JOURNAL OF MASS SPECTROMETRY
J. Mass Spectrom. 2008; **43**: 538–541
Published online 11 December 2007 in Wiley InterScience
(www.interscience.wiley.com) DOI: 10.1002/jms.1353

JMS

JMS Letters

Dear Sir,

A two-matrix system for MALDI MS analysis of serine phosphorylated peptides concentrated by Fe_3O_4/Al_2O_3 magnetic nanoparticles

α-Cyano-4-hydroxycinnamic acid (CHCA) is often selected as the matrix-assisted laser desorption/ionization (MALDI) matrix for peptide analysis. However, CHCA is recognized as a hot matrix,[1–5] which is seldom used in the analysis of phosphopeptides. This is because extensive fragmentation from intact analytes is frequently observed in MALDI mass spectra when CHCA is used as the matrix.[6,7] However, fragmentation derived from phosphopeptides can provide data for confirmation of phosphorylation of unknown ions[7,8] after enrichment by the affinity probes. These results suggested an approach for confirmation of the presence of phosphorylated peptides simply by using CHCA as the MALDI matrix. 2,5-dihydroxybenzoic acid (DHB) is the most common matrix for MALDI MS analysis of phosphopeptides.[9,10] The detection limit of phosphopeptides using 2,5-DHB as the matrix is generally better than that of using CHCA. Unlike analytes homogeneously distributing among CHCA crystalline, inhomogeneous crystalline of 2,5-DHB/analyte, i.e. 'sweet spots', is often observed during MALDI sample preparation. Although it is time-consuming to search 'sweet spots' to obtain analyte signals, analytes are generally concentrated on specific spots, which, therefore, lower the detection limit of analytes when 2,5-DHB is used as the MALDI matrix.

Two matrix-systems have been used for improvement of MALDI MS results.[11,12] Laugesen and Roepstorff[11] have demonstrated that the mass spectral results with increased sequence coverage and spot-to-spot reproducibility for peptide mass mapping were obtained when the mixture of CHCA and 2,5-DHB was used as the MALDI matrix. Cotter and coworkers[12] have reported that homogeneous sample deposition, improved peak intensity and good mass resolution were obtained in MALDI MS analysis by modifying the MALDI target to be hydrophobic and using CHCA/2,5-DHB mixture as the MALDI matrix. We herein examine the results of using the mixture of 2,5-DHB and CHCA as the MALDI matrix for MALDI MS analysis of serine phosphopeptides after enrichment from tryptic digests of proteins using Fe_3O_4/Al_2O_3 as the affinity probes, which have been demonstrated very effectively in enriching phosphopeptides selectively from complex samples.[13] We expect that a low concentration of phosphopeptides could be characterized by using this two-matrix system for MALDI MS analysis.

