# Concentration and in Situ Detection of Peptides Using Liquid Matrix-Assisted Laser Desorption Ionization Matrixes

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Letters to *Analytical Chemistry*

# Concentration and in Situ Detection of Peptides Using Liquid Matrix-Assisted Laser Desorption Ionization Matrixes

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**A ternary component system composed of α-cyano-4-hydroxycinnamic acid/3-aminoquinoline/quinoline (CHCA/3-AQ/Q; at a weight ratio of 1:4:4) was used as an extraction solvent as well as a liquid matrix for matrix-assisted laser desorption ionization (MALDI) mass spectrometry analysis. Peptides in aqueous solutions were extracted, concentrated, and prepared for MALDI analysis in one step. Extracting peptides in aqueous solutions was analogous to dispersive liquid−liquid microextraction and completed in less than 2 min because CHCA/3-AQ/Q was dispersed rapidly into the aqueous phase by ultrasonication during extraction. The detection limit for peptides in aqueous solutions was as low as 1.25 nM for angiotensin I. Protein digests obtained from conventional MALDI analysis and the proposed method were compared with respect to sequence coverage. The new approach was applied to sample cleanup, preconcentration, and in situ analysis of protein digests in signal suppressing agents such as Tris buffer and urea.**

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is a highly effective analytical tool in biological research. The efficiency of MALDI is sensitive to the sample preparation procedure. Sample homogeneity is of priority concern in MALDI analysis.[1] Conventional MALDI-MS with its off-line sample preparation and predominant use of solid matrices, viz., α-cyano-4-hydroxycinnamic acid (CHCA) or 2,5-dihydroxybenzoic acid (DHB), has certain merits and limitations. An inherent limitation of using crystalline matrixes in MALDI-MS is commonly referred to as hot spot formation, causing inhomogeneity on the sample and ultimately incurring significant variations of signal intensities at various positions of the sample spot.[2]

Adopting liquid matrix systems is a promising approach among several others to improve the sample homogeneity. Such matrixes[3] can increase sample homogeneity as well as replace continuously desorbed material as it is ablated from the surface.[4,5] Many of the early liquid matrix systems used glycerol based matrixes[6−8] or 3-nitrohenzyl alcohol (3-NBA) as the matrix.[9,10] The feasibility of using ionic liquids as liquid matrixes for MALDI has received considerable interest in recent years.[11] Ionic liquids are potential matrixes for MALDI-MS owing to their unique properties such as negligible vapor pressure, excellent thermal stability, and miscibility with water and organic solvents. Such factors make ionic liquid matrixes valuable alternatives for applications in qualitative[12,13] and quantitative[14−16] MALDI-MS.

Sze et al.[4] developed vacuum stable, chemically doped liquid matrixes by mixing standard solid MALDI matrixes, DHB and CHCA, with viscous liquids such as glycerol and diethanolamine. The amount of DHB and CHCA that could be dissolved in glycerol was increased using the base 3-aminoquinoline as a solubilizing agent. This approach provides excellent shot-to-shot reproducibility and long-term analyte signal stability. Bogan et al.[17] established rapid and confident protein and peptide identifications at the low femtomole level using liquid UV-MALDI matrixes. Later, Cramer and Corless[5] described the feasibility of applying the liquid matrix containing a ternary mixture of CHCA, 3-aminoquinoline, and glycerol to proteomic analysis. This liquid matrix significantly reduces the extent of unspecific ion signals (low mass and cation adduct formation) in peptide mass fingerprints and enhances the protein identification by peptide mass mapping of in-solution and

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in-gel digests. The same group recently optimized liquid matrix formulation and explored the stability for measurement of quantitative peptide abundance.[18]

Sample preparation procedures such as desalting and concentration are essential for successful MS analysis of biological samples. Many studies have examined the feasibility of applying methods such as solid phase microextraction (SPME),[19] liquid phase microextraction (LPME),[20] single drop microextraction (SDME),[21,22] and dispersive liquid−liquid microextraction (DLLME)[23] to preconcentration of analytes from various samples. These methods are simple to operation, quick, economical, as well as have a high enrichment factor and high recovery. Furthermore, liquid mciroextracton-related procedures with MALDI-MS have been applied successfully in peptides, proteins, environmental, and pharmaceutical analysis.[24,25] This study presents a new liquid matrix composed of α-cyano-4-hydroxycinnamic acid/3-aminoquinoline/quinoline (CHCA/3-AQ/Q) as a solvent for the extraction and preconcentration of peptides in aqueous samples before MALDI analysis. The samples were analyzed in situ after the extraction without an additional sample transfer. The extraction method is equivalent to conventional dispersive liquid−liquid microextraction (DLLME).[23] With the use of this approach, this study analyzes protein digests in the presence of signal-suppressing buffers and denaturants using CHCA/3-AQ/Q as a concentration solvent and a MALDI matrix.

