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Title:

Characterization of 6-bromoferulic acid as a novel common-use matrix for MALDI-TOF-MS

Short Title:

Usability of halogenated ferulic acids as MALDI matrices

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Rationale: Ferulic acid (FA) is a standard matrix used for analyzing proteins. In this study, the ability of a halogenated FA to serve as an effective MALDI matrix was investigated. Various halogenated FAs were synthesized, and the characteristics and performance of each were compared with those of the standard matrices CHCA and DHBA.

Methods: The abilities of 6-BFA, ferulic acid (FA), and eight other halogenated FA derivatives to ionize eight synthetic peptides were examined. Absorption measurements, MM2 structure optimizations, and proton affinity (PA) calculations were also performed for 6-BFA and FA. The suitabilities of these compounds as MALDI matrices for lipids, sugar chains, polymers, cyanocobalamin, synthetic peptides, and tryptic peptides originating from two types of serum proteins were also tested.

Results: The 6-position of FA was found to be the best site for introducing a bromine because the generated compound allowed facile detection of cyanocobalamin and several peptides. 6-BFA exhibited good sensitivity for large peptides (3–5 kDa) and peptides containing acidic amino acids or proline. 6-BFA was also shown to be a suitable matrix for MS/MS analysis when using MALDI time-of-flight (TOF) mass spectrometry (MS) with a quadrupole ion trap (QIT) system.

Conclusions: The properties of 6-BFA as a MALDI matrix differed from those of DHBA and CHCA. 6-BFA appears to be a useful matrix for de novo sequencing using MALDI-QIT-TOF-MS.

Introduction

Ferulic acid (FA) has a relatively long history as a MALDI-TOF-MS matrix and in protein analyses. The advantage of FA is that it can be used for detecting proteins with molecular weights ranging from 10 to 150 kilodaltons (kDa); for instance, FA is often used to identify bacteria.[1-3] Although α-cyano-4-hydroxycinnamic acid (CHCA)[4,5] is mainly used as a matrix in the field of peptide analysis, its halogenated forms exhibit superior sensitivity and selectivity for peptides. For example, the halogenated CHCA compound 4-chloro-α-cyanocinnamic acid (ClCCA), which is formed by substituting the hydroxyl group at the 3-position on the benzene ring with a chloride, gives rise to higher quality mass spectra and higher sensitivity relative to what can be obtained with CHCA.[6,7] Thus, halogenation may change the properties of the original matrix. However, halogenated matrices for protein analysis have not yet been examined. Because FA and sinapinic acid (SA) are typical matrices for protein analysis, in this study, we synthesized nine halogenated derivatives of FA and compared their suitabilities for MALDI ionization with that of unmodified FA. FA was also selected because it has a readily substitutable site on its benzene ring, and it is easier to derivatize than SA. It was thus hypothesized that FA would be more effective than SA for examining the effects of various halogen substitutions. The results show that the most effective substitution involved introducing bromine at the 6-position of the benzene ring of FA. Notably, the properties of FA as a matrix changed drastically upon 6-bromination, and the halogenated species was suitable for analyzing peptides with molecular weights (*Mw*) ranging from 1 to 5 kDa. However, the brominated compound was not as suitable a matrix as FA for analyzing proteins. Nevertheless, examining the properties of this matrix for biomolecule detection is expected to be valuable for further investigations. The proton affinity (PA) of 6-BFA was determined, and the PA was smaller than those of FA and CHCA but larger than that of ClCCA.[8] There are many MALDI matrices currently commercially available, and the appropriate matrix differs depending on the type of molecules being detected, the range of molecular weights, and the charges of the molecular ions. Thus, increasing the diversity of available matrices and ionization methods is important for increasing the utilization of MALDI-TOF-MS by facilitating simple and rapid analyses. In this paper, we report a new compound, 6-BFA, that may have a variety of uses based on its distinct properties.

Methods

Chemicals and commercial peptides

CHCA and DHBA were obtained from Shimadzu GLC Co. Ltd. (Kyoto, Japan). Ammonium bicarbonate, acetonitrile (CH_3CN, HPLC grade), trifluoroacetic acid (TFA), and 25% TFA (amino acid sequence grade) were obtained from FUJIFILM Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Adrenocorticotropic hormone (18-39) (ACTH), bradykinin (1-7) (BK), oxidized B chain of insulin (INS-B), BSA, and human serum Tf were obtained from Sigma-Aldrich Japan (Tokyo, Japan). Angiotensin II (AG-II), ovine corticotropin releasing factor (CRF), sET, substance P (SUB-P), [D-Arg1,D-Pro2,D-Trp7,9,Leu11]-substance P (aSUB-P), tertiapin (TTP), rat urotensin-II (UTS-II), human proadrenomedullin N-terminal 20 peptide (PAMP), and $(Pro\text{-}Pro\text{-}Gly)_{10}$ ($(PPG)_{10}$) were purchased from Peptide Institute, Inc. (Osaka, Japan). A Sequencing-Grade Modified Trypsin Kit was obtained from Promega (WI, USA). 2-Bromo-4-hydroxy-3-methoxybenzaldehyde, *tert*-butyl diethylphosphonoacetate, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), and phthalic anhydride were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), and pyridine (specially prepared solvent) was obtained from Pierce (IL, USA). Cyanocobalamin was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

Synthesis of 6-BFA

6-BFA was prepared from 2-bromo-4-hydroxy-3-methoxybenzaldehyde in four steps, and the structure is shown in Figure 1 (G). The key reaction, a Horner–Emmons–Wadsworth olefination[9-12] with *tert*-butyl diethylphosphonoacetate in the presence of DBU, converts the acetylated aromatic aldehyde into the corresponding protected 6-BFA. This reaction is followed by the removal of the *tert*-butyl ester in TFA and removal of the acetyl group with potassium carbonate in methanol to give 6-BFA in 64.5% overall yield. The product was recrystallized from MeOH to give highly pure 6-BFA: mp 245-248 °C (mp 229-230 °C in reference 13). Spectral data for 6-BFA: IR (KBr) 3387, 1666, 1604, 1504, 1411 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ 3.82 (s, 3 H), 6.52 (d, J = 16 Hz, 1 H), 7.02 (s, 1 H), 7.38 (s, 1 H), 7.75 (d, J = 16 Hz, 1 H), 10.1 (br s, 1 H), 12.3 (br s; 1 H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 56.0, 110.7, 116.2, 118.9, 119.0, 124.0, 141.7, 147.9, 148.0, 167.7; ESI-MS m/z 274 (M^+ + 2, 5.4), 272 (M^+, 5.5), 193 (100), 178 (25.9), 133 (18.6). Anal. Calcd for $C_{10}H_9BrO_4$: C, 43.98; H, 3.32. Found: C, 44.12; H, 3.38.

Matrix solutions and MALDI-TOF-MS analyses

6-BFA (1 mg) was suspended in 300 μL of CH_3CN in a small glass sample tube with a screw cap and shaken for approximately 10 min at room temperature. The tube was then left to stand for approximately 10 min to obtain a saturated solution of 6-BFA. The supernatant was diluted with 3 volumes of CH_3CN and used as the matrix solution. The matrix solution (1 μL) and 1 μL of the sample solution were mixed well in a 0.2 mL tube and applied to the target plate. CHCA and DHBA were each dissolved in a solution comprising 0.1% TFA in 50% CH_3CN and 0.1% TFA in 33.3% CH_3CN.

All spectra were obtained manually or automatically on an AXIMA-CFR instrument with a laser wavelength of 337 nm or on an AXIMA-QIT spectrometer (Shimadzu Biotech, Kyoto, Japan) with a laser wavelength of 355 nm. In the automatic MS/MS analyses, a circular well-type raster was used as the irradiation point for 500 laser shots. The properties of 6-BFA and DHBA were compared by changing the collision-induced dissociation (CID) value of the AXIMA-QIT in 8 steps (50, 100, 125, 150, 175, 200, 250, and 300), and automatic analyses were performed at each CID value.

To determine the detection limit of 6-BFA, a 2 pmol/μL solution of aSUB-P was prepared as a reference (internal standard), and 0.25, 0.5, 1.0, and 1.5 pmol/μL solutions of SUB-P were prepared as analytes (sample). The reference solution (1 μL), 1 μL of the analyte solution, and 2 μL of the matrix solution were mixed well, and 2 μL of this solution was applied to the target plate. UTS-II and TTP were prepared following the same procedure as a reference material and an analyte, respectively.

