REGULAR PAPER

Reduction of Cu(II) and Riboflavin in DIOS Mass Spectrometry

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Understanding ion-molecular reactions is suggested to be essential to delineating the mechanisms of matrix-assisted laser desorption/ionization (MALDI) and related ionization methods. To examine the implications of matrix-analyte reactions in the reduction observed in MALDI, copper(II) chloride and riboflavin were analyzed by desorption/ionization on porous silicon (DIOS) mass spectrometry. This system does not require a matrix for soft laser desorption/ionization. The two susbstances were reduced by DIOS as well, and the reduction of riboflavin was accelerated using water as a solvent, as reported in MALDI. The results indicated that target-analyte electron transfer occurs in LDI, although the possibility of matrix-analyte electron transfer in MALDI cannot be excluded.

1. Introduction

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) provides the molecular weights of labile or large molecules, and is currently an indispensable method of analyzing biomolecules and synthetic polymers.[1)–6)] Despite its widespread use, the mechanism and process of MALDI are not fully understood, in contrast to another soft ionization method, electrospray ionization (ESI).[7)–11)] However, as physical data such as ionization potentials and gas-phase acidities and basicities accumulate, and as the roles of matrix–matrix, matrix–analyte, and analyte–analyte reactions in both primary and secondary ionization events can be evaluated in more detail, it is now suggested that the secondary ion-molecule reactions in the plume are important for understanding MALDI spectra.[8), 9)]

Earlier studies have suggested that reduction is an inherent feature of MALDI; Zhang *et al*. reported that copper(II) ion was reduced by laser desorption ionization with or without a matrix.[12)] As reported by Itoh *et al*., flavin-containing compounds, riboflavin, riboflavin 5′-phosphate, and flavin-adenine dinucleotide, were reduced by MALDI and fast atom bombardment (FAB), whereas such reduction was not observed in the ESI mass spectrum.[13)] Furthermore, the relative intensities of $[M+2]^+$ and $[M+3]^+$ ions to $[M+1]^+$ ions were dependent on the matrix species used for MALDI.[13)] Also, photo-induced dissociation followed by reduction was suggested in the MALDI-MS of nitrotyrosine, in which the NO_2 moiety is partially changed into an amine (NH_2).[14), 15)] In the case of *S*-nitrosocysteine, only the reduced form (SH), not the SNO form, was observed in the MALDI mass spectra of the SNO-containing peptides and proteins, while the intact molecular ion species with SNO could be identified by the ESI mass spectrum.[16), 17)]

Different mechanisms, electron capture[7)] and charge transfer from the matrix[8)], have been proposed for the reduction in MALDI. Recently, it was reported that free electrons are formed by photoelectric emission from the metal/dielectric-substance interface.[18), 19)] The naked metallic target is not the source of photoelectrons, because the work function of metals is greater than the photon energy for MALDI. The presence of matrix molecules or analytes on the metal enhances the emission *via* band bending and the associated reduction in work function.[18), 19)] Thus, electron transfer between target and analyte is another important factor which must be considered in explaining the reduction by laser desorption/ionization.

Siuzdak *et al*. reported a method, laser desorption/ionization on porous silicon, termed DIOS.[20)] Porous silicon is a UV-absorbing semiconductor with a large surface area and is produced through electrochemical anodization or chemical etching of crystalline silicon. In DIOS, analytes are deposited on DIOS plates, and the "assisting" chemical matrix is not used for ionization. Thus, the DIOS mass spectra are free from the cluster ions of the matrix, and the scanty background signals facilitate detection of the ions derived from small molecules. The applications reported to date cover a wide variety of compounds including peptides, natural products, small organic molecules and hydrophilic synthetic polymers.[20)–29)] The DIOS mechanism, which is poorly understood, is presumed to be based on a porous scaffold retaining solvent and analyte molecules, and on the UV absorptivity that affords the transfer of laser energy to analytes.[20), 21)] The ionization process would be simple compared with that of MALDI, since no chemical reactions between analytes and the sample matrix are involved. This prompted us to investigate the reduction of Cu(II) ions and riboflavin in DIOS to delineate the mechanism of MALDI and related soft laser desorption/ionization.

