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Improvement in the Detection of Low Concentration Protein Digests on a MALDI TOF/TOF Workstation by Reducing α-Cyano-4-hydroxycinnamic Acid Adduct Ions

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Alpha-cyano-4-hydroxycinnamic acid (α-CHCA) as a matrix facilitates the ionization of proteins and peptides in a matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometer. The matrix itself also ionizes and so do its sodium and potassium adducts. Matrix clusters and metal ion adducts interfere with peptide ionization and peptide mass spectrum interpretation. These matrix adducts are significantly reduced with addition of ammonium monobasic phosphate or ammonium dibasic citrate to the matrix and sample deposited onto the MALDI target. The reduction of matrix adducts results in the increase of peptide intensity and signal-to-noise ratio as well as in improvement of peptide ionization for samples deposited onto the target at levels of 10 fmol or below. These improvements were particularly significant in the detection of peptides at amol levels when reduced amounts of matrix were also used.

KEY WORDS: Matrix clusters, cluster reduction, MALDI sensitivity, ammonium phosphate, ammonium citrate.

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Alpha-cyano-4-hydroxycinnamic acid (α-CHCA) has been widely used as matrix to facilitate the ionization of proteins and peptides in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).[1,2] However, sodium and potassium ions induce formation of α-CHCA adducts. These elements are present in solvents and buffers, and are extracted from many plastics that are used for sample preparation and storage. Matrix adducts are ubiquitous in MALDI-TOF mass spectra, and are particularly evident at low sample concentrations. Thus, detection of low abundance, low mass analytes often becomes problematic. To remedy this situation matrix additives[3] can be used to scavenge or exchange metal ions. In this work, reduced matrix adducts were observed in mass spectra by addition of ammonium monobasic phosphate ($NH_4H_2PO_4$) or ammonium dibasic citrate [$(NH_4)_2C_6H_6O_7$] to the matrix/sample.* Another observed benefit from the addition of ammonium monobasic phosphate to samples was an increase in the intensity and the signal-to-noise ratio of peptide peaks in MALDI-TOF mass spectra.

MATERIALS AND METHODS

Bovine serum albumin (BSA) and *Escherichia coli (E.coli)* β-galactosidase (both from Sigma Chemical Company, Milwaukee, WI) were digested with bovine trypsin (Sigma). These samples were diluted with 50:50 water–acetonitrile with 0.1% trifluoroacetic acid (TFA) to a series of concentrations from 0.2 fmol/mL to 1 pmol/mL. Solutions of various concentrations of ammonium monobasic phosphate and ammonium dibasic citrate (Sigma) were prepared by dissolving each salt in deionized water. A 5-mg/mL α-CHCA MALDI matrix was prepared by dissolving recrystallized α-CHCA (Sigma) in 50:50 water–acetonitrile with 0.1% TFA which contained 0–50 mM ammonium phosphate or 0–50 mM ammonium citrate. The MALDI samples were prepared by mixing one portion of protein digest sample with

*Ammonium dibasic citrate has been used as an additive for the analysis of phosphopeptides by MALDI-TOF-MS.[4]

nine portions of α-CHCA solution before spotting on the MALDI plate. A 2-mg/mL α-CHCA matrix was prepared in the same way as the 5-mg/mL α-CHCA matrix, which contained 10 mM ammonium phosphate.

MS and tandem mass spectrometry (MS/MS) data were acquired with the Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF optics.[5] For MS/MS spectra, the collision energy was 1 keV and the collision gas was air. The interpretation of both the MS and MS/MS data was carried out using the GPS Explorer software (Applied Biosystems, Framingham, MA).

RESULTS AND DISCUSSION

The α-CHCA matrix adducts are typically most abundant in the range of *m/z* 800–1100 of a MALDI mass spectrum, which is part of the mass range used for protein identification by both peptide mass fingerprinting (PMF) and MS/MS peptide sequencing. These MALDI matrix adduct signals are generally not dominant in relatively concentrated samples (e.g., 1 pmol deposited onto the MALDI target). However, they become quite dominant at analyte concentrations in the low femtomole levels or below. This results in complicated mass spectra and in difficulty with data interpretation, especially when the latter must be carried out in automated mode. An example of this effect was in the MALDI mass spectrum (Fig. 1A) of 500 amol *E. coli* β-galactosidase digest prepared in the standard manner by mixing with α-CHCA. The matrix adduct ions were reduced substantially with the addition of ammonium phosphate to the matrix, as shown in Figure 1B. In the latter spectrum several β-galactosidase peptides were observed that had not been detected in the former spectrum, which suggests that the addition of ammonium phosphate facilitated peptide ionization. These mass spectra were interpreted with GPS Explorer software using the MASCOT search engine for protein identification by PMF. The MASCOT score from the sample with ammonium phosphate was higher than that from the sample without ammonium phosphate (Fig. 2), thus increasing the confidence in the correct identification of the proteins.