Preparation of phthaloyl angiotensin II (ptAG-II)

First, 22.2 mg of phthalic anhydride was dissolved in 10 mL of pyridine. This solution was then diluted 100 times with pyridine for use in the phthaloyl reaction. Then, 200 pmol of dry AG-II was prepared in a 0.5 mL Eppendorf tube, and the AG-II was dissolved in 20 μL of water. Next, 20 μL of the dilute phthalic anhydride solution was added to the tube, and the mixture was left to stand for 4 h. The reaction mixture was dried on a centrifugal evaporator, and the solid residue was dissolved in 0.1% TFA for use in the MALDI-TOF-MS analysis.

Tryptic peptide preparation of BSA and Tf

To prepare the tryptic peptides, BSA (87 nmol, approximately 6.1 mg) or Tf (approximately 7.0 mg) was dissolved in 806 μL of H_2O in a 1.5-mL Eppendorf tube and heated for 3 min in a 100 °C water bath and then cooled on ice. Acetic acid (20 μL, 50 mM, supplied in the enzyme kit) was mixed with sequencing-grade trypsin in a vial (20 μg of trypsin/vial) and mixed well to activate the trypsin. After standing for 15 min at 30 °C, the protein solution was added to the vial of trypsin, and 44 μL of 1 M ammonium bicarbonate buffer was added. The stoppered vial (containing a total of 870 μL of solution) was incubated overnight at 37 °C and then diluted with 3 volumes of H_2O. A 10–μL aliquot was dried using a centrifugal evaporator. All the dried samples were dissolved in 0.1% TFA.

Proton affinity of 6-BFA and FA

To estimate the PA values of 6-BFA and FA, electrospray ionization (ESI) mass spectrometry analysis was performed with a 4000 QTRAP LC/MS/MS system (SCIEX, Framingham, Massachusetts, USA). 4-Fluoroaniline, γ-butyrolactone, and 4-nitroaniline were chosen as the reference bases.

Results and discussion

In vitro assay of FA and 6-BFA

Ferulic acid (FA), originally isolated from herbs used in Chinese medicine, has attracted attention from medical professionals as an antioxidant because it reacts with free radicals.[14] It exerts antitumor activity and inhibits metastasis of breast cancer cells by regulating epithelial to mesenchymal transition,[15] and it exerts neuroprotective effects against cerebral ischemia/reperfusion-induced injury via antioxidant and antiapoptotic mechanisms.[16] Recently, FA was also shown to inhibit the clot retraction responsible for platelet development.[17] However, the difference the influence on the cell and the living body due to the introduction of a bromide onto FA is unclear. Therefore, we investigated the cytotoxicity of FA and 6-BFA by a 3-(4,5-dimethylthial-2 -thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay at concentrations of 1, 10, 50, 100 and 1,000 μM against PC12 cells. If the concentration of 6-BFA was low, i.e., less than 1,000 μM, no drop in the survival rate of these cells was observed. The cytotoxicity of 6-BFA was almost the same as that of FA (data not shown).

Characterization of halogenated FAs by MALDI-TOF-MS analysis

Various halogenated FAs substituted at the 2-, 5-, and 6-positions of the benzene ring were synthesized (Figure 1). The ionizing power of each of these compounds for peptide mixtures (comprising BK, AG-II, SUB-P, sET, $(PPG)_{10}$, ACTH, INS-B, CRF, and ptAG-II; Table S1) was assessed by MALDI-TOF-MS. The 5- and 6-fluoro derivatives did not induce peptide ionization, and the 2-fluoro derivative could not be synthesized. Although FA could ionize the peptides, its sensitivity was low, as shown in Figure 1 (A). The sensitivity of FA for $(PPG)_{10}$ was particularly strong, and this characteristic was notably lost in the 2- and 5-chloro derivatives, as shown in Figure 1 (B and C). Although the sensitivity of the 6-chloro derivative for some peptides was lower, its specificity for $(PPG)_{10}$ was maintained (Figure 1 (D)). The sensitivities to the 2- and 5-chloro derivatives were higher than those of the other derivatives, and a similar trend was observed for the brominated compounds, as shown in Figure 1 (E and F, H and I). Notably, the 6-halo derivatives exhibited the best sensitivity while maintaining specificity for $(PPG)_{10}$, as shown in Figure 1 (D, G, and J). The specificity of FA appears to result from the localization of acidic amino acids, and 6-BFA provided the best MALDI matrix in this regard (Figure 1 (G)). By comparison, the sensitivity of the iodinated derivatives was lower, as shown in Figure 1 (H, I, and J). The solution-phase absorbance spectra (Figure 2 (A)) of the 6-halogenated derivatives (3, 6, 9 and 12) showed more explicit bimodal distributions relative to that of FA, and the intensities of the first and second absorption maxima were different. These results seem to reflect the changes in the physical properties of FA caused by halogenation, and these changes may influence the performance of 6-BFA as a MALDI matrix. D. A. Allwood et al. reported solid-state UV optical absorption spectra of the common matrices CHCA, FA, SA and DHBA. These matrices had an absorption range from 300 to 400 nm, and their absorption properties matched well with the typical MALDI laser wavelengths of 337 and 355 nm.[18] Thus, although bromination altered the physical properties of FA, describing the physical property changes and their impact on the compound's success as a matrix is difficult.

The spectral data in Figure 2 were converted to molar absorption coefficients by assuming that the absorbance was related to only one type of molecule. The results were compared and are listed in the lower part of Figure 2 (B). The molar absorption coefficient of FA was the highest, and the values for 6-chloro FA (6-CFA) and 6-BFA were almost equal to that of FA. Notably, the coefficients of these compounds were 4–5 times higher than those of the other compounds, and the coefficients were in the following order: FA > 6-CFA > 6-BFA. Therefore, these compounds absorb light from a 337 nm laser more easily than the other compounds, and 6-BFA may be useful as a MALDI-TOF-MS matrix. Considering the charge distribution on the two O atoms in the COOH groups of the present FA derivatives, the values

of which were obtained by extended Hückel calculations, the extent of the negative charges on these two oxygen atoms would be in the following order: 6-BFA > 6-CFA > FA (cf. the charges are shown in Figure 2 (B)). However, the differences are small and do not seem to indicate that the ability of FA to serve as a matrix would change upon conversion to 6-BFA.

We also investigated the types of molecules that can be ionized by 6-BFA; specifically, phospholipids, sugar chains, polyethylene glycols, and cyanocobalamin were examined. 6-BFA did not ionize phospholipids at all and did not effectively ionize sugar chains or polyethylene glycols. However, it was able to ionize peptides and cyanocobalamin, as described below. The inability of 6-BFA to ionize phospholipids and sugar chains may result from its fragmentation during laser irradiation since no parent ions were observed in either positive or negative mode (although some fragment ions were observed). Polyethylene glycols provided $[M + H]^+$ ions, but scalariform peaks of the sodium adduct ions appeared over a wide range (data not shown).

Application to MALDI-TOF-MS and analysis of peptides

Ionization of the synthesized peptides

To confirm the utility of 6-BFA as a matrix material, we compared 6-BFA with CHCA, a matrix commonly used for MALDI-TOF-MS. The mass spectra of a synthesized peptide mixture are shown in Figure 3, and the ion peak intensities obtained using CHCA (A) and 6-BFA (B, C, and D) are compared. In addition, the amino acid sequences, *Mw*, and isoelectric point (*pI*) values of each peptide in this mixture are shown in Table S1. sET and the collagen fragment $(PPG)_{10}$[19] afforded strong peaks when 6-BFA was used as the matrix (Figure 3 (B)); the intensity was approximately 10 times greater than that obtained using CHCA despite there being no similarity in the amino acid contents or sequences of these two peptides. Similarly, $(PPG)_5$ provided stronger peaks when using 6-BFA instead of CHCA as the matrix (data not shown). In part, the sensitivity of 6-BFA for the peptides may be due to their Pro content. The overall content of acidic amino acids may also be a factor; indeed, sET contains one Asp, two Glu, and succinic acid residues in its N-terminal region. In contrast, the N-terminal amino acids of BK, SUB-P, and ACTH are Arg, and these three peptides are poorly ionized in the presence of 6-BFA. AG-II has one Asp residue at its N-terminus and may be poorly ionized because of the adjacent Arg residue. Figure 3 (C) shows the mass spectrum of a peptide mixture that did not contain $(PPG)_{10}$ obtained using 6-BFA as the matrix. The intensities of BK, AG-II, SUB-P, and ACTH all recovered to some extent. The other peptides may be ionized more efficiently because ion suppression was reduced by removing the $(PPG)_{10}$. The use of 6-BFA as a matrix resulted in higher-intensity peaks for the

