.
[k] This signal is also observed.

compare the behavior in the MALDI/TOF-MS of benzoindoles with that of pyridoindoles.

Similarly, in the family of β-carbolines (pyridoindoles), harmalol was shown to be a good matrix for proteins of low molecular mass (up to aprotinin), yielding minor satellite signals for ribonuclease. Figure 1 shows a detail of the mass spectrum of insulin using nor-harmane as matrix (negative mode) and Fig. 2 shows that for aprotinin (positive mode) using harmine as matrix. As is shown, the ratio of satellite-adduct signal intensity to molecular ion signal intensity is irrelevant with β-carbolines as the matrix. These findings are important for proposing β-carboline compounds as a new family of MALDI/TOF-MS matrix materials. A practical advantage of using matrices such as β-carbolines in ethanol–water containing 0.1% TFA (1 : 1, v/v) solution is due to their particular stability. In this solution, β-carbolines are present as protonated species whose photophysical and photochemical properties have been described elsewhere.[11–16] Using our typical protein (analyte) loading of 0.5 pmol on the probe tip, signals were obtained using both β-carbolines in the protonated form (solvent as above) and β-carbolines in the neutral form (solvent DMSO) and no significant differences were observed in the mass spectra obtained on the stainless-steel probe, using sample preparation methods A, B and C. When PVDF membrane slices were used for sample preparation (method D), both β-carboline species (protonated and neutral) yielded the same protein MALDI/TOF mass spectra in the positive mode.

The results of examining proteins with the phenylcarbonyl compounds selected in the present study have demonstrated that 2,4-DHOA, 2,5-DHOA, 2,4,6-THOA and 2,6-DHOBA can be used for most types of proteins in the positive mode and sometimes in both positive and negative modes (up to trypsin in Table 2). 2,6-DHOA and 3,5-DHOA exhibit very limited utility as matrices (Table 2). Recently, 2,6-DOHA[24] and 2,6-DHOBA[24] were described as MALDI/TOF-MS matrices for oligosaccharides (β-cyclodextrin, sucrose octaacetate, maltoheptaose, raffinose-5-hydrate, chitotetraose and dextran). The authors[24] recognized that the best single matrices that they use for the TOF-MS analysis of oligosaccharides were 2,6-DHOBA, gentisic acid, 3-amino-4-hydroxybenzoic acid and 3-aminoquinoline. Previously, 2,4,6-TOHA[25] had been identified as a non-carboxylic acid matrix which in combination with diammonium sulfate, diammonium hydrogencitrate or diammonium L-tartrate (matrix additives) was shown to be highly efficient for the desorption of natural and modified nucleotides.

Although anthranilic acid[7] and salicylic acid[26] have been checked previously as MALDI/TOF-MS matrices, we studied their behavior in order to compare under our experimental conditions their capability as a MALDI matrix with that of phenyl ketones, 2,6-DHOBA, β-carbolines and classical matrices (Table 2). In our hands, anthranilic acid was found to be an excellent protein matrix, producing strong signals, while salicylic acid showed a very limited capability as MALDI matrix.

It is interesting that although cytochrome *c* has a similar molecular mass (12 384) to ribonuclease A (13 700) and lysozyme (14 307), anthranilic acid, 2,6-DHOBA, harmol, harmalol and carbazole could not