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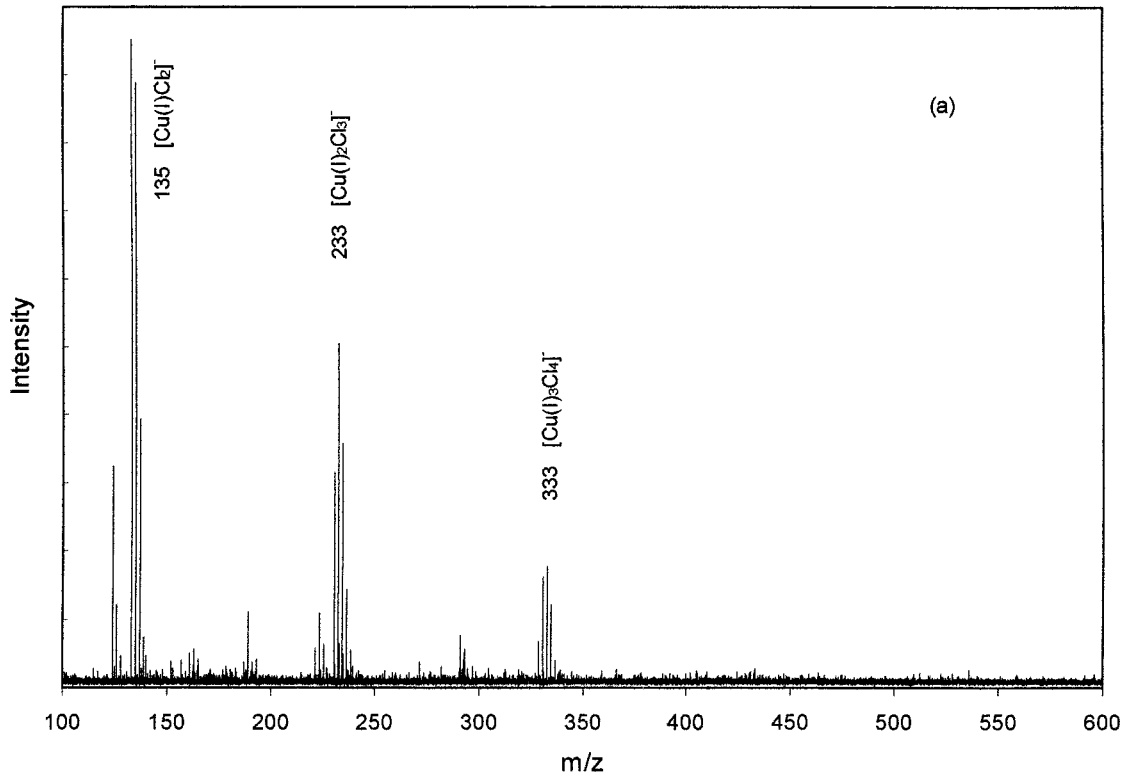


Fig. 1(a) See the caption of Fig. 1 on page 15.

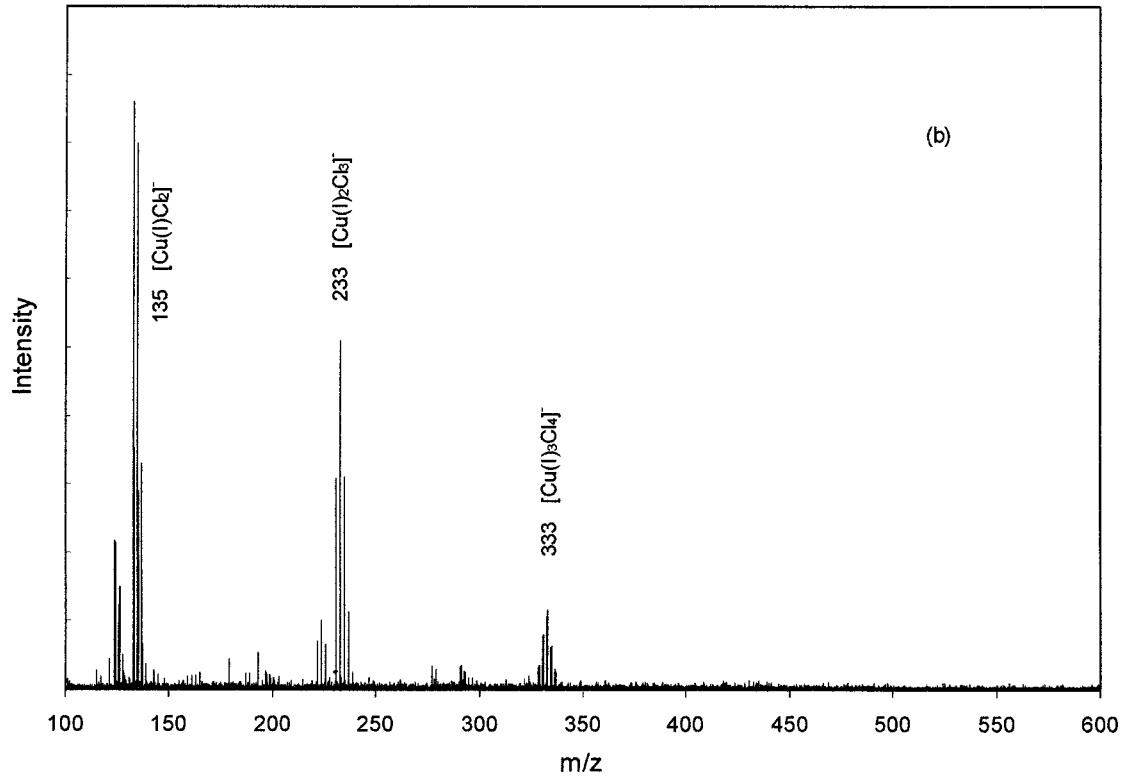


Fig. 1(b) See the caption of Fig. 1 on page 15.