## EXPERIMENTAL SECTION

**Liquid−Liquid Microextraction.** All the materials and reagents were used without further purification (see the Supporting Information). The CHCA/3-AQ/Q liquid matrix was prepared by dissolving CHCA, 3-aminoquinoline, and quinoline (1:4:n by weight, $n = 0, 1, 2, 4, 6, 8$) in a solution of acetonitrile containing 0.1% trifluoroacetic acid. The mixture was vortexed until all compounds were dissolved, then dried in a SpeedVac. To extract the analytes in an aqueous solution, 0.5−2 μL of liquid matrix CHCA/3-AQ/Q was used to extract the peptides from the sample solution by ultrasonication. Following extraction, phase separation was performed by centrifugation for 1 min at 13 krpm. Because of the relatively high viscosity of the liquid matrix in the lower phase, aqueous solutions in the upper phase could be easily removed by a pipet.

**Protein Digestion.** Protein standards in various buffered solutions were reduced by 5 mM dithiothreitol (DTT) at 56 °C for 1 h and, then, alkylated by 20 mM iodoacetamide (IAA) in the dark room for 1 h. Buffered solutions and their final concentrations are 25 mM $NH_4HCO_3$, 25 mM Tris plus 3.6 M urea, 50 mM Tris, and 25 mM Tris plus 100 mM guanidine thiocyanate. The 25 mM $NH_4HCO_3$ buffer was used in sequence coverage studies, while other buffers were used in signal suppression studies. Next, sample solutions were adjusted to pH 8.5. Finally, proteins were digested by trypsin at a 1:25 enzyme to protein molar ratio and 37 °C overnight in a water bath.

**Mass Spectrometry and Protein Identification.** Analyses were conducted using a Bruker Autoflex time-of-flight mass spectrometer (Bruker Daltonnics, Breman, Germany) equipped with a SCOUT 384 probe ion source. The system uses a pulsed nitrogen laser (wavelength 337 nm; model MNL 202-C; LTB Lasertechnik Berlin GmbH, Berlin, Germany) with an energy of 98 μJ/pulse. Samples were analyzed in reflectron mode with the acceleration voltage of 21 kV. Solid MALDI matrix was prepared by dissolving CHCA in a solvent mixture of 1:1 acetonitrile/0.1% TFA to saturation. A 0.5 μL aliquot of sample solution that was mixed with an equal volume of CHCA matrix solution was spotted on a target plate and dried before MALDI-MS analysis. For liquid matrix experiments, solutions containing liquid matrix CHCA/3-AQ/Q and analytes were directly placed onto a target plate and analyzed by MALDI-MS. Each mass spectrum was generated by averaging 200−300 summed scans. Data were acquired and analyzed using Flex Control (version 2.0) and Flex Analysis (version 2.2) softwares. Mass peak lists were submitted for peptide mass fingerprinting using the MASCOT search engine and searched against the MSDB database (taxonomy: all entries). The searching parameters were set as follows: a mass tolerance of 300 ppm, three missed-cleavage sites for myoglobin and one missed-cleavage site for other proteins, and variable modifications of cysteine carbamidomethylation and methionine oxidation.

## RESULTS AND DISCUSSION

This study investigated the feasibility of using liquid matrix as both a concentration solvent and a MALDI matrix. Sze et al. demonstrated that CHCA/3-AQ/glycerol mixed at 1:4:6 by weight produces spectra with sensitivity superior to most liquid matrixes but is comparable to the sensitivity obtained from conventional solid matrix preparation.[4] Although a useful matrix, the glycerol-based matrix is an inadequate extraction solvent for aqueous samples because of its high hydrophilicity. The matrix solution was made hydrophobic by replacing glycerol with quinoline. The dissolution of the hydrophobic phase into the aqueous phase was examined by mixing 40 μL of water with 1 μL of CHCA/3-AQ/Q via ultrasonication for 2 min. Following phase separation by centrifugation, adding one, two, and four parts (by weight) of quinoline to CHCA/3AQ produced an extraction solvent loss of 22 (±4)%, 13 (±3)%, 2.0 (±0.6)%, respectively. The percentage loss was estimated through three replicates by weighing the extraction solvent before and after the mixing. Moreover, adding quinoline more than 4 parts consistently yielded negligible solvent loss. Also, 4 parts of quinoline were added to the extraction solvent in this study.