The details of the preparation steps of Fe_3O_4/Al_2O_3 have been described elsewhere.[13] Proteins (1 mg) were prepared in an ammonium bicarbonate (Riedel-de Haën, Germany) aqueous solution (50 mM, 0.25 ml) containing urea (Riedel-de Haën, Germany) (8 M), followed by the incubation at 38 °C for 30 min. Dithiothreitol (0.4 mg) (Sigma, St. Louis, MO) was added to the solution and the mixture reacted at 45 °C for 1 h. After cooling to room temperature, the mixture was mixed with iodoacetic acid (1 mg) (Sigma, St. Louis, MO) prepared in aqueous ammonium bicarbonate (50 mM) in an ice bath. The sample vial was wrapped with aluminum foil and vortex-mixed for 2.5 h at room temperature. After completion of the reaction, an aqueous ammonium bicarbonate (50 mM, 0.5 ml, pH ~8.2) was added into the above mixture. The denatured protein solution (1 mg/ml = $\sim 4 \times 10^{-5}$ M) obtained above was digested enzymatically by mixing with trypsin (0.1 mg/ml) (Sigma, St. Louis, MO) prepared in ammonium bicarbonate (50 mM, pH ~8.2), at a weight ratio of 50 : 1, and incubated at 37 °C for 18 h. The protein digest with a concentration of 10^{-6} M was prepared directly by dilution with 0.15% trifluoroacetic acid (TFA)/water solution. However, the sample with a concentration of 5×10^{-11} M was prepared by diluting the original tryptic digest (4×10^{-5} M) to 5×10^{-10} M by ammonium bicarbonate (50 mM) followed by 10-fold dilution with 0.15% TFA/water solution. Fe_3O_4/alumina magnetic nanoparticles (40 mg/ml) were prepared in 0.15% TFA(Riedel-de Haën, Germany)/water under sonication for 30 min. The magnetic particle suspension (0.7 μl) was mixed vigorously with the tryptic digestion product solution (49.3 μl) by pipeting in and out of the sample vial for 30 s. The nanoparticles conjugated with their target species were aggregated on the sample wall by positioning a magnet to the outside of the sample vial so that the solution could be easily removed by pipette. The isolated particles were rinsed with 0.15% TFA solution (60 μl × 2) prepared in acetonitrile (Merck, Germany)/deionized water (1/1, v/v), mixing vigorously by pipeting in and out of the mixture in the vial. The rinsed nanoparticles were mixed with a MALDI matrix (0.7 μl, 30 mg/ml), which was prepared in an acetonitrile/deionized water (2/1, v/v) solution containing 1% phosphoric acid (Riedel-de Haën, Germany). The suspension was applied to a MALDI sample target. After the evaporation of the solvent, the sample was ready for Biflex III MALDI TOF MS (Bruker Daltonics, Germany) analysis. All the mass spectra were obtained from positive-ion mode.

Figure 1(a) displays the MALDI mass spectrum obtained using the Fe_3O_4/alumina magnetic nanoparticles to enrich phosphopeptides from the tryptic digest of chicken ovalbumin with CHCA as the matrix. The peaks marked with numbers 2088.91, 2512.10 and 2902.31, which are serine phosphopeptides, appear in the mass spectrum. The corresponding peptide sequences of these ions are listed in Table 1. However, three peaks marked with asterisks are derived from the phosphopeptide peaks by the loss of a HPO_3^-. Furthermore, all the fragment ions marked with asterisks have higher relative abundance than their corresponding phosphopeptides. In post-source decay (PSD) MALDI mass spectra, the fragmentations generated from their corresponding phosphopeptides generally lose a phosphoric acid (~98 Da).[14] Without using the PSD mode, all the dephosphorylated fragment ions derived from phosphopeptides by the loss of a HPO_3 group can be readily identified in the same mass spectrum using CHCA as the MALDI matrix. In this experiment, the Fe_3O_4/alumina magnetic nanoparticles conjugated with their target species were introduced into the mass spectrometer after mixing with CHCA. We suspected that serious fragmentation might be caused by the presence of the magnetic nanoparticles in the samples. Therefore, we removed the magnetic nanoparticles after mixing with CHCA solution and only the solution containing the eluted species was introduced into the mass spectrometer. Figure 1(b) displays