The matrix adducts in the range of *m/z* 800–1100 are apparently formed from α-CHCA tetramers or pentamers, sodium and potassium salts, and hydrates, the likely compositions of which and corresponding *m/z* values are listed in Table 1.* One possible explanation for the observed reduction of matrix adducts by ammonium phosphate is that these adducts are dissociated upon addition of this salt to the matrix–sample solution. This is illustrated by comparing the signal-to-noise ratios for the ions of two matrix adducts and two β-galactosidase tryptic peptides as a function of the ammonium phosphate concentration, as shown in Figure 3A. Ammonium dibasic citrate has a similar effect to that of ammonium monobasic phosphate, though ammonium phosphate can be used over a broader concentration range (1–50 mM) than ammonium citrate (0.5–10 mM) for the reduction of matrix adducts. However, the matrix adducts were not completely removed at ammonium citrate concentrations of 2 mM or lower, whereas peptide signals were dramatically decreased with the addition of ammonium citrate at concentrations of 5 mM or higher. The decrease of peptide signals, paradoxically, may be due to the lower solubility of ammonium monobasic phosphate in water compared with ammonium dibasic citrate. Thus, ammonium monobasic phosphate crystallizes and precipitates with the matrix–sample solution, but at concentrations of 20 mM or higher, a significant portion of ammonium dibasic citrate precipitates on top of matrix–sample crystals as the solvent evaporates. This supposition was confirmed by visual inspection of the samples on MALDI plates. Overall, ammonium citrate addition causes reduced peptide intensities and lower signal-to-noise ratios at concentrations of 5 mM or higher (Fig. 3B). By contrast, peptide signal-to-noise ratios increased 40–70% along with absolute peptide ion intensities with addition of 1–20 mM ammonium monobasic phosphate (Fig. 3B). Even though the peptide signal-to-noise ratio increased with addition of ammonium citrate at 0.5–2 mM concentration, the matrix adducts were not reduced dramatically.

Matrix adduct-related fragment ions are observed in a peptide MS/MS spectrum (Fig. 4A) if the matrix adduct ion *m/z* is close to the peptide ion *m/z* (Fig. 4B), which complicates the spectrum interpretation. However, this MS/MS spectrum interference is reduced with the addition of ammonium phosphate to the matrix–sample mixture (Fig. 5A), because the matrix adduct ion abundance is significantly reduced in the MS spectrum (Fig. 5B). This, in turn, improves the confidence in the sequence derived from the peptide MS/MS spectrum, whether the spectrum is interpreted de novo or used for database searching.

For very low concentration sample (e.g., 200 amol deposited on the MALDI target), along with ammonium phosphate addition, reduced matrix concentration further improves peptide ionization and peptide fragmentation. Three more peptides from a 200 amol BSA trypsin digest were observed with 2 mg/mL α-

*It is worth noting that the fractional mass of the matrix adducts is generally smaller than that of a typical isobaric peptide. This mass deficiency is due to the high oxygen content of these adducts.