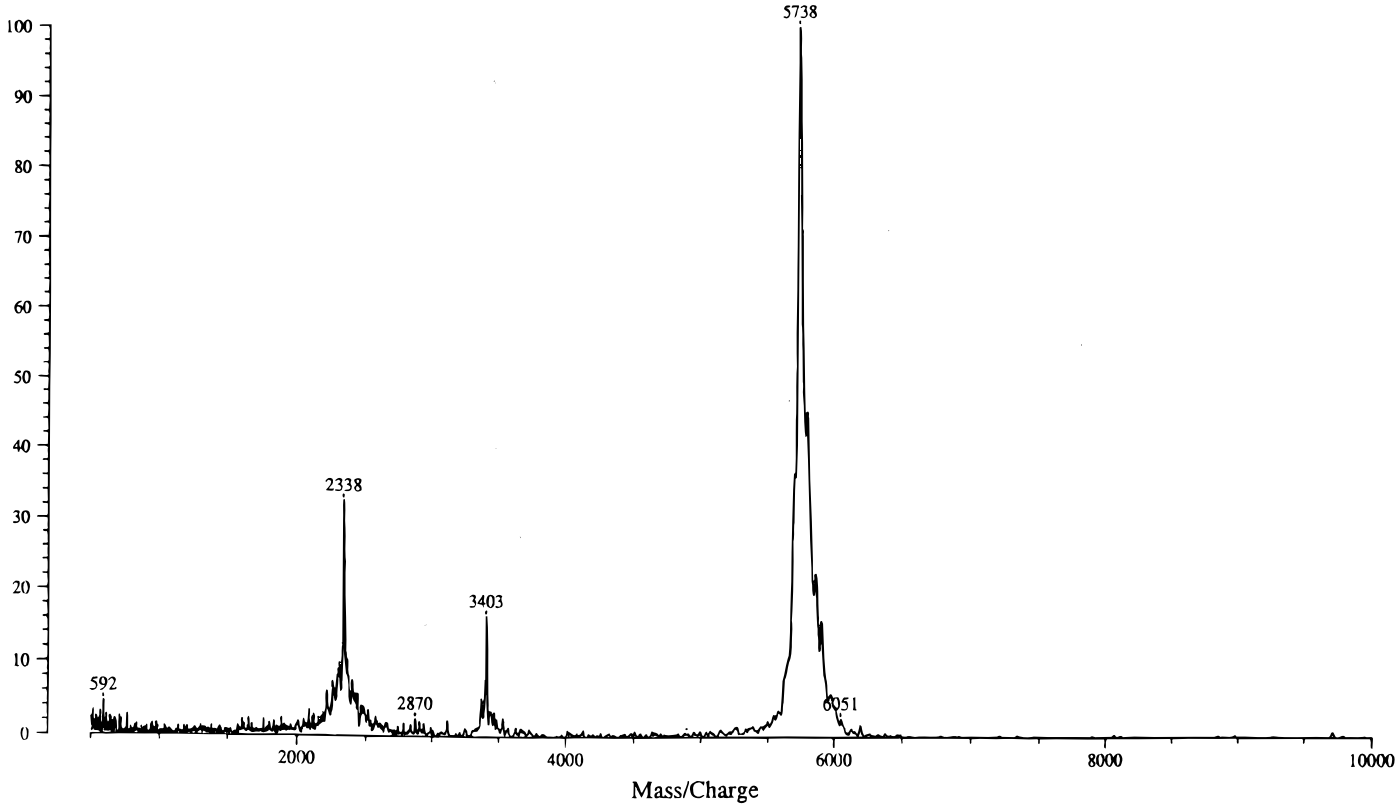

Figure 1. TOF mass spectrum of insulin ($M_r = 5738 \pm 4$ (measured)) using *nor*-harmane as matrix. The region of the spectrum shown corresponds to a mass range of 500–10 000. Conditions: $\lambda_{exc} = 337$ nm; see footnotes to Table 2.