2. Experimental

2.1 Materials

Methanol, ethanol, copper dichloride, riboflavin, nicotinic acid, trifluoroacetic acid (TFA), and hydrofluoric acid (46%) were purchased from Wako Pure Chemical (Osaka, Japan), and α-cyano-4-hydroxycinnamic acid (CHCA) and protoporphyrin IX were from Aldrich (Milwaukee, WI, USA). Deuterated reagents, methanol-d_4, deuterium oxide and TFA-d, were obtained from Cambridge Isotope Laboratories (Andover, MA, USA).

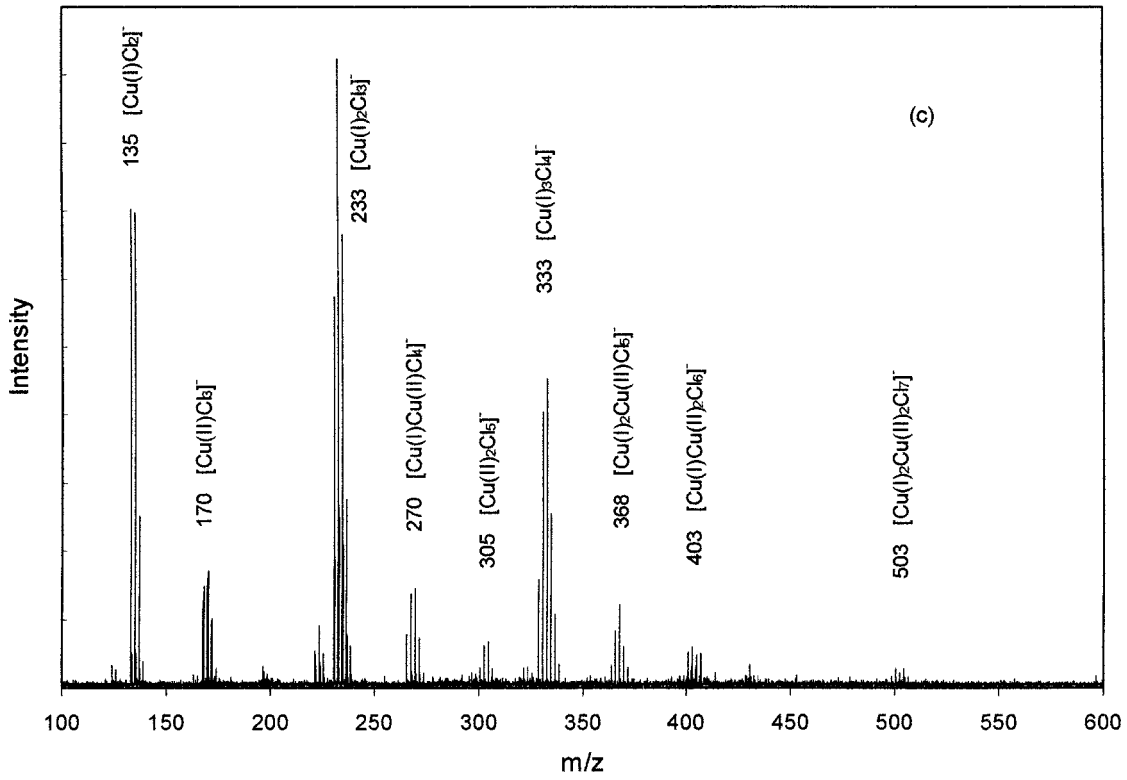


Fig. 1(c)