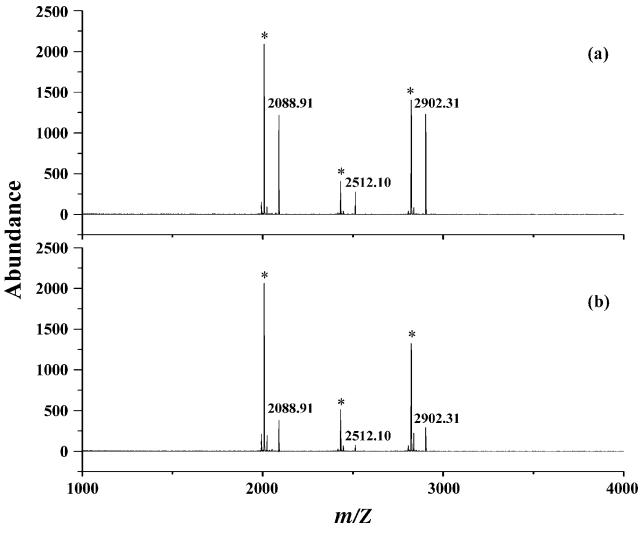

Figure 1. MALDI mass spectra obtained using Fe_3O_4/alumina magnetic nanoparticles to selectively enrich their target species from the tryptic digest of ovalbumin (1 μM, 50 μl). (a) The magnetic nanoparticles were incorporated in the MALDI sample. (b) The magnetic nanoparticles were not incorporated in the MALDI sample. CHCA was used as the MALDI matrix. Each mass spectrum was acquired from average of 300 laser shots.

*Correspondence to: Yu-Chie Chen, Department of Applied Chemistry and Institute of Molecular Science, National Chiao Tung University, Hsinchu 300, Taiwan. E-mail: yuchie@mail.nctu.edu.tw

Table 1. Peaks observed in the MALDI mass spectrum of Fig. 1

No.	Observed $[M+H]^+$	Theoretical $[M+H]^+$	Sequences
1	2088.91	2088.91	EVVGsAEAGVDAASVSEEFR (340–359)
2	2512.10	2512.12	LPGFGDsIEAQCGTSVNVHSSLR (62–84)
3	2902.31	2902.31	FDKLPGFGDsIEAQCGTSVNVHSSLR (59–84)

's' stands for phosphorylated serine. 'C' stands for carboxymethylated cysteine.

the MALDI mass spectrum from the sample used in Fig. 1(a), but with only the eluted CHCA solution introduced into the mass spectrometer. The intensities of the fragment ions marked with asterisks generated from the phosphopeptide ion peaks were even higher than those observed in Fig. 1(a). The intact pseudo-molecular ion peaks of phosphopeptides are barely observed. The fragmentations are even serious in the absence of the nanoparticles in the MALDI samples. That is, the presence of the nanoparticles in the MALDI sample can buffer the energy transfer and reduce the fragmentation. It will be easy to identify the presence of phosphopeptides if intact phosphopeptide ions and their fragmentations from loss of HPO_3^- can be simultaneously observed in the same mass spectrum. We can easily identify the phosphopeptides based on the appearance of phosphorylated/dephosphorylated ion pairs with the loss of a HPO_3^- group. The results suggest that the introduction of the nanoparticles in the MALDI samples and the use of CHCA as the matrix are helpful for obtaining intact phosphopeptides and their fragmentations with comparable intensities.

The phosphopeptides obtained from the tryptic digest of ovalbumin appearing in Fig. 1 are singly phosphorylated peptides on serine. We used a tryptic digest of β-casein as the sample to investigate the effect of CHCA on multiple-phosphorylated serine peptides. Figure 2 displays the MALDI mass spectrum obtained using the Fe_3O_4/alumina magnetic particles. A noticeable peak at m/z 1980.06 derived from the phosphopeptide peak at m/z 2061.82 (#33–48, FQsEEQQQTEDELQDK, 's' stands for phosphorylated serine) by the loss of a HPO_3^- appears in the MALDI mass spectrum using CHCA as the matrix. The phosphopeptide peak at m/z 3122.25 (#1–25, RELEELNVPGEIVEsLsssEESITR, 's' stands for phosphorylated serine) as shown in Fig. 2 contains four phosphorylation sites. Additionally, a series of peaks appears at m/z 3040.22, 2958.88, 2877.21 and 2795.75, derived from the phosphopeptide peak at m/z 3122.25, which contains four phosphorylated serines, by loss of one, two, three and four HPO_3^- groups, respectively. From these results, the number of phosphorylation sites can be counted in the mass spectrum simply using CHCA as the MALDI matrix.