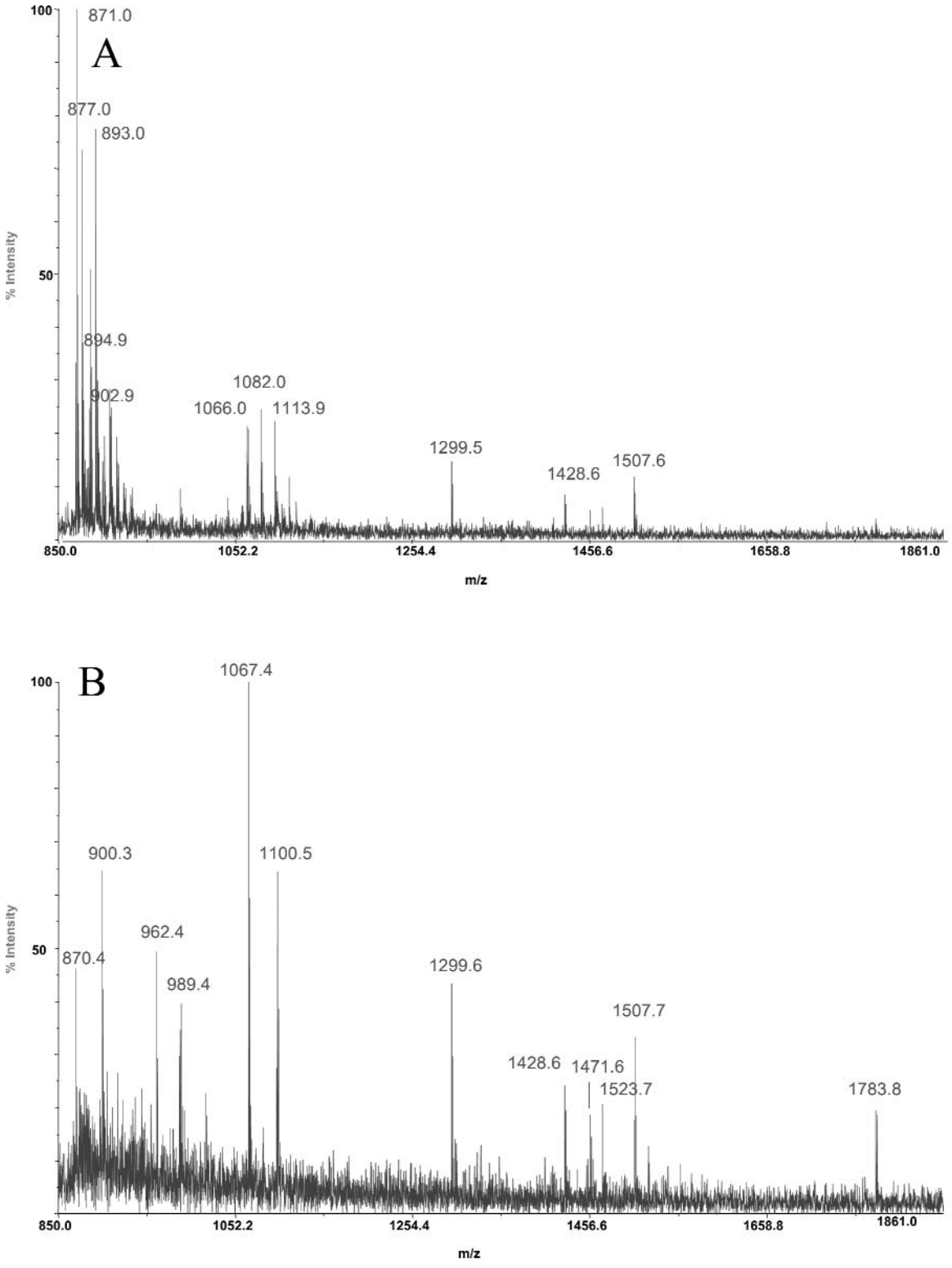

FIGURE 1

MALDI-TOF mass spectra of of 500 amol *E. coli* β-galactosidase digest in α-CHCA only **(A)** and with the addition of 10 mM ammonium monobasic phosphate to the sample–matrix mixture **(B).** Note the significant reduction or complete elimination of matrix cluster adducts in the range of up to *m*/*z* ~1200, and the noticeable improvement in the overall peptide ion signal strength.

A

Rank	Protein Name	Accession No.	Protein Score	Protein C. I. %
4	(P00722) BETA-GALACTOSIDASE (EC 3.2.1.23)	BGAL_ECOLI	12	0

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence
1067.4905	1067.4352	-0.0553	-52	158	166	WVGYGQDSR
1299.6228	1299.5565	-0.0663	-51	943	952	ELNYGPHQWR
1507.6958	1507.6121	-0.0837	-56	962	973	YSQQQLMETSHR

Rank	Protein Name	Accession No.	Protein Score	Protein C. I. %
1	(P00722) BETA-GALACTOSIDASE (EC 3.2.1.23)	BGAL_ECOLI	56	98.863

Peptide Information

B

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence
870.4257	870.4077	-0.018	-21	552	557	YWQAFR
962.4802	962.4509	-0.0293	-30	381	388	QNNFNAVR
1067.4905	1067.4567	-0.0338	-32	158	166	WVGYGQDSR
1100.5807	1100.5428	-0.0379	-34	44	52	TDRPSQQLR
1299.6228	1299.5883	-0.0345	-27	943	952	ELNYGPHQWR
1428.6865	1428.651	-0.0355	-25	15	26	DWENPGVTQLNR
1507.6958	1507.6609	-0.0349	-23	962	973	YSQQQLMETSHR

FIGURE 2

Mascot results for protein identification by peptide mass fingerprinting of a β-galactosidase tryptic digest analyzed by MALDI-TOF/TOF-MS without the addition of ammonium monobasic phosphate **(A)** and with addition of the ammonium monobasic phosphate **(B).**