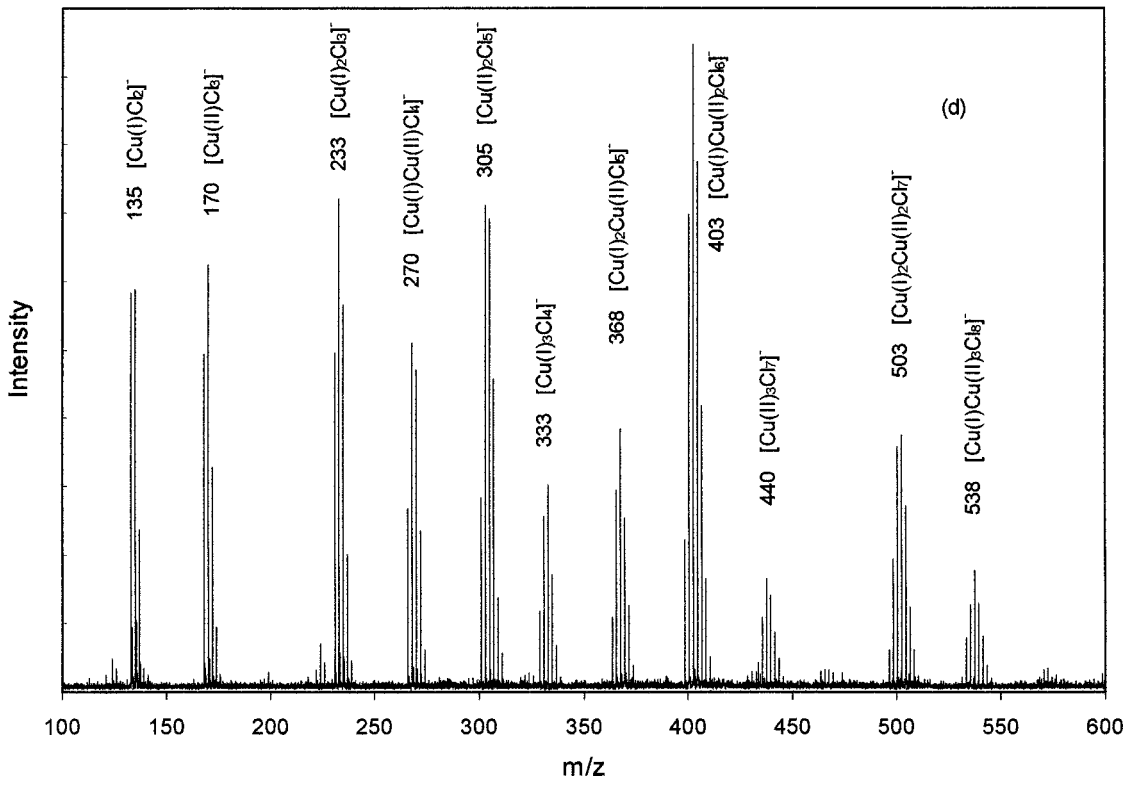
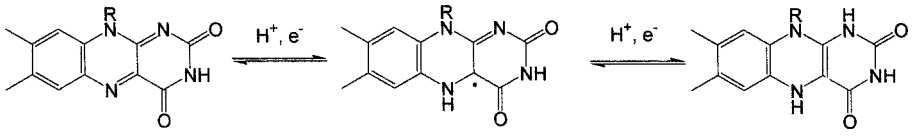


Fig. 1(d)

Fig. 1. DIOS TOF mass spectra of $CuCl_2$ in negative ion mode at various concentrations of $CuCl_2$: (a) 0.01 mg/mL, (b) 0.05 mg/mL, (c) 0.1 mg/mL, and (d) 0.2 mg/mL.

2.2 Preparation of DIOS plate and MS

The DIOS plate was prepared according to the procedures described by Siuzdak.[20), 21)] Briefly, a silicon (100) wafer of n-type and $\rho = 1$–$3\ \Omega$/cm resistivity was anodically etched (32 mA/cm^2) in a 1 : 1 (v/v) solution of ethanol–HF (46%) under exposure to white light (40



Scheme 1. Process of riboflavin reduction.

mW/cm^2) for 1 min. After etching, the DIOS plate was washed in ethanol, and dried *in vacuo*.

Copper dichloride was dissolved at 0.01–0.2 mg/mL in 50% methanol, and riboflavin was dissolved at 0.01 mg/mL in methanol or in a 0.1% trifluoroacetic acid and 50% methanol solution. Each 0.5 μL aliquot of $CuCl_2$ or riboflavin solution was deposited on a DIOS plate and dried at room temperature.

DIOS mass spectra were acquired in reflectron mode using a Voyager-DE Pro Time-of-Flight (TOF) mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a pulsed nitrogen laser (337 nm). Monoisotopic peaks of the ion species of CHCA, $[M+H]^+$ at *m/z* 190.05, $[2M+H]^+$ at *m/z* 379.09, $[3M-3H+4Na]^+$ at *m/z* 656.06, were used as calibrants for positive mode measurement. Monoisotopic peaks of nicotinic acid $[M-H]^-$ at *m/z* 122.02, CHCA $[M-H]^-$ at *m/z* 188.03, and protoporphyrin IX $[M-2H+Na]^-$ at *m/z* 583.23 were used as calibrants for negative mode measurement.

3. Results and Discussion

In the direct LDI of $Cu(II)Cl_2$ without using sample matrix, Cu(II) ions were reduced to Cu(I) on a metallic sample-target, while cluster ions composed of three oxidation states of copper, *i.e.*, Cu(0), Cu(I), and Cu(II), were observed when a nonmetallic polyetheretherketone (PEEK) target was used.[12] These observations suggested electron transfer from metallic target to analytes to be involved in the reduction of Cu(II).[11] In MALDI, on the other hand, the reduction of Cu(II) by electron transfer from matrix molecules as well as the target should be taken into account. Therefore, it was interesting to examine the reduction of Cu(II) by DIOS, since porous silicon is a semiconductor and the sample matrix is not used. As shown in the DIOS spectra of $CuCl_2$ at the concentration of 0.01 and 0.05 mg/mL in the negative ion mode, the intense signals at *m/z* 135, *m/z* 233, and *m/z* 333 represented $[Cu(I)Cl_2]^-$, $[Cu(I)_2Cl_3]^-$, and $[Cu(I)_3Cl_4]^-$, respectively, whereas the cluster ions containing Cu(II), which were abundant on