A conventional DLLME is based on a ternary component solvent system in which an extraction solvent and a disperser solvent are injected into the aqueous solution.[23] The disperser solvent that is soluble in the extraction solvent should be miscible in the aqueous phase. After the injection, the mixture becomes a cloudy solution. The resulting dispersed fine droplets of extraction solvent are centrifuged to the bottom of the reaction vessel and then collected. In this study, aqueous samples were mixed with the proposed liquid matrix (CHCA/3-AQ/Q) by sonication and produced milk yellow emulsions owing to the dispersion of the liquid matrix into the aqueous phase. The


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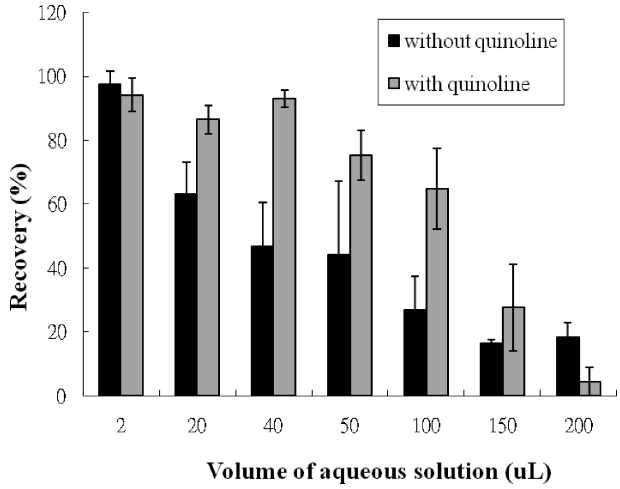


**Figure 1.** Recovery rate of Ang I in an aqueous solution using 2 $\mu$L of CHCA/3AQ/Q (1:4:4) vs various volumes of aqueous solution.

mixtures were then separated into two phases by centrifugation for 1 min at 13 krpm. Therefore, the proposed liquid−liquid microextraction method (LLME) is analogous to DLLME.

The peptide, Ang I, was selected as a test compound and quantified by adding Sub P as an internal standard. The calibration curve was generated by performing MALDI analysis, in which CHCA/3-AQ/Q (1:4:4) was used as a matrix. The plot of abundance ratios of Ang I to Sub P (0.5 pmol) versus the amount of Ang I (ranging from 0.1 to 2.5 pmol) yielded a satisfactory linear correlation with a correlation coefficient ($R^2$) of 0.989 (based on three replicates).

The three-component matrix CHCA/3-AQ/Q should have an ionic characteristic owing to the reaction of the carboxylic group in CHCA with the amine group in 3-AQ, subsequently producing a carboxylic anion and primary ammonium cation. The NMR spectrum of the liquid matrix reveals the absence of a signal corresponding to a carboxylic proton, thus supporting this assumption. The feasibility of recovering Ang I from various volumes of an aqueous solution using 2 $\mu$L of extraction solvents was investigated. CHCA/3-AQ solvent systems with and without quinoline were used. Ang I recovered from extraction was quantified by adding Sub P (0.5 pmol) into the liquid matrix after extraction. Figure 1 shows the recovery rates calculated according to the above-mentioned calibration curve. The liquid−liquid extraction using the solvent containing quinoline (CHCA/3-AQ/Q = 1:4:4) produced a recovery rate exceeding 90% when the aqueous/organic volume ratio was up to 20:1. The recovery rate maintained more than 60% until the volume ratio was 50:1 and later decreased with an increasing volume of aqueous solutions. Adding quinoline is essential in maintaining the hydrophobicity of the extraction solvent. Figure 1 also shows the recovery rate of Ang I using CHCA/3-AQ (1:4) as an extraction solvent. The recovery rate decreased significantly with an increasing volume of the aqueous sample solution. The rapid decrease of the recovery rate is attributed to the dissolution of the extraction solvent into the aqueous phase.