TABLE 1

Possible Compositions of Matrix Adduct Ions

Adduct nominal *m/z*	Composition	Adduct calculated monoisotopic (^{12}C) *m/z*
833	$[A_2(A\text{-}H)_2K_2]H^+$	833.0900
839	[A(A-H)$_3$Na2K]H+	839.0981
841	$[A(A\text{-}H)_3Na_3(H2O)]H^+$	841.1347
855	$[A(A\text{-}H)_3NaK_2]H^+$	855.0720
861	$[(A\text{-}H)_4Na_3K]H^+$	861.0800
865	$[A_4(H_2O)_6]H^+$	865.2418
871	$[A(A\text{-}H)_3K_3]H^+$	871.0460
877	$[(A\text{-}H)_4Na_2K_2]H^+$	877.0540
887	$[A_2(A\text{-}H)_2K_2(H_2O)_3]H^+$	887.1218
893	$[(A\text{-}H)_4NaK_3]H^+$	893.0279
897	$[(A\text{-}H)_4Na_3K(H_2O)_2]H^+$	897.1012
901	$[(A\text{-}H)_4Na_3K(H_2O)]Na^+$	901.0726
903	$[(A\text{-}H)_4Na_4\ (H_2O)_2]Na^+$	903.1092
911	$[(A\text{-}H)_4NaK_3\ (H_2O)]H^+$	911.0385
917	$[(A\text{-}H)_4Na_2K_2\ (H_2O)]Na+$	917.0465
919	$[(A\text{-}H)_4Na_3K(H_2O)_2]Na+$	919.0831
1066	$[A(A\text{-}H)_4Na_2K_2]H^+$	1066.0966
1082	$[A(A\text{-}H)_4NaK_3]H^+$	1082.0705
1106	$[(A\text{-}H)_5Na_3K_2(H_2O)]H^+$	1106.0891

A, α-cyano-4-hydroxycinnamic acid neutral molecule ($C_{10}H_7NO_3$).

A-H, deprotonated α-cyano-4-hydroxycinnamic acid neutral molecule ($C_{10}H_6NO_3$).

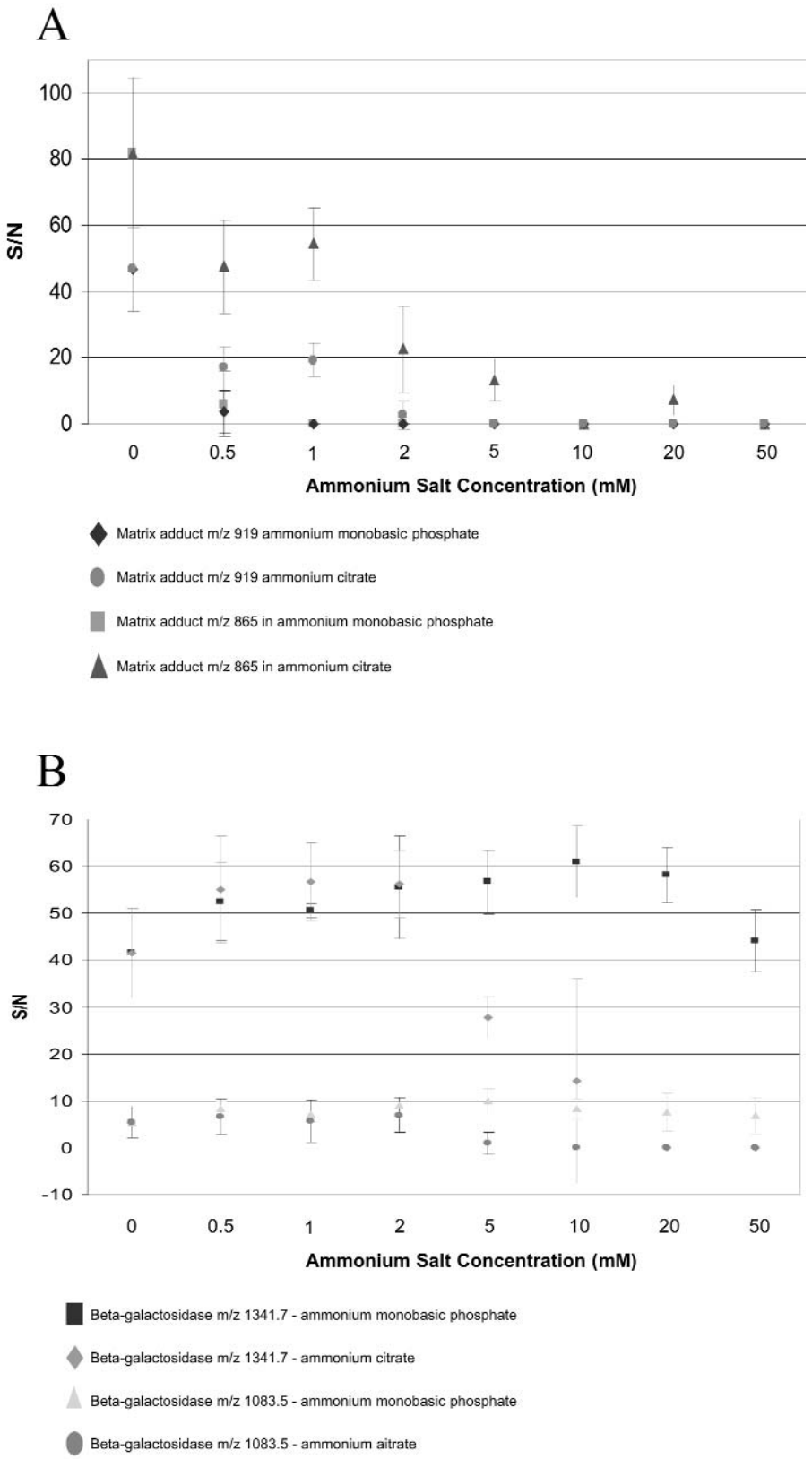

FIGURE 3

Suppression of matrix adducts **(A)** and enhancement of peptide signals **(B)** as a function of concentration of ammonium salts added to the matrix–sample mixture. Each data point represents six replicate analyses.