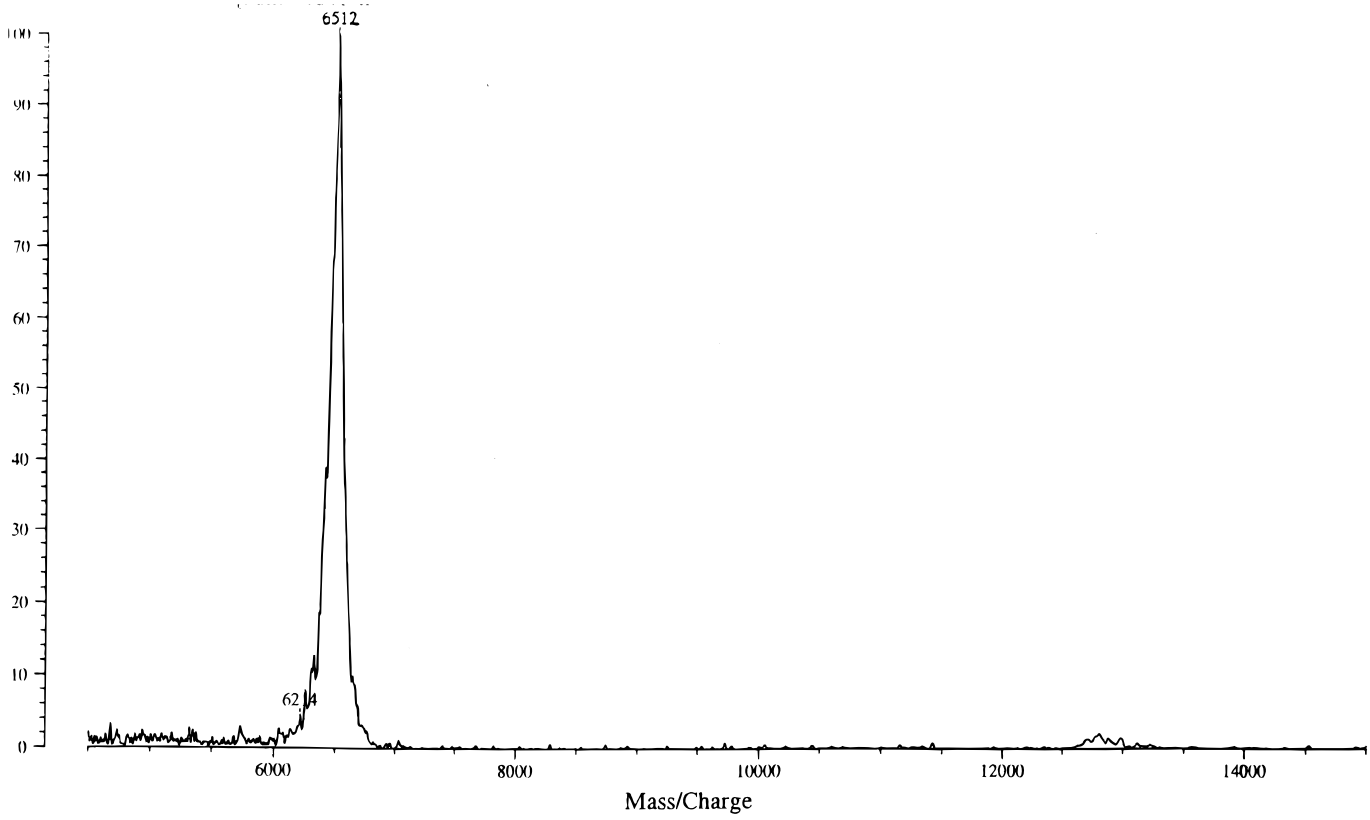

Figure 2. TOF mass spectrum of aprotinin (M_r = 6512 ± 5 (measured)) using harmine as matrix. The region of the spectrum shown corresponds to a mass range of 4500–15 000. Conditions: λ_{exc} = 337 nm; see footnotes to Table 2.

produce gaseous ionic cytochrome *c* molecules. The higher number of positively charged residues per molecule of cytochrome *c* (24)[27] compared with ribonuclease (18)[27] and lysozyme (18)[27] might account for this result.

Systematic check of mass dependence in sulfated oligosaccharide analyses

In order to study the mass dependence and functional group dependence of useful matrices in sulfated oligosaccharide analyses, matrices which have been found to work most satisfactorily for proteins (Table 2) were checked (Table 3). As shown in Tables 1 and 3, the entire β-carboline family was useful as MALDI matrices, and 1-nitrocarbozole was the only partially useful carbazole derivative of the many examined.

In addition, the classical matrices that proved to be useful are CHC, sinapinic acid, gentisic acid and 6-ATT, Gentisic acid and 6-ATT were the most useful matrices among the classical matrices studied (Table 3).

As can be seen in Table 3, the sulfated oligosaccharides analyzed were detected in general as anions, in the negative mode, suggesting that the presence of sulfate groups in the structure stabilizes the corresponding anionic form. Even when classical matrices were used, the anionic oligosaccharide molecules were preferentially formed.

Although the phenylcarbonyl compounds studied proved to be useful matrices for proteins in general (Table 2), no signals were observed when they were checked as possible MALDI matrices for sulfated oligosaccharides in both negative and positive modes. The minor acidic and basic properties of these compounds and the special interaction between the carbonilic and hydroxylic moieties at 2- and 6-positions (*ortho* positions) (intramolecular hydrogen bond in the ground state and photoenolization in the electronic excited state[10]) would account for such results.