larger peptides, such as INS-B and CRF (Figure 3 (C)). INS-B contains two oxidized Cys residues with one of those residues being in the N-terminal region, possibly making that region very acidic. The N-terminal region of CRF also seems relatively acidic because it contains one Glu residue and one Asp residue. $(PPG)_{10}$ may absorb a large amount of energy, preventing its ionization. Thus, 6-BFA may allow highly sensitive analysis of large peptides such as INS-B and CRF (Figure 3 (C)). ACTH may give rise to a higher-intensity ion than other peptides containing N-terminal Arg residues because of its higher molecular weight. AG-II is a notable peptide in this experiment because the second basic Arg residue is thought to inhibit the effect of the N-terminal acidity caused by Asp. For this reason, we investigated the effect of a carboxyl group in the peptide by adding phthalic anhydride to the N-terminus of AG-II, and phthaloyl AG-II and normal AG-II were analyzed by MALDI-TOF-MS using 6-BFA as the matrix (Figure 3 (D)). Clearly, the sensitivity of the matrix for phthaloyl AG-II was high, but this phenomenon was reversed when the matrix was CHCA (data not shown). Thus, in smaller peptides, the increased acidity of the N-terminal region seems to be an effective strategy for increasing the sensitivity of the 6-BFA matrix. 6-BFA is less prone to desensitization relative to CHCA because its sensitivity for acidic peptides is excellent, and in the CHCA matrix, desensitization is caused by peptide acidification by succinylation and phthaloylation. The *pI* value for each peptide is shown in Table S1. The interactions between the matrix and the peptide do not always depend on the *pI*. For instance, although the *pI* values of $(PPG)_{10}$ and ACTH are almost identical, the sensitivities of the matrices for these ions differ. Therefore, the arrangement of acidic or basic amino acids may be a more important factor than the *pI* when small peptides are used. In addition, the Pro content and the position of these residues seem to be important factors.

Analyses of tryptic peptides from native proteins

To examine the applicability of the matrices studied herein for native peptide analysis, BSA and Tf tryptic peptides were tested; the results are shown in Figure 4. Figures 4 (A) and (B) show the MS data of the BSA peptides obtained using 6-BFA and CHCA, respectively; likewise, Figures 4 (C) and (D) show the results for the Tf' peptides. The detected peptides of interest and their amino acid sequences are listed in Table S2 (A) and (B). Peptides B1 and B3 (Figure 4 (A) and Table S2 (A)) gave rise to slightly more intense peaks using the 6-BFA matrix. The presence of two acidic amino acids is thought to enhance ionization, as described in the previous section. Although T1 and T2 (Figure 4 (D) and Table S2 (B)) contain acidic amino acids (Asp and Glu, respectively) at their N-termini, they gave rise to intense peaks when using CHCA as the matrix. This result suggests that the C-terminal Arg residue strongly influence the sensitivity for peptides with a *Mw* less than 1500.[20-23] The sensitivities of CHCA for T3, T4, T6, T7, and T8 were better even though these peptides

contain several acidic amino acids (Figure 4 (D) and Table S2 (B)). However, these peptides also contain basic amino acids in both their N- and C-terminal regions. Therefore, the sensitivities of CHCA for these peptides appear better than those of 6-BFA; the same explanation may be applied to B2, B7, and B11 (Figure 4 (B) and Table S2 (A)). In these cases, the His residues may influence sensitivity since peptides T4, T6, B2, and B7 (Figure 4 (D), Table S2 (B), (B), and Table S2 (A), respectively) contain His residues in their N-terminal regions. By comparison, the 6-BFA-specific peptide T5 contains four acidic amino acids and an N-terminal Glu-Asp- sequence (Figure 4 (C) and Table S2 (B)). The acidity of the N-terminus seems to be responsible for the good sensitivity of 6-BFA for this peptide. In addition, Lys is the C-terminal amino acid of T5, T9, T13 (Figure 4 (C) and Table S2 (B)), B1, B3, B5, B8 and B9 (Figure 4 (A) and Table S2 (A)). On this basis, 6-BFA is therefore thought to be superior for ionizing peptides that include Lys and His but not Arg. Peptides B4 and B6 were detected from both matrices. These results can be attributed to the fact that B4 has Arg as its C-terminal amino acid, an Asp is present in its N-terminal region, and one Glu is present in its C-terminal region; thus, this peptide can be detected from either matrix but with different ion intensities (Figure 4 (A), (B), and Table S2 (A)). B6 gives rise to a relatively intense signal, but it was difficult to detect as it possesses a C-terminal Arg residue and internal His and Lys residues; B6 is likely detectable because its Glu-Asp sequence is located far from its His and Lys residues (Figure 4 (A) and Table S2 (A)). Although T10 possesses characteristics that should make it detectable with 6-BFA (i.e., an N-terminal Glu residue in addition to three Glu, one Asp, and three Pro residues), T10 was specific to CHCA. T10 also contains two Lys, two His, and one Arg as basic amino acids; accordingly, this peptide contains five positive charges and one negative charge. The positions of the basic amino acids (i.e., two His and one Arg) in the C-terminal region, however, led to high sensitivity of CHCA for this peptide. Similarly, T11 contains both acidic amino acids (an N-terminal Asp in addition to three Asp, two Glu, and two Pro residues) and basic amino acids (a C-terminal Arg, another Arg, two Lys, and two His residues) (Figure 4 (D) and Table S2 (B)). The higher sensitivity of 6-BFA for T11 can be understood in terms of four structural features: an Asp residue in the N-terminal region, only one Arg in the C-terminal region, two Pro residues, and a *Mw* greater than 3000. Although T13 contains no acidic amino acids in its N-terminal region, its C-terminal residue is Lys, the peptide contains two Asp and two Pro residues and it has a *Mw* greater than 3000; therefore, this peptide produced high-intensity peaks from the 6-BFA matrix (Figure 4 (D) and Table S2 (B)). T12 is a special case because it is two peptides joined by Cys–Cys (S–S) bonds, and each peptide has Arg as its C-terminal residue (Figure 4 (D) and Table S2(B)). This peptide also possesses four acidic and five basic amino acids; thus, there is no overall negative charge, and the resulting peak intensity was not high. The large *Mw* and the presence of two Pro residues seem to allow the sensitive detection of this peptide from 6-BFA as the matrix, which may be

very useful for the structure analysis of proteins. B12 is also joined by one S–S bond, and this peptide has an overall negative charge because of the presence of four Asp, three Glu, two Pro, and three Lys residues. B12 seems to result in intense peaks with 6-BFA as the matrix because of its large *Mw* and acidity (Figure 4 (A) and Table S2 (A)). B14 is similar to B12, but it does not satisfy the above assumptions completely because of its very basic N-terminal region (on account of the Arg-His fragment); notably, the C-terminal residues of these two peptides are Lys and Arg (Figure 4 (A) and Table S2 (A)). Both peptides possess one Asp, four Glu, three Pro, two Arg, three Lys, and one His residue; therefore, they did not have strong overall negative charges. The analyses of synthetic and native peptides suggest that a large *Mw* is another important factor and that the effects of positive and negative charge localization may be overshadowed by the presence of a large number of peptide bonds. In addition, the presence of Pro is likely important because many peptides detectable with 6-BFA as the matrix contain Pro; thus, 6-BFA, like FA, is sensitive to $(PPG)_{10}$, as shown in Figures 1 and 3. In particular, the 6-BFA-specific peptides B13, B14, T11, and T13 contain 2–4 Pro residues (Figure 4 (A), (C), Table S2 (A) and (B), respectively).