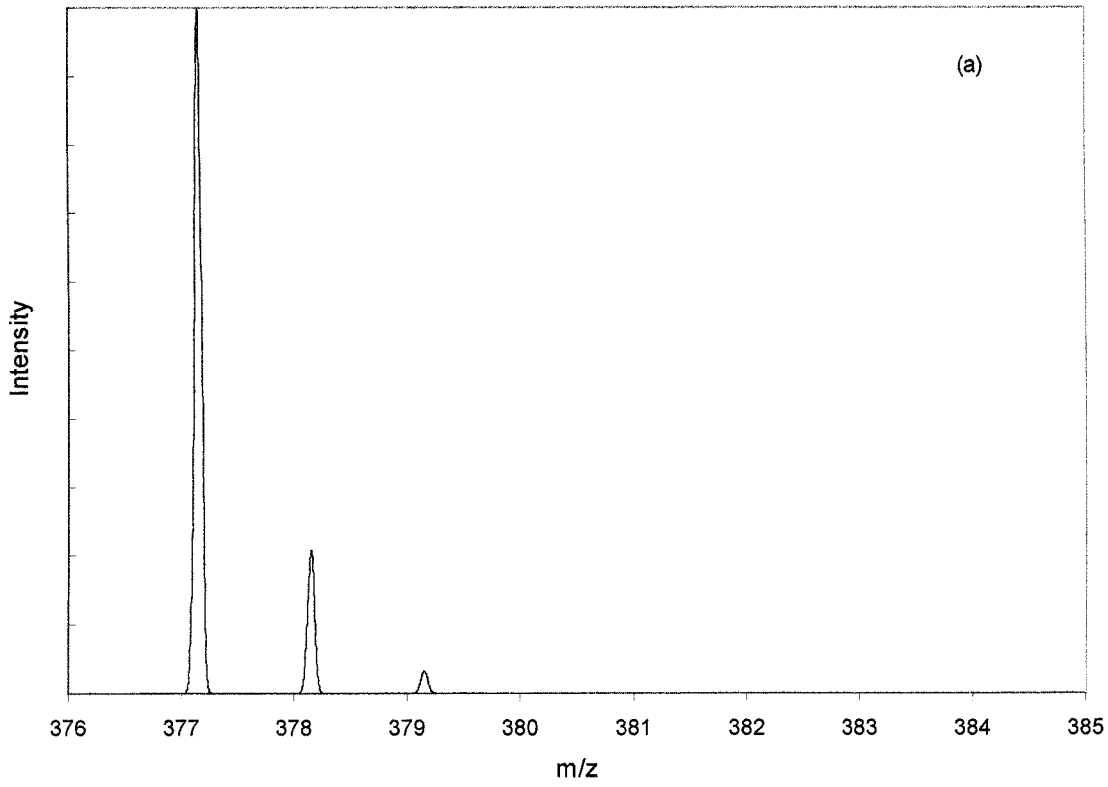


Fig. 2(a) See the caption of Fig. 2 on page 19.

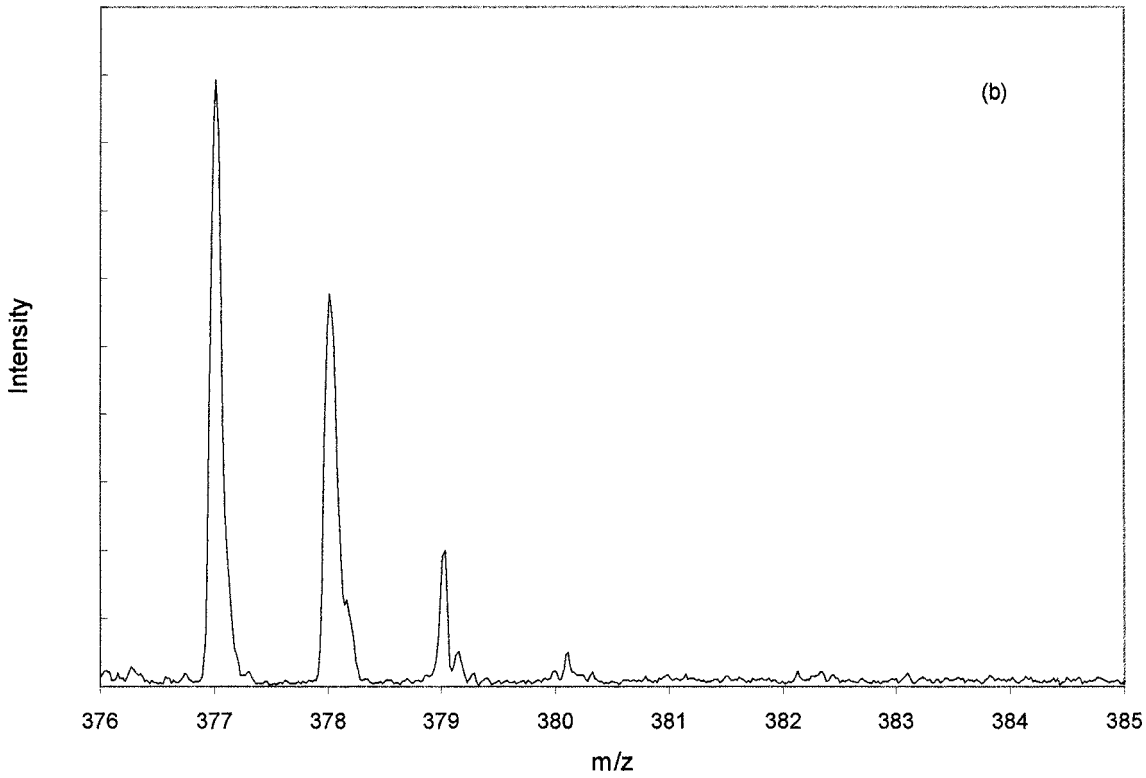


Fig. 2(b) See the caption of Fig. 2 on page 19.