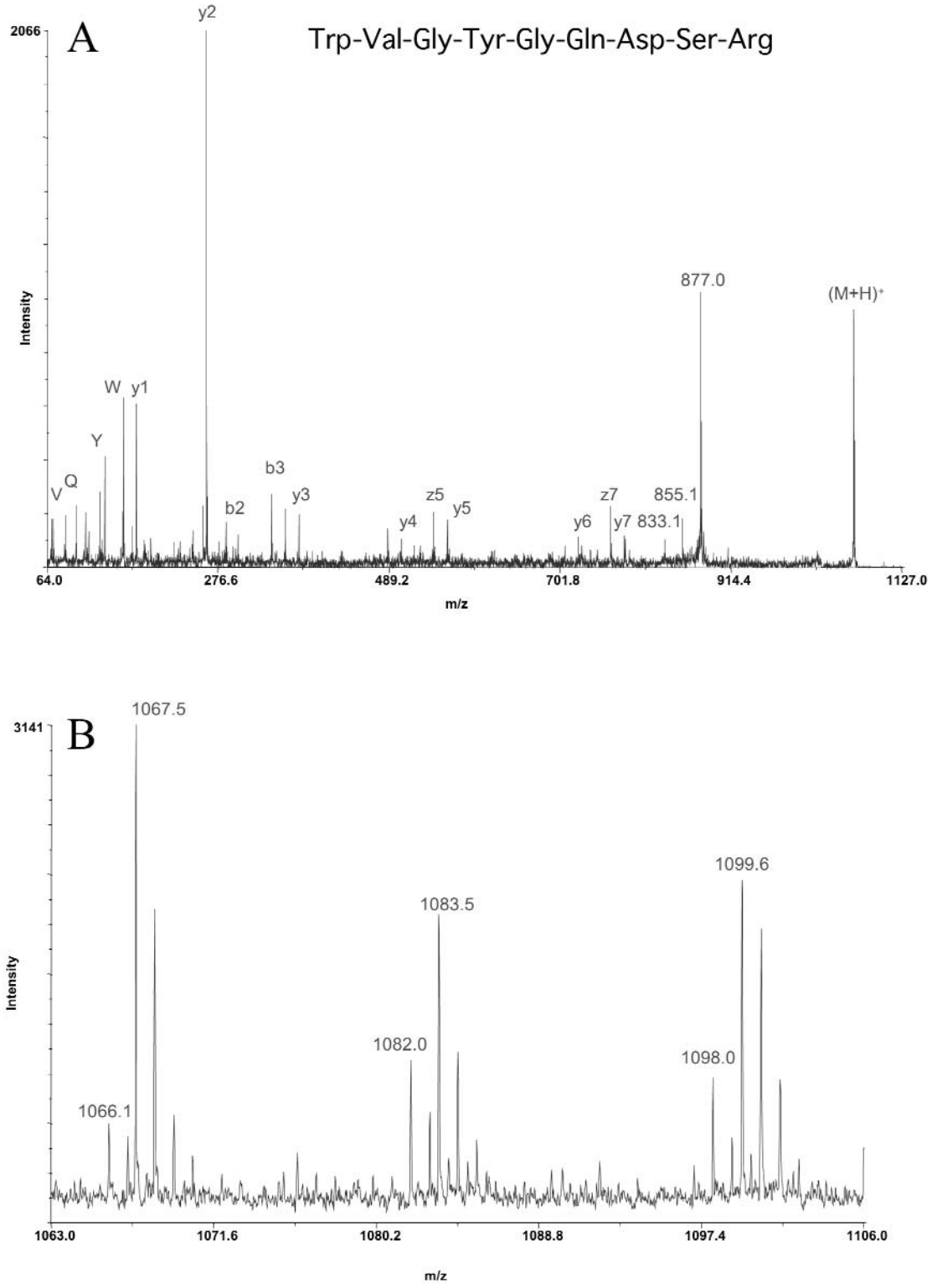

FIGURE 4

MS/MS spectrum of peptide WVGYGQDSR [$(M+H)^+$ *m/z* 1067.5] **(A)**, obtained by digestion of β-galactosidase with trypsin. Note the abundant ions at *m/z* 833.1, 855.1, and 877.0, all of which are fragments from the matrix adduct ion of *m/z* 1066.1 which is very close in mass to the peptide precursor ion **(B).**