Analyses of vitamin B_{12}

Figure 5 shows the spectra of cyanocobalamin obtained using 6-BFA (A) and CHCA (B). Because the cobalt ion of cyanocobalamin has a CN ligand that is easily removed by ionization, the MH ion was difficult to detect. It was previously reported that CHCA does not provide a 1355.5 $[M + H]^+$ ion but that ClCCA does provide a relatively intense $[M + H]^+$ ion.[24,25] No mass ion at 1218.5 was reported in these previous studies, suggesting that its presence may depend on the measurement conditions. The properties of 6-BFA may be intermediate between those of CHCA and ClCCA since the use of 6-BFA as a matrix provided a stronger $[M + H]^+$ peak relative to what was obtained with CHCA.

Crystallization of 6-BFA on a target plate

The crystals of 6-BFA and CHCA are presented in Figures 6 (A) and (B), respectively. 6-BFA generally consisted of sharp, uniform, needle-like crystals, whereas CHCA was granular. This difference seems to be reflected in the quality of the spectra;[26] indeed, crystal homogeneity is known to result in better-quality MS spectra.[27]

Proton affinity analyses

We estimated the PA values of 6-BFA and FA based on the method of Mirza et al. (Figure 7);[28] the reported PA value of FA is 879 kJ/mol. In this study, the PA values of 6-BFA and FA were calculated to be 203.9 kcal/mol (853.2 kJ/mol) and 207.2 kcal/mol (867.1

kJ/mol), respectively, and the value for 6-BFA is closest to that for DHBA (855.2 kJ/mol) and is in the range of known PA values of commonly used matrices.[29] It is possible that the good sensitivity of 6-BFA for acidic peptides and small basic peptides without Arg in either the N- or C-terminal region is caused by its reduced PA.

Lu I-C et al.[30] reported the ion/neutral ratio predicted by using the thermal proton transfer model for MALDI mechanisms under typical laser wavelengths, and the ratios of CHCA, FA, SA and DHBA were measured. The ion-to-neutral ratio of CHCA was approximately three orders of magnitude higher than the ratios of the other matrices, and the maximum surface temperature of CHCA before desorption was approximately 2 to 3 times higher than that of the other matrices. However, the difference in the Gibbs free energies of these matrices was only approximately 25%. Therefore, thermally induced proton transfer is the dominant ion generation reaction that occurs in the MALDI processes. However, CHCA and DHBA seem to be thermodynamically more stable than FA and SA in this range. 6-BFA is assumed to be as stable as DHBA because of its PA value and applicability to MALDI-QIT-TOF-MS, as described later.

Additionally, Lu I-C et al. [31] reported that the thermal proton transfer model enables prediction of the increase in total ion intensity as a function of the concentration and PA of an analyte, and a total intensity increases with the PA values. When an analyte (A) with a high PA is added into the matrix (M), the follow reaction readily occurs. $A_l + M_l \leftrightarrows (A + H)^+_l + (M - H)^-_l$ (a subscript l indicates liquid phase).

It is thought that this reaction is most likely to occur, but it may be difficult to rationalize the unique ionizing power of 6-BFA (i.e., the influence of acidic amino acid residues in the vicinity of the N-terminal and the enhanced ionization of Pro-containing peptides) based on this platform because many of the peptides sensitive to 6-BFA have low theoretical PA values. The relationship between the analyte PA and the sensitivity of 6-BFA seems to deviate from what is typically seen with MALDI-TOF-MS matrices.

Relationship between peptide concentration and ionization intensity

We next used an internal standard method to prepare a quantitative standard curve to determine the detection limit and the ideal sample/matrix ratio (Figure 8),[32] as described in the Methods section. SUB-P was used as the analyte, and aSUB-P was used as the reference. The N-terminal amino acid of both peptides is Arg, and 6-BFA is therefore less sensitive than CHCA for these peptides. However, the results obtained using 6-BFA (Figure 8 (A)) and CHCA (Figure 8 (B)) do not differ significantly and show relatively good linearity and correlation coefficients. These results suggest that no remarkable damping of the sample peak will occur even for small sample-to-matrix ratios; thus, the described method of preparing the 6-BFA solution for use as a matrix is appropriate. In addition, 6-BFA resulted in less

scattering than CHCA (Figure 8), probably because the sample molecules were distributed more uniformly in the 6-BFA matrix. Indeed, the CHCA matrix exhibits localized ionization sites, whereas the 6-BFA matrix provides uniform peak intensities throughout the target plate. The S/N ratio for 6-BFA at low concentrations is superior to that of CHCA, which was also evident in the experiments using UTS-Π (reference) and TTP (analyte) in which Arg was not present or only one Arg was present in the middle of the peptide (Figure S3 for the standard curve of UTS-II versus TTP).

Using 6-BFA for MS/MS analysis

Because 6-BFA was suggested to be a cold matrix, it was used for de novo sequencing by MALDI-QIT-TOF-MS, and the results were compared to those obtained with DHBA as the matrix. CHCA and ClCCA are inappropriate matrices for MALDI-QIT-TOF-MS (data not shown), and cold DHBA is currently the best matrix for MS/MS analysis using QIT. Therefore, the QIT system is already well suited for using DHBA, and no parameter changes were needed. Typical data obtained in the MS/MS analyses of the synthetic peptide ACTH are shown in Figures 9 (A) and (B). The CID values were automatically increased stepwise from 50 to 300, and spectra were collected at each step; the raster is shown in Figure 9 (C). Figures 9 (A) and (B) show the spectra obtained using 6-BFA and DHBA, respectively, with automatic analyses. 6-BFA may provide lower-quality data for peptides containing an N- or C-terminal Arg residue, but the results shown in Figures 9 (A) and (B) are generally comparable. This result also indicates that 6-BFA is suitable for use in sequencing by MALDI-QIT-TOF-MS and suggests once more that like DHBA, 6-BFA is a cold matrix.[33] In addition, because the PA of 6-BFA is similar to that of DHBA, as mentioned above, 6-BFA could be used for this MS2 analysis (as DHBA). The ACTH spectrum obtained using 6-BFA was identified in a Mascot search as that of a peptide belonging to 'Other (include plasmid and artificial sequence) Taxonomy'. The same analyses were performed for the commercial peptides PAMP, $(PPG)_{10}$, TTP, and UTS-II, and 6-BFA provided satisfactory results (data not shown).

Next, 6-BFA was applied to MS/MS analyses of trypsin-digested Tf; the results are shown in Figures 9 (D), (E), and (F). The data show two characteristic peptide ions, T5 and T7 (Figure 4). The MS/MS spectrum of T5 and the Mascot search results are shown in Figures 4 (C) and (D) and Table S2 (B). Table S2 (B) shows the fragment ions for predicting the amino acid sequence, which covered the entire sequence; based on these fragments, T5 was selected as the only candidate (*EDLIWELLNQAQEHFGK*). Peptide T7 was not recognized as a tryptic peptide of Tf in the Mascot database search. We therefore conducted MS/MS analysis of this peptide using 6-BFA (Figure 9 (F)). The results suggest that peptide T5 was converted to T7

by elimination of the N-terminal Lys residue, which may have been caused by incomplete (~50%) cleavage of the C- and N-terminal Lys residues by trypsin (-*GG**K**ED*-). Importantly, the presence of this Lys influences the sensitivity of the peptide ionization by 6-BFA or CHCA, which is consistent with the finding that a basic amino acid in the N- or C-terminal region decreases the sensitivity of the detection of the peptide ionization by 6-BFA.

Conclusions

To date, no investigations of the use of halogenated FA derivatives as MALDI matrices have been reported. Highly pure 6-BFA was conveniently synthesized and evaluated as an effective and practical MALDI matrix. The results show that the optimal matrix depends on the properties of the analyte. 6-BFA provides good results for acidic peptides containing an acidic amino acid in the N- and/or C-terminal regions and for large peptides, and it is applicable for de novo sequencing by MALDI-QIT-TOF-MS. By comparison, 6-BFA shows low sensitivity for basic peptides containing a basic amino acid in the N- or C-terminal regions and for small peptides. Notably, 6-BFA shows high sensitivity to proline-rich peptides, a characteristic also seen in unsubstituted FA, and this allows the detection of peptides containing proline residues. 6-BFA is suggested to be a cold matrix that is suitable for proteome analysis, but its application in peptide mass fingerprinting analysis requires further study. The ionization efficiency is affected by the matrix purity, and the commonly used matrix CHCA exhibits good efficiency in this regard. Overall, 6-BFA will contribute to proteomic analysis in peptide mass fingerprinting methods in the near future.