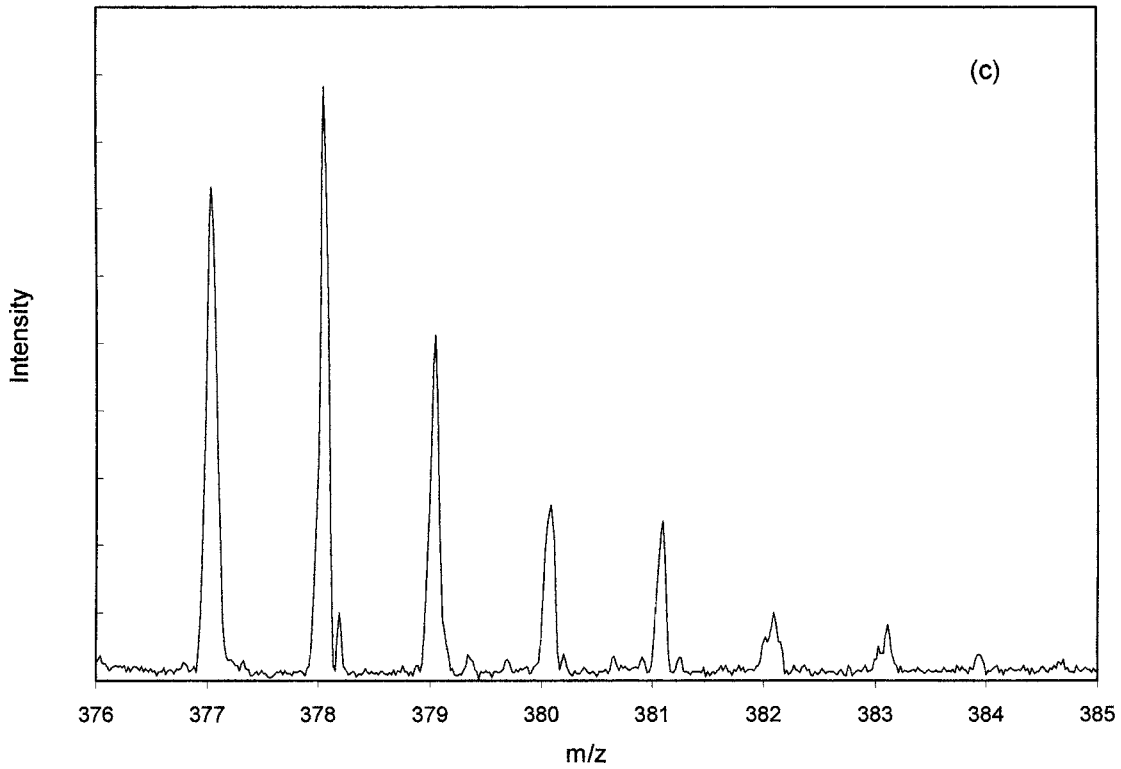


Fig. 2(c) See the caption of Fig. 2 on page 19.

a PEEK target in an earlier study,[12] were not observed. [Figs. 1a, b] The result indicated that Cu(II) ions were reduced on a silicon target, as with a metallic target, due to electron transfer from the DIOS plate. However, the cluster ions containing Cu(II), *i.e.*, $[Cu(II)Cl_3]^-$ (*m/z* 170), $[Cu(I)Cu(II)Cl_4]^-$ (*m/z* 270), $[Cu(II)_2Cl_5]^-$ (*m/z* 305), $[Cu(I)Cu(II)_2Cl_6]^-$ (*m/z* 403), $[Cu(I)Cu(II)_3Cl_8]^-$ (*m/z* 538), became prominent, as the $CuCl_2$ concentration increased, indicating that a thick sample crystallite can suppress the electron transfer from the target to the analyte as reported in MALDI and LDI.[12), 18), 19)]