The number of phosphorylation sites could be readily obtained using CHCA as the matrix. However, the detection limit for phosphopeptides using CHCA is generally higher than that using

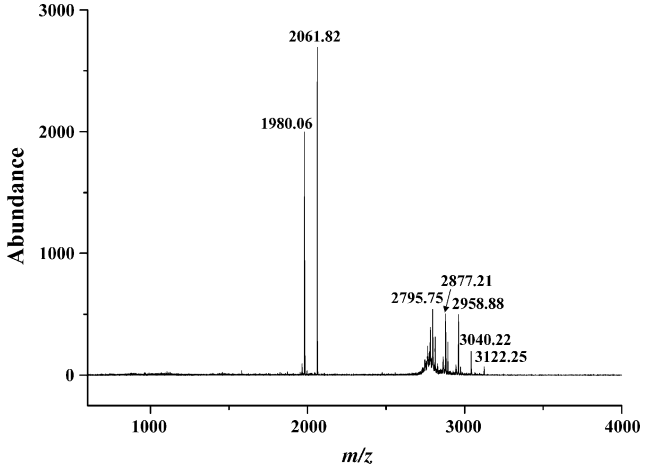

Figure 2. MALDI mass spectrum obtained using Fe_3O_4@alumina magnetic nanoparticles to selectively enrich their target species from the tryptic digest of β-casein (2 μM, 50 μl). CHCA was used as the MALDI matrix.

2,5-DHB as the MALDI matrix. This may be because the analyte molecules are homogeneously distributed among CHCA. On the other hand, 'sweet spots' are commonly observed in the MALDI sample and lower concentrations of analytes can be observed in the MALDI mass spectra when using 2,5-DHB as MALDI matrix. That is, analyte molecules can concentrate on specific crystalline form of 2,5-DHB although it requires efforts to search for the 'sweet spots'. 2,5-DHB generally forms needle-like crystals, while CHCA forms grain-like crystals. When these two matrices in acetonitrile/1% phosphoric acid (2/1, v/v) were mixed, both needle-like and grain-like crystals were observed in the sample when the solvent evaporated. Figure 3(a–c) display the photographs of the sample containing Fe_3O_4/alumina-target species with 2,5-DHB at concentrations of 30, 15 and 7.5 mg/ml, respectively. Figure 3(d) displays a photograph of the sample containing Fe_3O_4/alumina-target species and the mixture of 2,5-DHB (15 mg/ml) and CHCA (15 mg/ml). Needle-like crystals derived from 2,5-DHB are observed in the sample wells when the sample is mixed with 2,5-DHB alone (Fig. 3(a–c)), or when mixed with the 2,5-DHB/CHCA mixture (Fig. 3(d)). The crystal quantities decreased as the concentration of 2,5-DHB decreased, and the crystals in the sample well of Fig. 3(c) were barely detectable. Furthermore, the presence of CHCA in the mixture does not affect the formation of crystalline of 2,5-DHB (Fig. 3(d)). We were able to easily find analyte signals by aiming the laser along the needle-like crystalline spots. However, when we replaced CHCA with sinapinic acid (SA), the needle-like crystalline forms derived from 2,5-DHB did not appear as the solvent evaporated (Fig. 3(e)), perhaps these two matrices were miscible.