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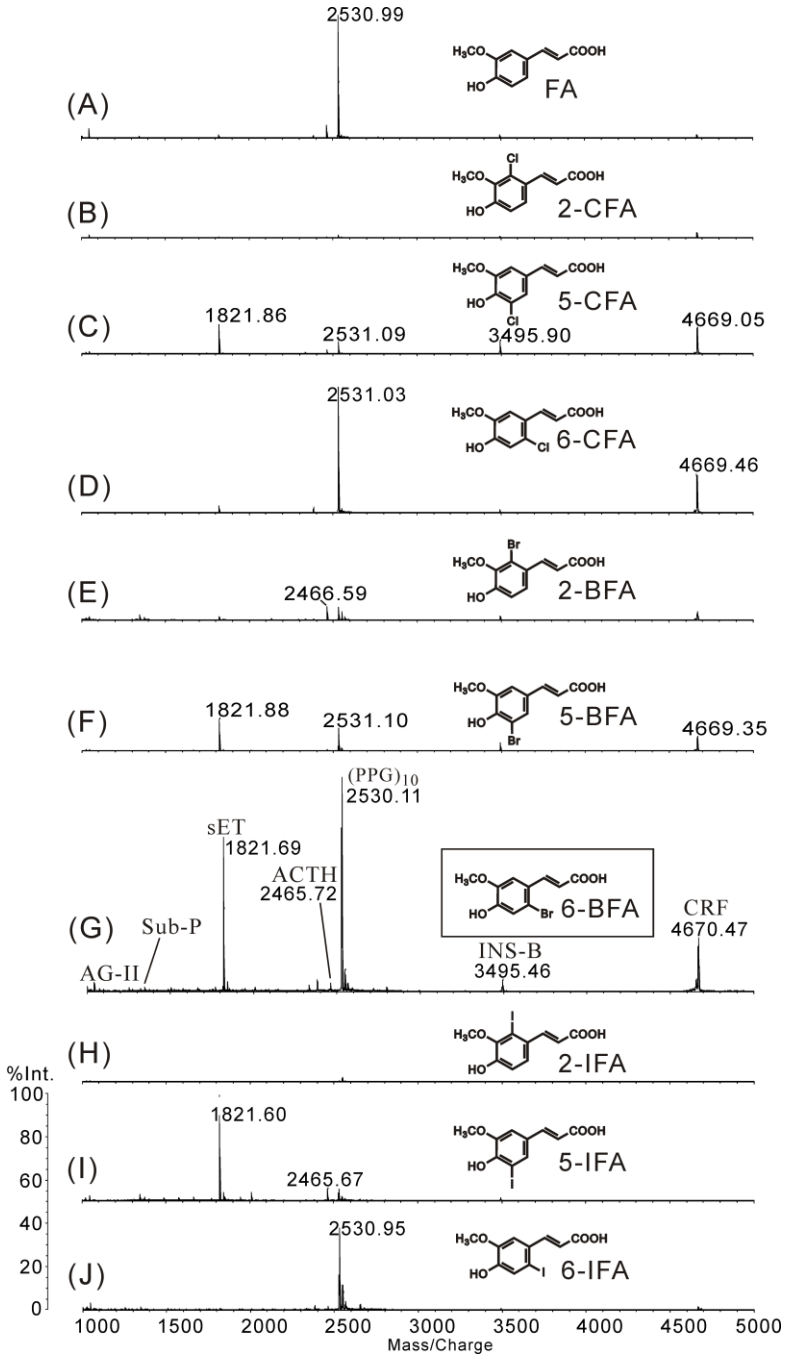
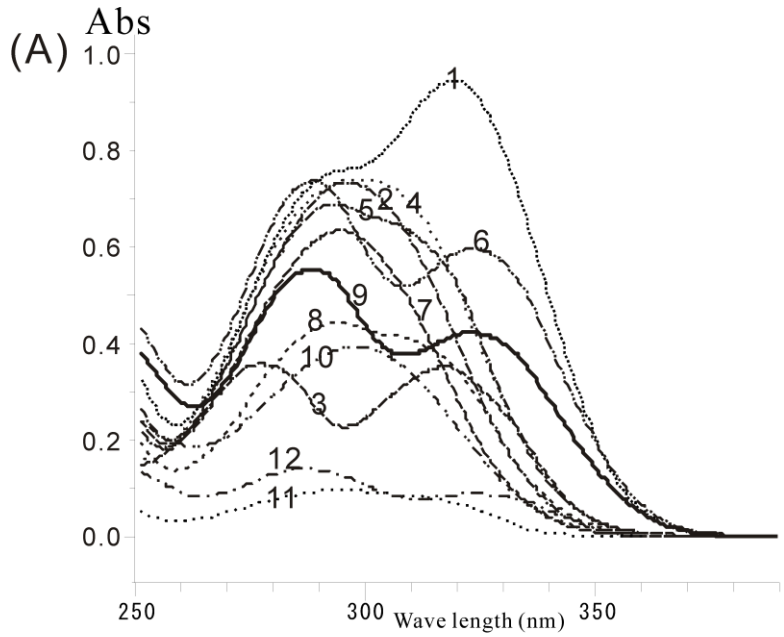


Figure 1

MALDI-TOF-MS spectra of the peptide mixture recorded using (A) FA; (B–D) 2-, 5-, and 6-chloro FA, respectively; (E–G) 2-, 5-, and 6-bromo FA, respectively; and (H–J) 2-, 5-, and 6-iodo FA, respectively.

All of the spectra were normalized to lane (G), and the structure of 6-BFA is shown in a square in this lane. FA and eight halogenated derivatives were tested as MALDI matrices, and eight peptides were used as analytes (see Figure 3 (D), except pt-AGII). (G) 6-BFA exhibited satisfactory sensitivity for the detection of sET, $(PPG)_{10}$, INS, and CRF.

(A)



(B)

	Mw	Conc. [mmol]	Abs. 337 nm	Molar Absorptivity ε[mol^{-1}· L· cm^{-1}]	Charge values (Huchel)* O (C=O)	O (-OH)
1: FA	194.18	25.75	0.593	23030	-0.704029	-0.209849
2: 5-FFA	212.17	23.57	0.157	6662	-0.705225	-0.203220
3: 6-FFA	212.17	23.57	0.166	7044	-0.718871	-0.209453
4: 2-CFA	228.63	21.87	0.097	4435	-0.714424	-0.208079
5: 5-CFA	228.63	21.87	0.153	6996	-0.704568	-0.202900
6: 6-CFA	228.63	21.87	0.466	21308	-0.718681	-0.209030
7: 2-BFA	273.08	18.31	0.058	3168	-0.712414	-0.206837
8: 5-BFA	273.08	18.31	0.103	5625	-0.704268	-0.202760
9: 6-BFA	**273.08**	**18.31**	**0.326**	**17805**	**-0.724069**	**-0.240268**
10: 2-IFA	320.08	15.62	0.059	3777	-0.710101	-0.205572
11: 5-IFA	320.08	15.62	0.014	896	-0.704110	-0.202728
12: 6-IFA	320.08	15.62	0.067	4289	−0.712212	-0.206014

*: The MM2 calculation for the structure optimization was performed by the extended Huckel method by using Chem3D (Ver. 13.0).

Figure 2

Absorption spectra of FA and halogenated FA derivatives (A) and charges on the oxygen atoms in the COOH groups of these materials (tabulated in (B)).

The spectra were recorded in methanol at a concentration of 5 μg/mL (U2190, Hitachi High-Technologies Co., Tokyo Japan).