The reduction of riboflavin ($M=376$) in MALDI was

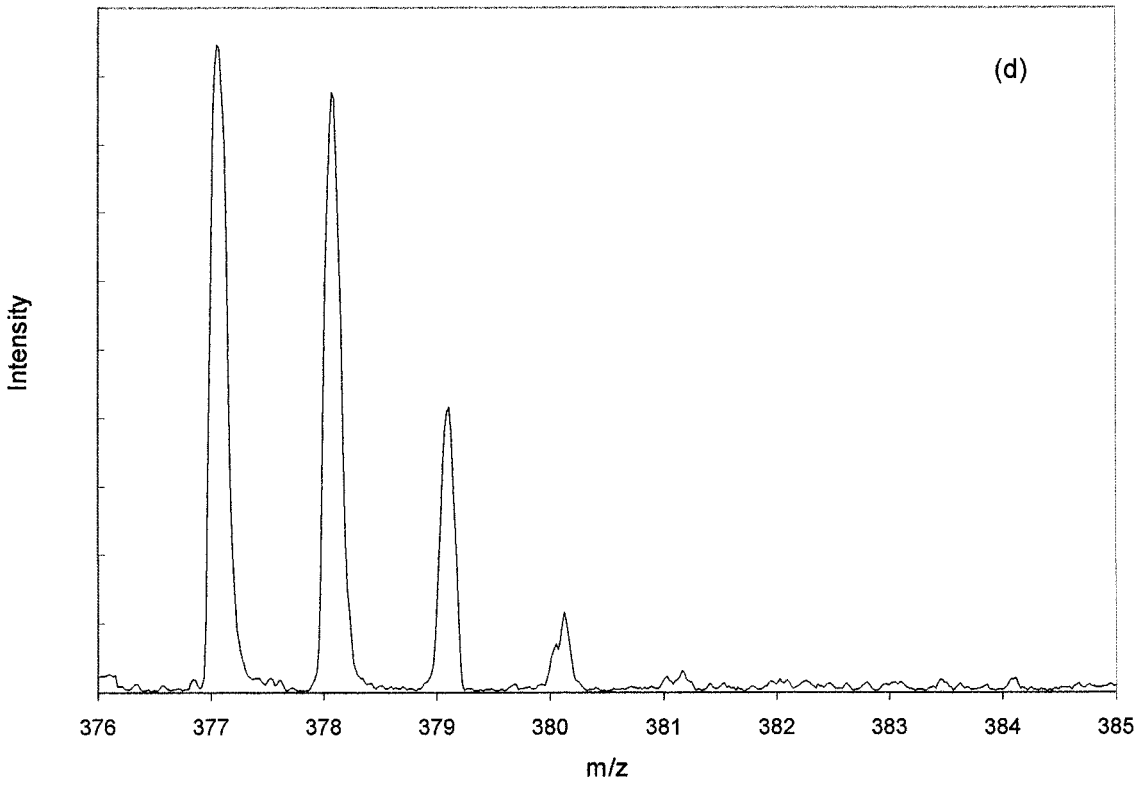


Fig. 2(d) See the caption of Fig. 2 on page 19.

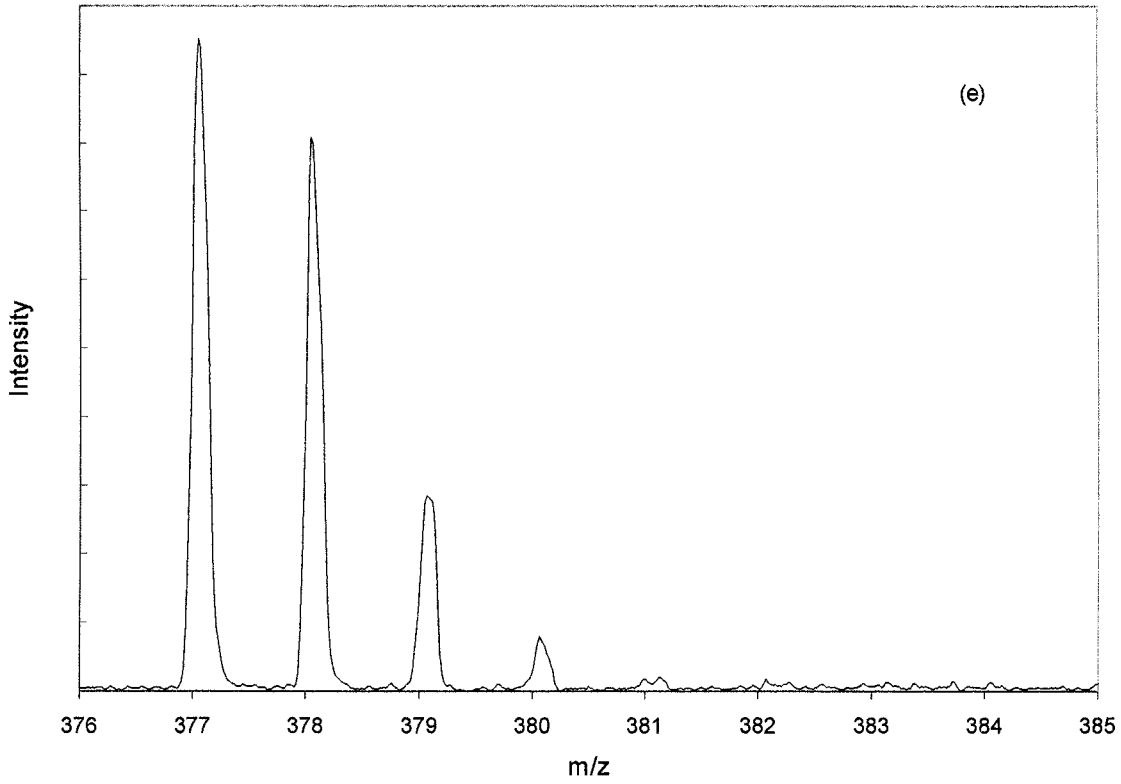


Fig. 2(e) See the caption of Fig. 2 on page 19.