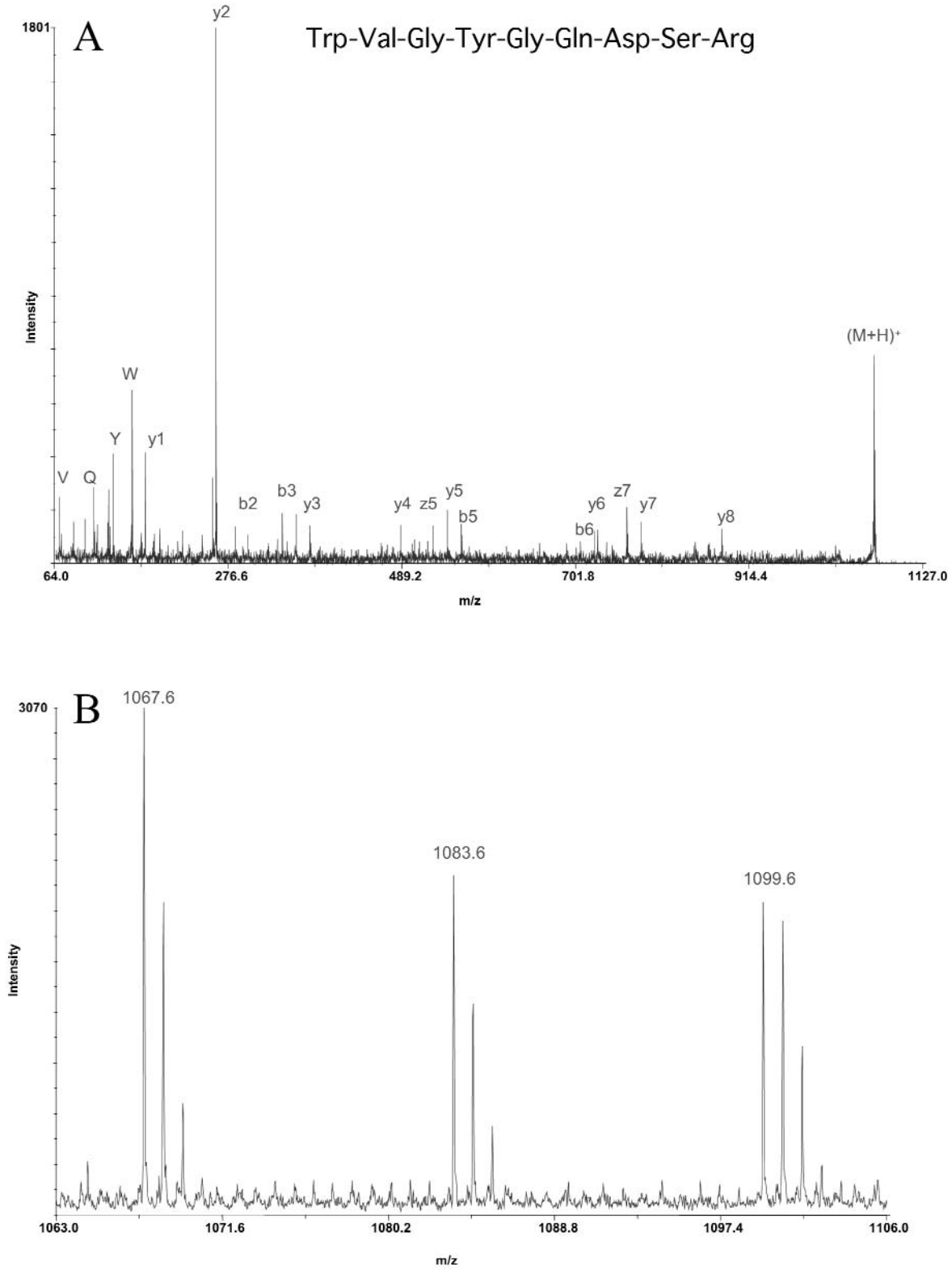

FIGURE 5

MS/MS spectrum of the β-galactosidase peptide WVGYGQDSR [$(M+H)^+$ *m/z* 1067.5] **(A).** In this case ammonium monobasic phosphate had been added to the matrix–sample mixture, thus practically eliminating the matrix adduction ions in the mass spectrum **(B).** The MS/MS spectrum no longer contains fragments derived from matrix adduct ions, unlike the spectrum obtained from a sample analyzed without the ammonium salt addition (Fig. 4).