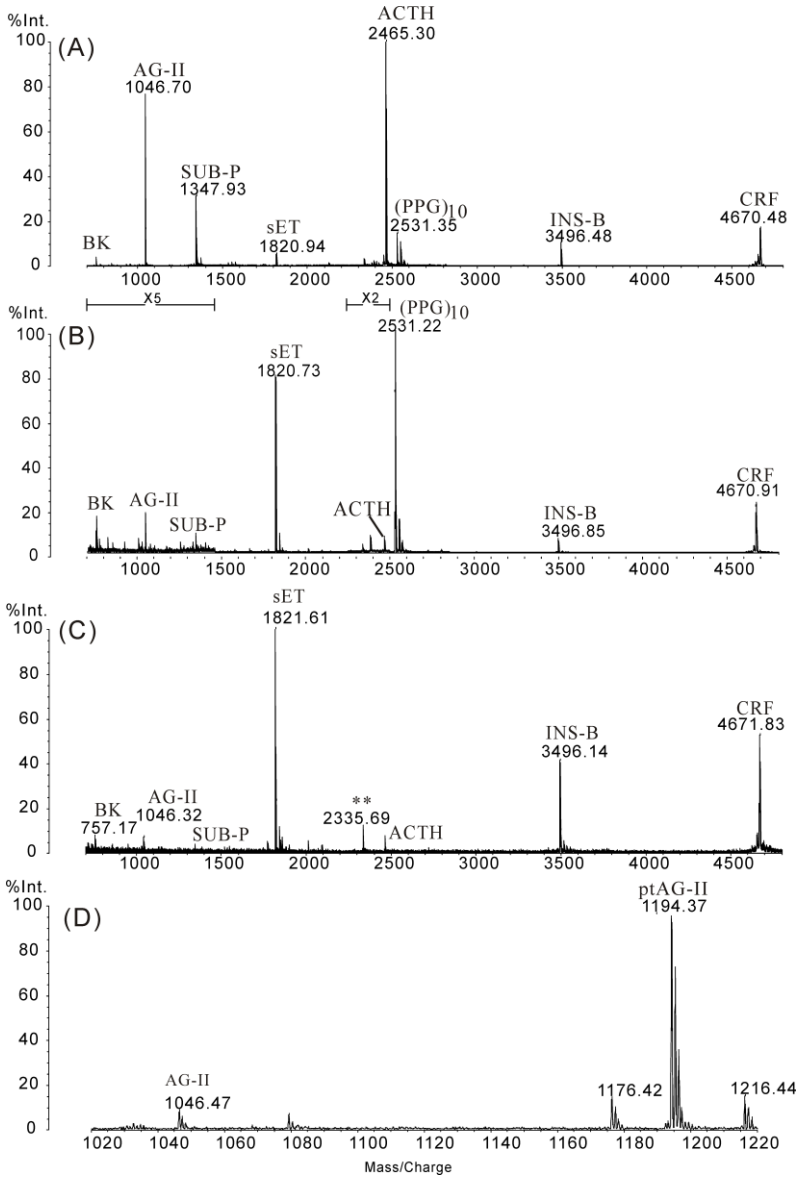


Figure 3

MALDI-TOF-MS spectra of the peptide mixture obtained using CHCA and 6-BFA. (A) and (B) show the ion intensities of the peptides obtained with 6-BFA (B, C, and D) and CHCA (A) as the matrices. The amino acid sequences, *Mw*, and *pI* values of each peptide are listed in Table S1. 6-BFA (B) exhibited strong sensitivity to sET and $(PPG)_{10}$. CHCA (A) exhibited strong sensitivity to AG-II, SUB-P, and ACTH. (C) shows the results obtained when $(PPG)_{10}$ was removed from the peptide mixture used in (A) and (B). The sensitivity of the matrices to BK, AG-II, SUB-P, and ACTH were recovered, and 6-BFA, but not CHCA, exhibited good sensitivity for INS-B and CRF (A). *m*/*z* 2335.69 (**) seems to be the dianion of CRF in (C). Synthesized ptAG-II with the addition of a carboxyl group clearly gave rise to more intense peaks than native AG-II (D). These results suggest that in this case, the improvement in sensitivity is related to the acidity of the N-terminal region.

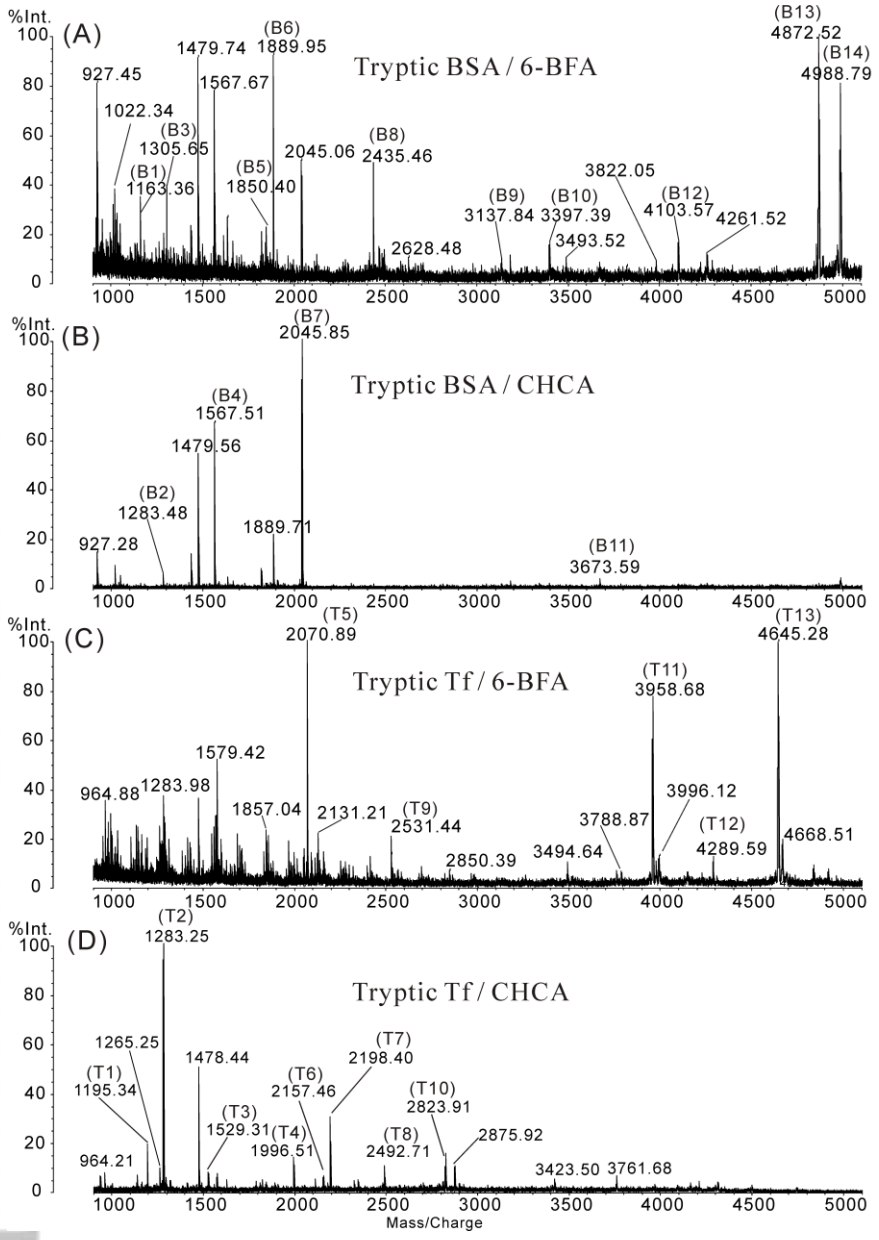


Figure 4

MALDI-TOF-MS spectra of tryptic BSA (A and B) and Tf (C and D) peptides obtained using 6-BFA (A and C) and CHCA (B and D). The amino acid sequences of the identified peptides and positions in each protein are also shown (Table S2 (A and B)).

The identification was performed based on searching the Mascot database. 6-BFA (A and C) provided high-intensity peaks for large peptides with *Mw* values greater than 3000. The peaks marked with B1–14 (Table S2 (A)) and T1–13 (Table S2 (B)) in each spectrum has been attributed to specific peptides. The numbers in square brackets indicate the position of the peptides in these proteins and are based on the amino acid sequence information obtained by searching the Mascot database.