Figure 4(a–c) present the MALDI mass spectra of the samples obtained when Fe_3O_4/alumina probes were used to selectively enrich their target species from a solution (50 μl) containing the tryptic digest of β-casein (50 pM) using 2,5-DHB at concentrations of 30 , 15 and 7.5 mg/ml, respectively, as the MALDI matrix, Fig. 4(d) and (e) were obtained using a mixture of 2,5-DHB (15 mg/ml) and CHCA (15 mg/ml), and a mixture of 2,5-DHB (15 mg/ml) and SA (15 mg/ml) as the matrix, respectively. There were no analyte peaks appearing in Fig. 4(a) as such a low concentration (50 pM) of sample was used for analysis. With a decrease in the concentration of 2,5-DHB, a phosphopeptide peak at m/z 2061.82 derived from β-casein appears in the mass spectra (Fig. 4(b) and (c)). However, it was difficult to find the 'sweet spots' since only a few needle-like crystals derived from 2,5-DHB could be found on the sample well (Fig. 3(b) and (c)). When a mixture of 2,5-DHB and CHCA was used as the matrix, a high abundance peak at m/z 2061.82 appeared in the mass spectrum (Fig. 4(d)) and its dephosphorylated peptide peak at m/z 1980.76 with a weak intensity also appear in the same mass spectrum. Furthermore, it was very easy to obtain the analyte signals by directing the laser along the needle-like crystals. We believe that the presence of CHCA in the matrix is helpful for the formation of crystals of 2,5-DHB. It could be confirmed by examining the crystals in Fig. 3(d), which showed more crystals than in Fig. 3(b), even though the same amount of 2,5-DHB had been deposited in both wells. Furthermore, based on our experimental results, 2,5-DHB and CHCA are immiscible in the solution, which helps the crystallization of 2,5-DHB. Additionally, we found that phosphopeptides cocrystallize with 2,5-DHB, but not with CHCA in the two-matrix mixture. Therefore, even though the concentration of the sample was reduced to ~50 pM, analyte signals could be obtained along the few needle-like crystals derived from 2,5-DHB after enrichment by our affinity probes. The detection limit was therefore reduced to ~2.5 fmol. When the mixture of 2,5-DHB and SA was used as the matrix, there were no needle-like crystals observed on the sample well (Fig. 3(e)). Thus, no analyte signals were observed in the MALDI mass spectrum (Fig. 4(e)). The results indicate that a 2,5-DHB/CHCA mixture is useful for the analysis of low concentrations of phosphopeptides.

DOI: 10.1002/jms

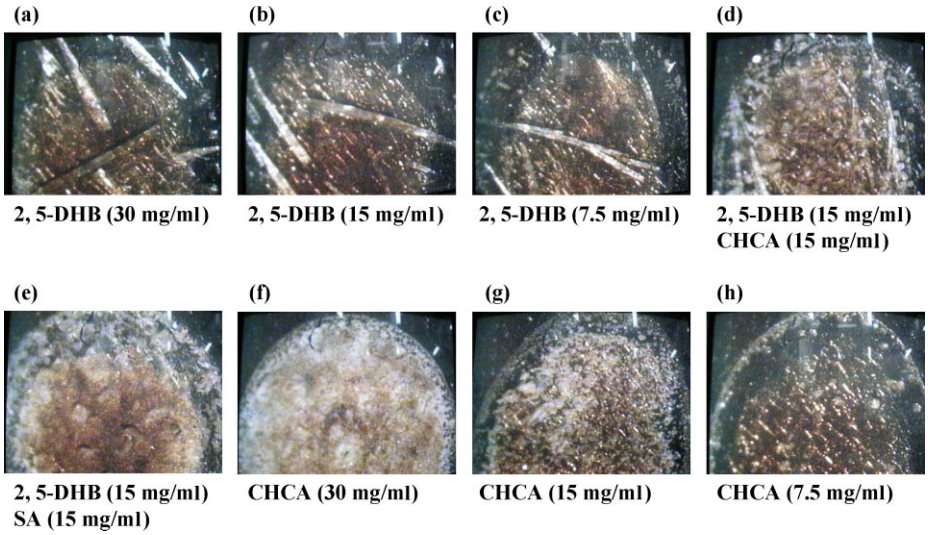

Figure 3. Photographs of the MALDI samples containing Fe_3O_4@alumina-target species using (a) 2, 5-DHB (30 mg/ml, 0.4 μl), (b) 2,5-DHB (15 mg/mL, 0.4 μl), (c) 2,5-DHB (7.5 mg/ml, 0.4 μl), (d) the mixture of 2,5-DHB (15 mg/ml) and CHCA (15 mg/ml, 0.4 μl), (e) the mixture of 2,5-DHB (15 mg/ml) and SA (15 mg/ml), 0.4 μl, (f) CHCA (30 mg/ml, 0.4 μl), (g) CHCA (15 mg/ml, 0.4 μl), and (h) CHCA (7.5 mg/ml, 0.4 μl), as the MALDI matrix. All the matrices contained 1% phosphoric acid.