reported by Itoh *et al*., who attributed it to the transfer of protons and electrons from matrix molecules to riboflavin as shown in Scheme 1.[13] In order to examine the involvement of sample matrix as a source of electrons, riboflavin was analyzed by DIOS-MS. Riboflavin was dissolved in a 50% methanol/0.1% TFA solution, placed on a DIOS target and dried. As shown in Fig. 2 b, the intensities of $[M+2]^+$ (*m/z* 378) and $[M+3]^+$ (*m/z* 379) relative to that of $[M+1]^+$ (*m/z* 377) were obviously high compared with the calculated isotopic distribu-

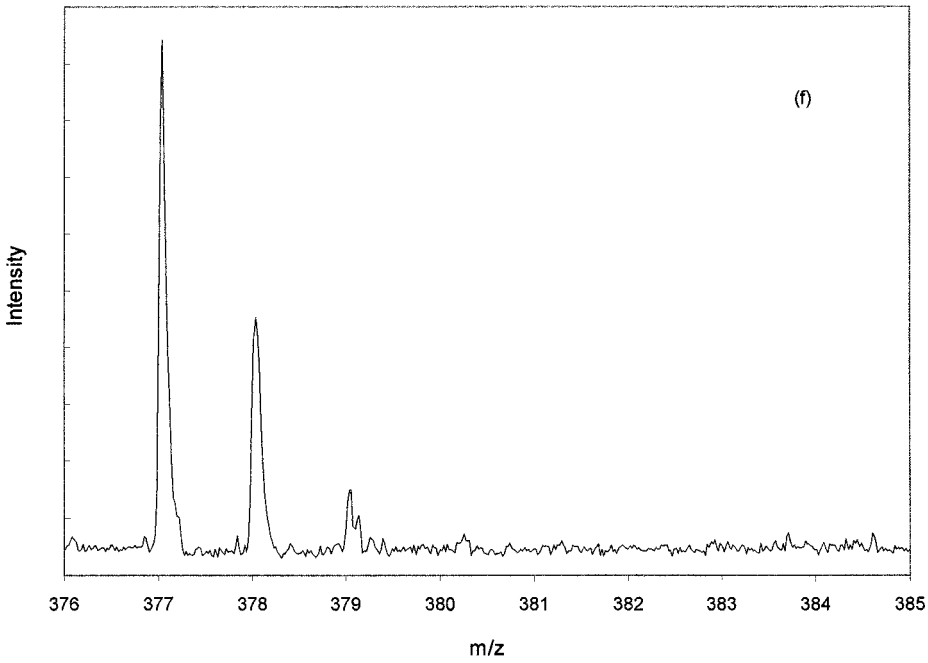


Fig. 2(f)

Fig. 2. DIOS TOF mass spectra of riboflavin in positive ion mode. (a) theoretical isotope distributions of $[M+H]^+$. Riboflavin was dissolved in methanol/H_2O/TFA (b), methanol/D_2O/TFA (c), methanol-d_4/H_2O/TFA (d), methanol/H_2O/TFA-*d* (e), and methanol/TFA (f).

tion of intact $[M+H]^+$. The result indicated that riboflavin was reduced in DIOS. A line of studies regarding the MALDI mechanism has focused on the electron transfer from matrix molecules to analytes in the desorption plume.[7), 8), 13)] More recently, electron transfer from the sample target to analytes has been proposed as well.[12), 18), 19)] Our results support the latter mechanism underlying the reduction in MALDI and related soft laser desorption/ionization methods.

Obviously, the proton source is a key factor affecting the reduction of riboflavin in MALDI.[13)] To further investigate proton involvement, riboflavin was analyzed by DIOS-MS using various deuterated solvents: methanol-d_4 / H_2O / TFA, methanol / D_2O / TFA, and methanol/H_2O/TFA-*d*. In either case, the intensities of $[M+2]^+$ and $[M+3]^+$ relative to $[M+1]^+$ were high compared with the control, indicating the incorporation of deuterium (Figs. 2c–e). The effect was most significant with the use of deuterium oxide. Thus, water was the major proton donor in this experimental setting, where it predominated TFA and dissociated into H^+ and OH^- more easily. Finally, to confirm the role of water, riboflavin was dissolved in a methanol/TFA solution and measured by DIOS-MS. As shown in Fig. 2f, reduction was significantly suppressed, indicating that, as a solvent, water affects the reduction of riboflavin in LDI.

4. Conclusion

Copper and riboflavin are reduced by DIOS as observed in MALDI. This indicates that target-analyte electron transfer occurs in LDI including MALDI, although the possibility of matrix-analyte electron transfer in MALDI cannot be excluded. Water plays a role in the reduction of riboflavin by DIOS.

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