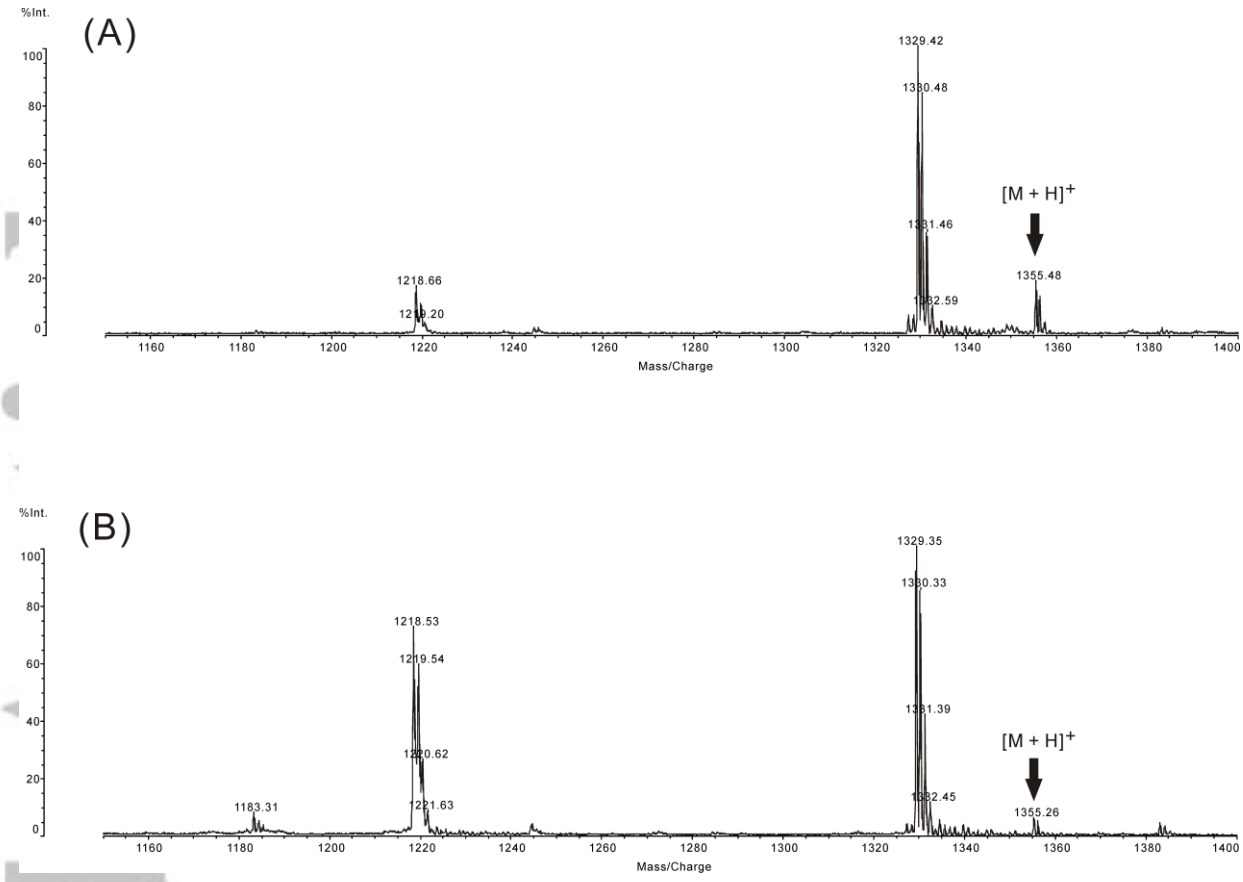


Figure 5

MALDI-TOF-MS spectra of cyanocobalamin with 6-BFA (A) and CHCA (B) as the matrices. 6-BFA afforded a relatively strong $[M + H]^+$ ion peak, as shown by the arrows, whereas when CHCA is used as the matrix, these signals were very weak.

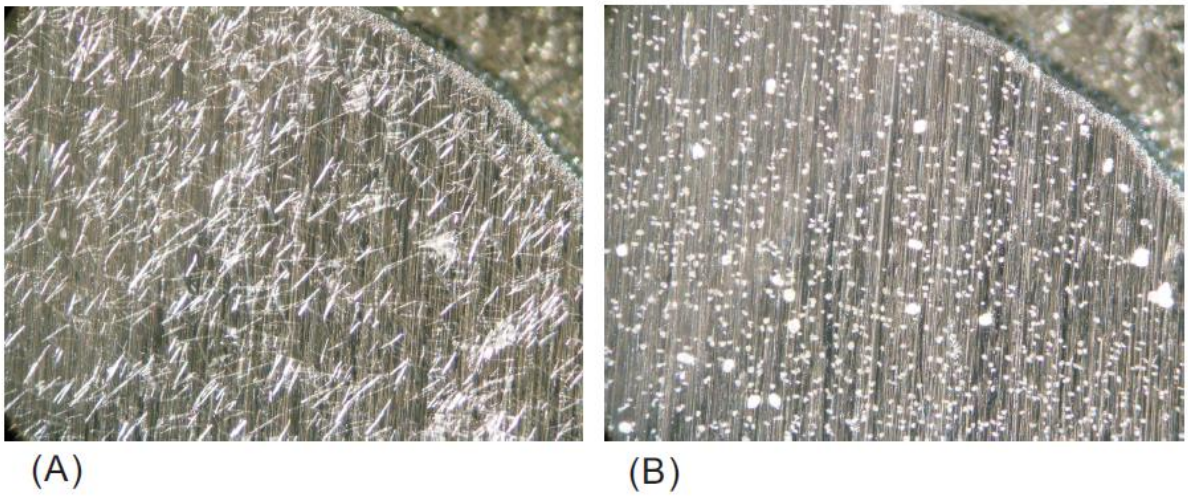


Figure 6

(A) and (B) show the crystals of 6-BFA (sharp, needle-like crystals) and CHCA (granular crystals), respectively, on a target plate.

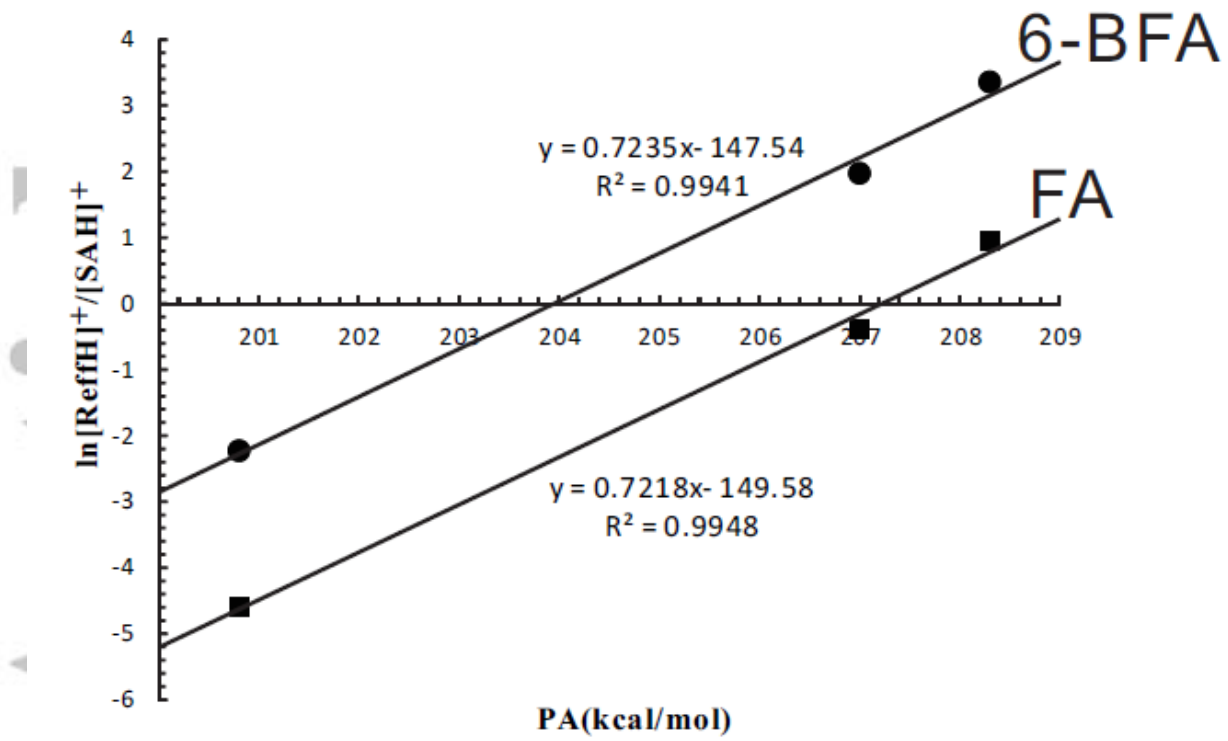


Figure 7

A logarithm plot of the product ion ratios for FA and 6-BFA based on the method of Mirza et al.[25]

The collision energies were 5, 6, 7, 8, 9, and 10 eV. γ-Butyrolactone, 4-fluoroaniline, and 4-nitroaniline were chosen as the reference bases (A). The graphs in (B) and (C) were constructed to determine the detection limit and optimal sample/matrix ratios.