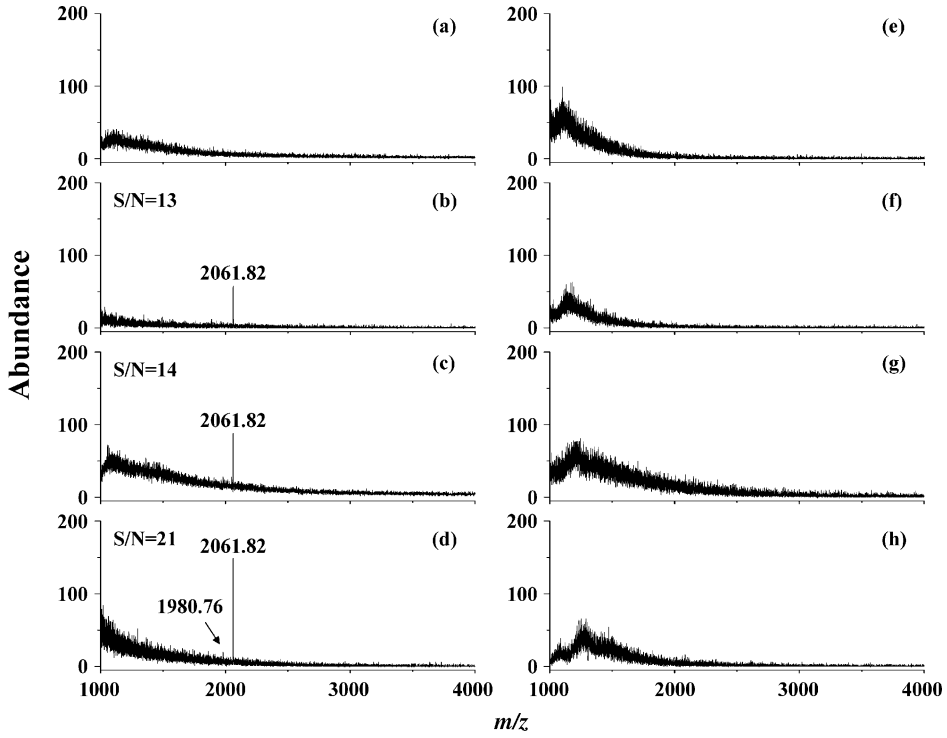

Figure 4. MALDI mass spectra of the sample obtained using Fe_3O_4@alumina affinity probes to selectively enrich their target species from the tryptic digest of β-casein (50 pM, 50 μl) by using (a) 2,5-DHB (30 mg/ml, 0.4 μl), (b) 2,5-DHB (15 mg/ml, 0.4 μl), (c) 2,5-DHB (7.5 mg/ml, 0.4 μl), (d) the mixture of 2,5-DHB (15 mg/ml) and CHCA (15 mg/ml), 0.4 μl, and (e) the mixture of 2,5-DHB (15 mg/ml) and SA (15 mg/ml), 0.4 μl, (f) CHCA (30 mg/ml, 0.4 μl), (g) CHCA (15 mg/ml, 0.4 μl), and (h) CHCA (7.5 mg/ml, 0.4 μl), as the MALDI matrix. All the matrices contained 1% phosphoric acid.

In conclusion, fragmentation of phosphopeptides revealing in the MALDI mass spectra can be enhanced by using CHCA as the MALDI matrix. By incorporation of our nanoparticles into CHCA during MALDI sample preparation, the intensities of the fragment ions and their corresponding phosphopeptides are comparable, which is useful for determining phosphorylation. On the basis of the CHCA-enhanced fragmentation, the number of phosphorylation sites of an enriched phosphopeptide containing multiple phosphorylation sites can be readily determined. When a mixture of 2,5-DHB and CHCA as the matrix was used, the detection limit was reduced to ~2.5 fmol. This approach seems quite promising for the analysis of phosphopeptides.