LD/TOF-MS of the new matrices

Nitrogen LD/TOF-MS results for those compounds that work as MALDI matrices are shown in Table 4. As we described elsewhere, the β-carbolines studied show strong absorbance at 337 nm, and both neutral and protic forms are highly efficient fluorescent species.[12–16,28] In the present work, we were able to measure the emission and excitation fluorescence spectra from the solid film prepared on the stainless-steel probe and on the PVDF membranes. Thus, we could characterize the films as formed by protonated β-carbolines or neutral β-carboline molecules. The striking similarity of the TOF mass spectra obtained from both kinds of films suggests that, in the positive mode, only the intact molecular ion and the corresponding dimeric form are observed (Fig. 3) while only some fragments from the dimeric structure are observed in the negative mode (Table 4). As can be seen in Table 4, when ethanol–water containing 0.1% TFA (1 : 1, v/v) was used as solvent and the trifluoroacetate of the protonated β-carboline is present in the matrix–analyte deposit (protonated β-carbolines were characterized by their fluorescence emission and excitation spectra), the

Table 3. Dependence of matrices for MALDI on the molecular mass of sulfated oligosaccharide analytes[a]

Matrix	Sulfated oligosaccharide molecular mass 549	712	1570	1733
Pyridoindoles:	–	–	–	–
β-Carbolines:[b]	–	–	–	–
nor-Harmane[b]	–	–	–	–
Harmane[b]	–	–	–	–
Harmine[b]	–	–	–	–
Harmol[b]	–	–	–	–
Harmine on PVDF[c]	–	–	–	–
Harmaline[b]	–	–	–	–
Harmalol[b]	–	–	–	–
Benzoindoles:				
Carbazoles:				
Carbazole[d]	0	0	0	0
2-Hydroxycarbazole[d]	0	0	0	0
1-Nitrocarbazole[d]	–	–	0	0
Phenylcarbonyl compounds:				
Acetophenones:				
2,4-DHOA[b]	0	0	0	0
2,5-DHOA[b]	0	0	0	0
2,6-DHOA[b]	0	0	0	0
3,5-DOHA[b]	0	0	0	0
2,4,6-THOA[b]	0	0	0	0
Benzoic acids:				
Salicylic acid[b]	0	0	0	0
2,6-DHOBA[b]	0	0	0	0
Anthranilic acid[b]	0	0	0	0
Classical matrices:				
CHC[e]	+/–	–	–	0
Sinapinic acid[e]	0	0	+/–	+/–
Gentisic acid[e]	+/–	–	–	–
Gentisic acid/gramicidin S[f]	+/–	–	–	–
6-ATT[e]	–	–	–	–
6-ATT/gramicidin S[f]	–	–	–	–
Indoleacrylic acid[e]	0	0	0	0

[a] Shown are no spectrum signal (0), spectrum obtainable in the positive mode (+), in the negative mode (–) and both modes (+/–). Data were obtained in the linear mode of the TOF-MS, and oligosaccharide (sulfated carrageenans from seaweeds) samples were prepared as solutions in Milli-Q-grade water and placed on a stainless-steel probe tip as films according to method A (see Experimental). Solvent for matrices, ethanol–water containing 0.1% TFA (1:1, v/v). $\lambda_{exc} = 337$ nm.
[b] 2.5 mg ml^{-1}.
[c] Samples as films placed on PVDF membranes according to method D (see Experimental).
[d] 2.5 mg ml^{-1} of DMSO.
[e] 10 mg ml^{-1}.
[f] Using gramicidin S as internal standard for mass calibration.

signal corresponding to the trifluoracetate anion was also observed. This signal was not detected when methanol, ethanol, DMSO and water–acetonitrile were used as solvents (methods A–C). Because no fragmentation was observed even when different solvents were used, it is apparent that these compounds are very stable. The reproducibility of the spectra and the high stability of the molecular ion made these compounds useful as MALDI matrices. Additionally, as mentioned previously, the amount of adduction is negligible in both proteins and sulfated oligosaccharide analyses.

Similarly, very simple spectra are obtained in carbazole, 2-hydroxycarbazole and 1-nitrocarbazole under nitrogen laser desorption conditions (Table 4). As β-carbolines, carbazoles show the intact molecular ion as a strong signal in both positive and negative modes, together with a small signal corresponding to the dimeric structure. Only 1-nitrocarbazole shows an important fragment from the molecular ion owing to the loss of the NO_2 group (m/z 166).