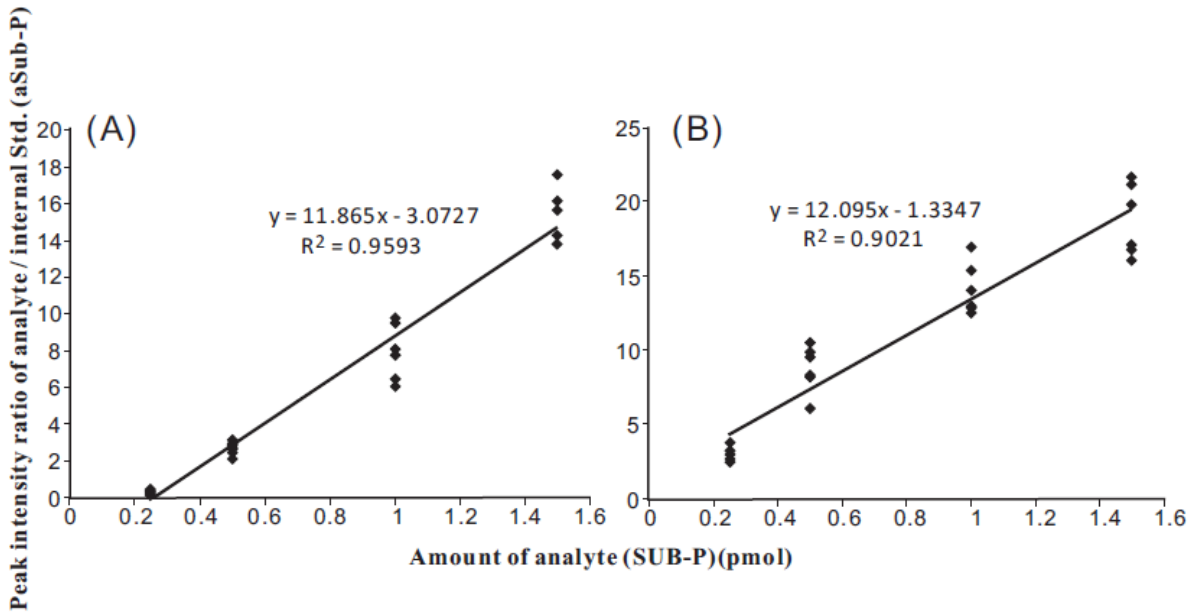
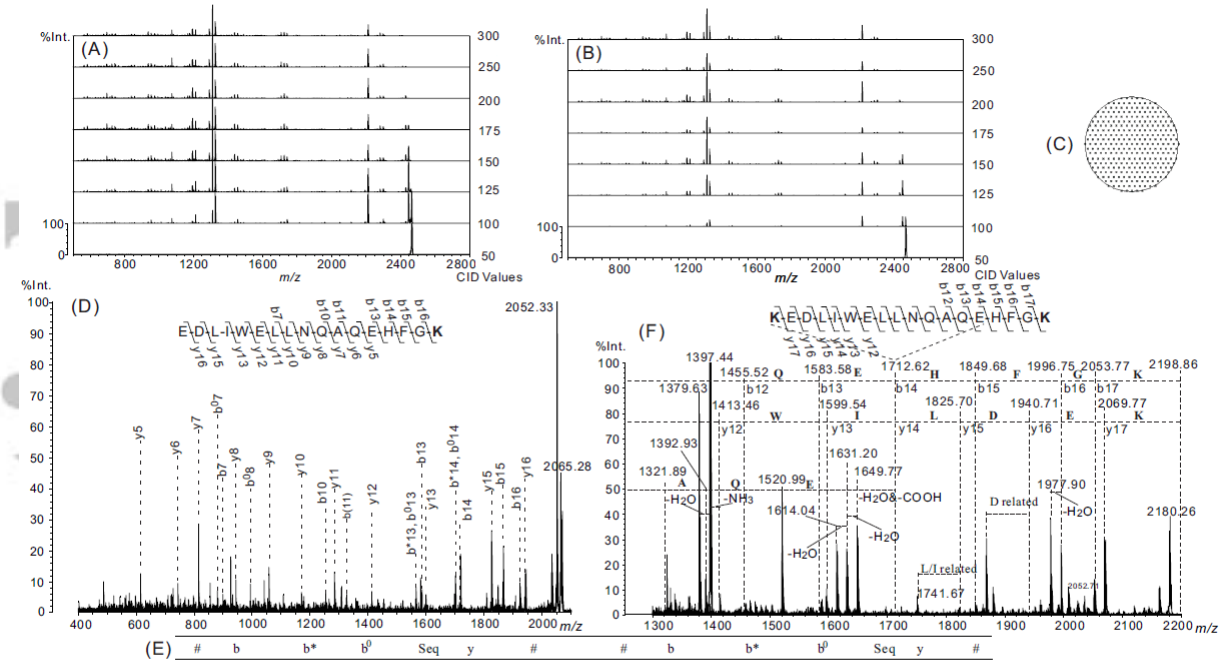


Figure 8

Quantitative plots similar to the standard curves of 6-BFA (A) and CHCA (B) generated using an internal standard.

SUB-P (*RPKPQQFFGLM*) was used as the analyte, and aSUB-P (*dRdPKPQQdWFdWGL*) was used as the reference. The slope in the curve for 6-BFA is smaller than that for CHCA because 6-BFA is not sensitive to peptides with an N-terminal Arg. However, 6-BFA provides better linearity than CHCA. The changes in the mass spectra as a function of analyte concentration are shown in Figure S1 for 6-BFA and S2 for CHCA with their S/N values.



(E)

#	b	b*	b^0	Seq	y	#	#	b	b*	b^0	Seq	y	#
1	130.1499		112.0393	E		17	9	1126.5779	1109.5514	1108.5673	N	***1058.5014***	9
2	245.0768		227.0662	D	***1940.9865***	16	10	***1254.6365***	1237.6099	1236.6259	Q	***944.4585***	8
3	358.1609		340.1503	L	***1825.9595***	15	11	***1325.6736***	***1308.6470***	***1307.6630***	A	***816.3999***	7
4	471.2499		453.2344	I	1712.8755	14	12	1453.7322	1436.7056	1435.7216	Q	***745.3628***	6
5	657.3243		639.3137	W	***1599.7914***	13	13	***1582.7748***	***1565.7482***	***1564.7642***	E	***617.3042***	5
6	786.3668		768.3563	E	***1413.7121***	12	14	***1719.8337***	***1702.8071***	***1701.8231***	H	488.2616	4
7	***899.4509***		***881.4403***	L	***1284.6695***	11	15	***1866.9021***	1849.8755	1848.8915	F	351.2027	3
8	1012.5350		***994.5244***	L	***1171.5854***	10	16	***1923.9236***	1906.8970	1905.9130	G	204.1343	2
							17				K	147.1128	1

Bold italic under line numbers are matches ions

Figure 9

The phased MS/MS spectra of ACTH with 6-BFA (A) or DHBA (B) and the MS/MS spectra of peptides T5 (D) and T7 (F) shown in Table S2.

In (A) and (B), the CID values obtained in the MS/MS analyses changed by 8 fold, and these spectra were normalized at CID 300. A total of 500 laser shots were performed at specific irradiation locations, and constant measurement conditions were maintained by programmed automatic analysis using a raster, as shown in (C). The fragmentation of ACTH when using 6-BFA started at a lower CID value, and the set range of CID values was wider than that for DHBA. The sensitivity of 6-BFA for ACTH was not strong, but the MS/MS spectra were equivalent to those obtained with DHBA.

In (D) and (F), the MS/MS spectra of the T5 peptide with *m/z* 2070.89 are shown in Table S2, and the *m/z* assignments were based on the Mascot search results. The partial MS/MS spectrum of the T7 peptide with *m/z* 2198.40 is shown in Figure 4 (D). The T5 peptide was 6-BFA specific, as shown in Figure 4 (C). This peptide was acidic and contained Glu and Asp residues in the N-terminal region. The matched MS/MS fragment ions are listed in (E). The results suggested that the *m/z* 2198.40 ion was converted to the *m/z* 2070.89 ion by elimination of Lys from the N-terminal region.