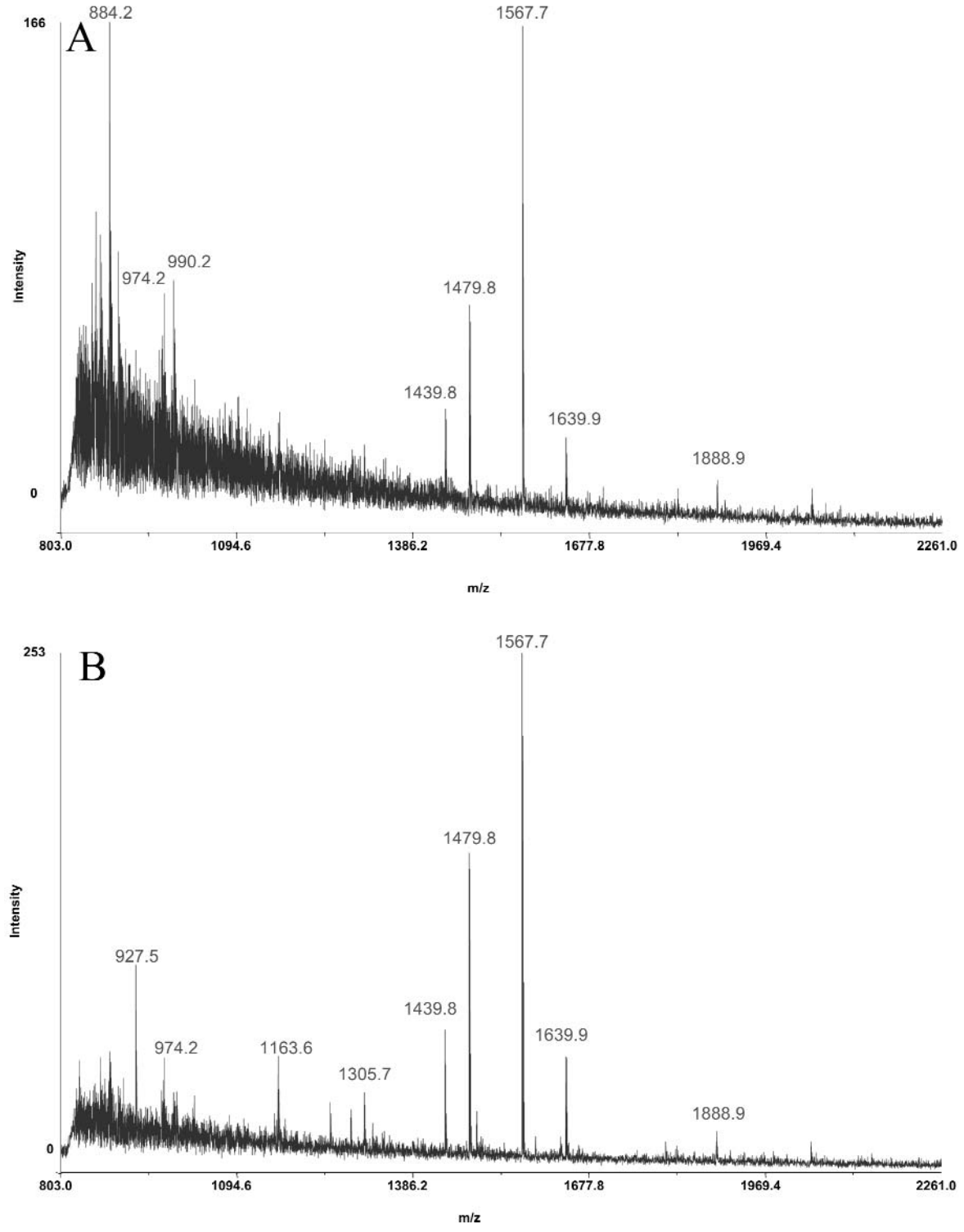

FIGURE 6

MS spectra of a BSA digest, acquired with an α-CHCA matrix concentration of 5 mg/mL **(A)** and 2 mg/mL **(B).** Note that at the 2-mg/mL matrix concentration, more peptide signals are present and with better signal-to-noise ratios. Ammonium monobasic phosphate (10 mM) was added to both preparations.