We have described previously the photodimerization and the electron impact mass spectra of β-carbolines[11] and carbazoles.[17–21] During the present study, we also examined the LD/TOF-MS of all the other benzoindoles (carbazoles) mentioned in Table 1, although they are not useful MALDI matrices.[27]

A surprising result was observed when LD/TOF-MS ($\lambda_{exc} = 337$ nm) was applied to phenylcarbonyl compounds. Although all of them show their molecular ion and typical fragmentations in the electron impact mass spectra,[30] only 2,6-DHOA, 2,4,6-THOA and 2,6-DHOBA, i.e., all phenylcarbonylic compounds with hydroxylic moieties present in both *ortho* positions, showed TOF-MS signals. This result suggests that there is not a direct relationship between desorption, ionization and stability in the gaseous state of organic molecules of low molecular mass after excitation at 337 nm (LD/TOF-MS) and their capability to work as useful MALDI/TOF-MS matrices. No signals were detected in LD/TOF-MS ($\lambda_{exc} = 337$ nm) for 2-hydroxybenzophenone and 2,4-dihydroxybenzophenone.

The relationships between the properties of the ground and electronic excited states using the LD/TOF-MS and the possible usefulness as MALDI matrices of the compounds in Table 1 are under investigation.

CONCLUSION

As shown in Table 2, β-carbolines and hydroxyphenylcarbonyl compounds, including 2,6-dihydroxybenzoic acid, are very useful matrices for proteins, the results obtained being similar to those observed by using classical matrices such as CHC, sinapinic acid, gentisic acid, indoleacrylic acid and 6-ATT. When sulfated oligosaccharides were studied with the same matrices (Table 3), the β-carboline family was shown to be the only type useful as MALDI matrix. As we suggested previously, a practical advantage of using β-carbolines in acidic solutions is not only their photochemical stability, but also their vacuum stability. Samples prepared with these matrices remain stable under vacuum and in the open air with no significant decrease in signal intensity or quality. Also, they can be used consecutively in TOF-MS, producing very strong and stable signals of either positive (protonated) or negative (deprotonated) protein molecule ions and only negative (deprotonated) sulfated oligosaccharide molecule ions. Additional advantages of these new matrices are their solubility in different organic solvents (e.g., DMSO, ethanol, methanol, propanol, butanol, acetonitrile) and their possible use under a wide range of experimental conditions.

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Table 4. LD/TOF-MS signals of β-carbolines, carbazoles and phenylcarbonyl compounds useful as MALDI matrices in the positive and negative modes[a]

Matrix	M_r	*m/z* (relative intensity, %) Positive mode	Negative mode
Pyridoindoles:			
β-Carbolines:			
nor-Harmane	168	168 (100), 334 (10)	112 (90), 166 (100), 225 (10)
Harmane	182	182 (100), 362 (12)	112 (100), 224 (60), 246 (21)
Harmine	212	212 (100), 423 (10)	112 (100), 224 (80), 246 (20)
Harmol	198	198 (100)	112 (100), 225 (30), 247 (55)
Harmaline	214	214 (100), 426 (8)	112 (100), 224 (31), 246 (46)
Harmalol	198	198 (100), 396 (9)	112 (100), 160 (80), 204 (18)
Benzoindoles:			
Carbazoles:			
Carbazole	167	166 (100), 330 (10)	164 (100)
2-Hydroxycarbazole	183	182 (100), 359 (6)	180 (100), 358 (6)
1-Nitrocarbazole	210	166 (100), 210 (100)	209 (100), 416 (8)
Phenylcarbonyl compounds:			
Acetophenones:			
2,4-DHOA	152	No[b]	No
2,5-DHOA	152	No	No
2,6-DHOA	152	153 (100)	152 (100)
3,5-DHOA	152	No	No
2,4,6-THOA	168	169 (100)	168 (100)
Benzoic acids:			
2,6-DHOBA	154	155 (100), 174 (8)	154 (100)

[a] Spectra were obtained by loading a drop of the β-carboline, carbazole or phenylcarbonyl compound solution (5 mg per 2-ml; solvent, ethanol–water containing 0.1% TFA (1 : 1, v/v) on the stainless-steel probe. $\lambda_{exc} = 337$ nm.
[b] No signals were observed in TOF-MS.