Acknowledgements

We would like to thank the National Science Council (NSC) of Taiwan and MOE-ATU (96W821G021 and 96W801H109) for financially supporting this research.

Yours,

CHENG-TAI CHEN and YU-CHIE CHEN*
Department of Applied Chemistry and Institute of Molecular Science, National Chiao Tung University, Hsinchu 300, Taiwan

References

1. Kjellström S, Jensen ON. Phosphoric acid as a matrix additive for MALDI MS analysis of phosphopeptides and phosphoproteins. *Analytical Chemistry* 2004; **76**: 5109.
2. Kokubu M, Ishihama Y, Sato T, Nagasu T, Oda Y. Specificity of immobilized metal affinity-based IMAC/C18 tip enrichment of phosphopeptides for protein phosphorylation analysis. *Analytical Chemistry* 2005; **77**: 5144.
3. Yang XF, Wu HP, Kobayashi T, Solaro RJ, van Breemen RB. Enhanced ionization of phosphorylated peptides during MALDI TOF mass spectrometry. *Analytical Chemistry* 2004; **76**: 1532.
4. Schulz E, Karas M, Rosu F, Gabelica V. Influence of the matrix on analyte fragmentation in atmospheric pressure MALDI. *Journal of the American Society for Mass Spectrometry* 2006; **17**: 1005.
5. Stimson E, Truong O, Richter WJ, Waterfield MD, Burlingame AL. Enhancement of charge remote fragmentation in protonated peptides by high-energy CID MALDI-TOF-MS using ''cold'' matrices. *International Journal of Mass Spectrometry* 1997; **169**: 231.
6. Gorman JJ, Ferguson BL, Nguyen TB. Use of 2,6-dihydroxyacetophenone for analysis of fragile peptides, disulphide bonding and small proteins by matrix-assisted laser desorption/ionization. *Rapid Communications in Mass Spectrometry* 1996; **10**: 529.
7. Kapková P, Lattová E, Perreault H. Nonretentive solid-phase extraction of phosphorylated peptides from complex peptide mixtures for detection by matrix-assisted laser desorption/ionization mass spectrometry. *Analytical Chemistry* 2006; **78**: 7027.
8. Annan RS, Carr SA. Phosphopeptide analysis by matrix-assisted laser desorption time-of-flight mass spectrometry. *Analytical Chemistry* 1996; **68**: 3413.
9. Stensballe A, Jensen ON. Phosphoric acid enhances the performance of Fe(III) affinity chromatography and matrix-assisted laser desorption/ionization tandem mass spectrometry for recovery, detection and sequencing of phosphopeptides. *Rapid Communications in Mass Spectrometry* 2004; **18**: 1721.
10. Hart SR, Waterfield MD, Burlingame AL, Cramer R. Factors governing the solubilization of phosphopeptides retained on ferric NTA IMAC beads and their analysis by MALDI TOFMS. *Journal of the American Society for Mass Spectrometry* 2002; **13**: 1042.
11. Laugesen S, Roepstorff P. Combination of two matrices results in improved performance of MALDI MS for peptide mass mapping and protein analysis. *Journal of the American Society for Mass Spectrometry* 2003; **14**: 992.
12. Gundry RL, Edward R, Kole TP, Sutton C, Cotter RJ. Disposable hydrophobic surface on MALDI targets for enhancing MS and MS/MS data of peptides. *Analytical Chemistry* 2005; **77**: 6609.
13. Chen CT, Chen WY, Tsai PJ, Chien KY, Yu JS, Chen YC. Enrichment of phosphopeptides and phosphoproteins from complex samples using magnetic particles coated with alumina as the concentrating probes for MALDI MS analysis. *Journal of Proteome Research* 2007; **6**: 316.
14. Hoffmann R, Metzger S, Spengler B, Otvos Jr L. Sequencing of peptides phosphorylated on serines and threonines by post-source decay in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Mass Spectrom.* 1999; **11**: 1195.

DOI: 10.1002/jms