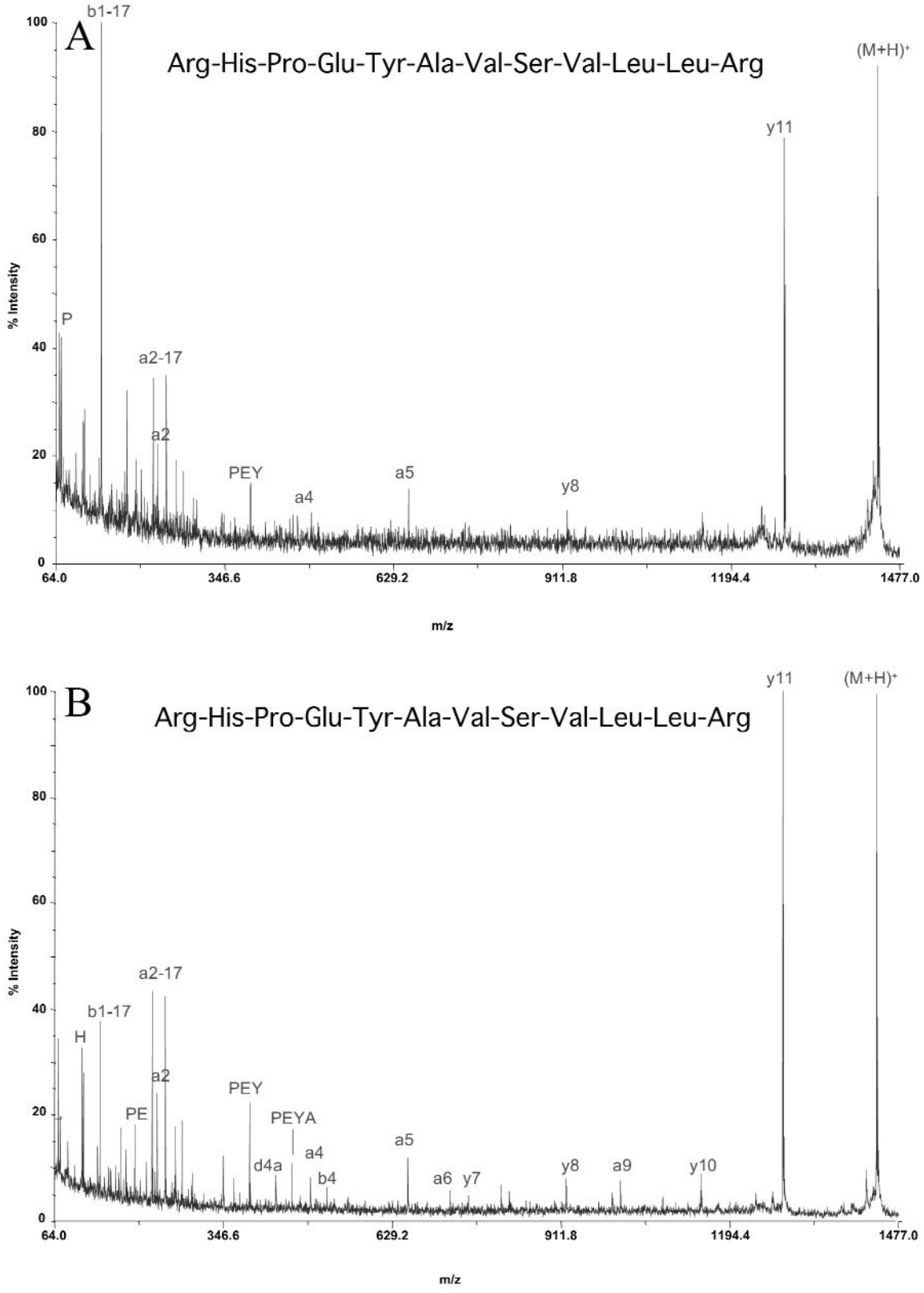

FIGURE 7

MS/MS spectra of BSA peptide RHPEYAVSVLLR [$(M+H)^+$ *m/z* 1439.8], from the sample analyzed with an α-CHCA matrix concentration of 5 mg/mL **(A)** and 2 mg/mL **(B).** Note that the improvement in MS signal for the lower matrix concentration sample (Fig. 6) is evident in the MS/MS spectra as well, with the 2 mg/mL sample yielding sufficient fragment ions to identify the peptide unambiguously as belonging to BSA by database search.

CHCA (Fig. 6B) than with 5 mg/mL α-CHCA (Fig. 6A); ammonium phosphate had been added to both matrix preparations. By database searching using Mascot,[6] BSA was identified as the top protein hit for both matrix concentrations. However, the score returned by Mascot was below the significant threshold for the sample analyzed in 5 mg/mL matrix (43 vs 62), whereas for the sample analyzed in 2 mg/mL matrix the Mascot score was well above the significant threshold (73 vs 62), and so the confidence in the correct protein identification obtained from the latter results is significantly greater. Also, more complete peptide sequence information, due to the presence of more abundant and numerous MS/MS fragment ions from these peptides, was obtained from the MS/MS spectra than from the sample analyzed in 2 mg/mL matrix, allowing for positive confirmation of the protein identification results (Fig. 7).

Addition of ammonium monobasic phosphate to protein digests deposited on MALDI targets can be complemented by the use of this salt as a modifier in reversed-phase high performance liquid chromatography (HPLC) mobile phases during fractionation of protein digests. Ammonium phosphate can facilitate the separation of peptides eluted from a reversed phase HPLC column.[7] With this approach, HPLC fractions, mixed with matrix and deposited directly onto MALDI plates for mass spectrometric analysis, may benefit from the better separation of peptides and the effect of ammonium phosphate in reducing or eliminating interfering matrix clusters with sodium and potassium ions which are often present in samples separated by HPLC.

CONCLUSION

Both ammonium monobasic phosphate and ammonium dibasic citrate reduce formation of α-CHCA adducts and increase the peptide signal-to-noise ratio when added to the matrix–sample mixture. Ammonium monobasic phosphate can be used in a wider concentration range than ammonium citrate, benefitting both, and it is our preferred matrix additive. These effects are enhanced by the use of α-CHCA matrix at a reduced concentration for peptide samples deposited onto the MALDI target at the amol level, which further improves sensitivity and, apparently, also enhances the fragmentation of peptides.

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