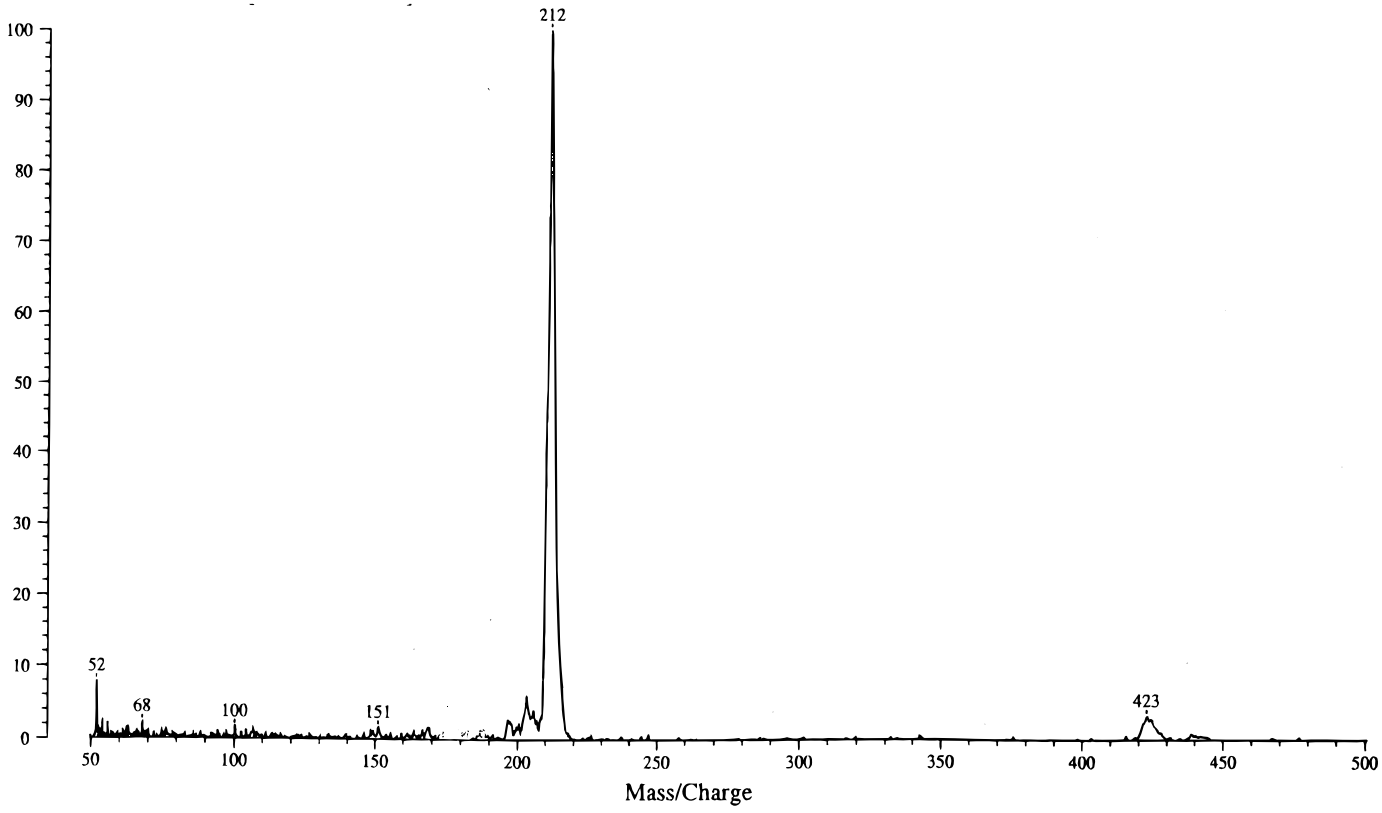

Figure 3. Positive mode LD/TOF mass spectrum of harmine. Conditions: $\lambda_{exc} = 337$ nm; solvent, ethanol–water containing 0.1% TFA (1 : 1, v/v); concentration, 2.5 mg ml^{-1}; total harmine solution loaded, 0.5 μl; 50 laser shots summed.

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