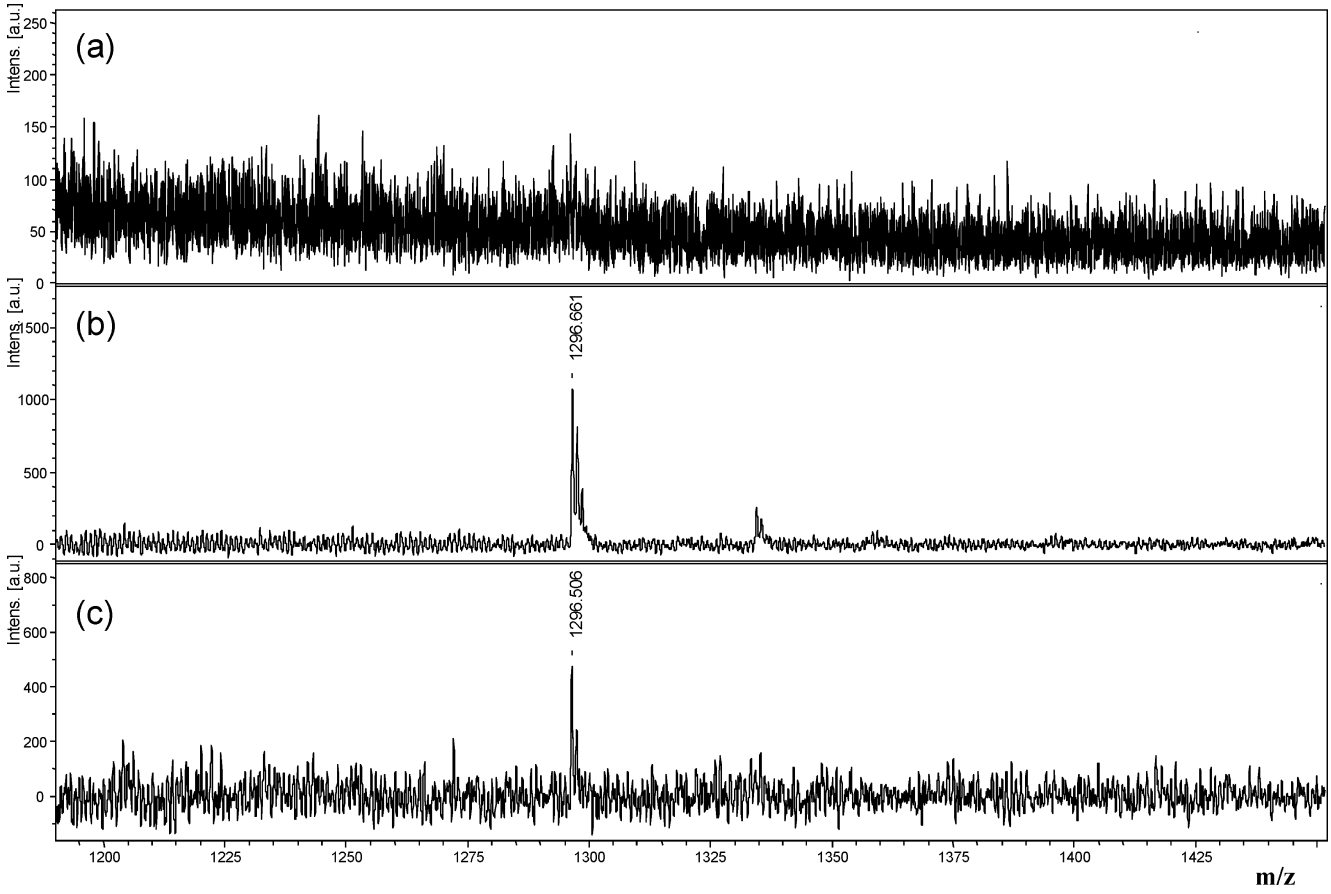


**Figure 2.** MALDI mass spectra of Ang I: (a) The peptide (5 nM, 0.5 $\mu$L) was analyzed using a CHCA matrix. (b) The peptide (5 nM, 20 $\mu$L) was extracted using 0.5 $\mu$L of CHCA/3-AQ/Q prior to in situ MALDI analysis. (c) The peptide (1.25 nM, 20 $\mu$L) was analyzed using the same approach as described in part b.

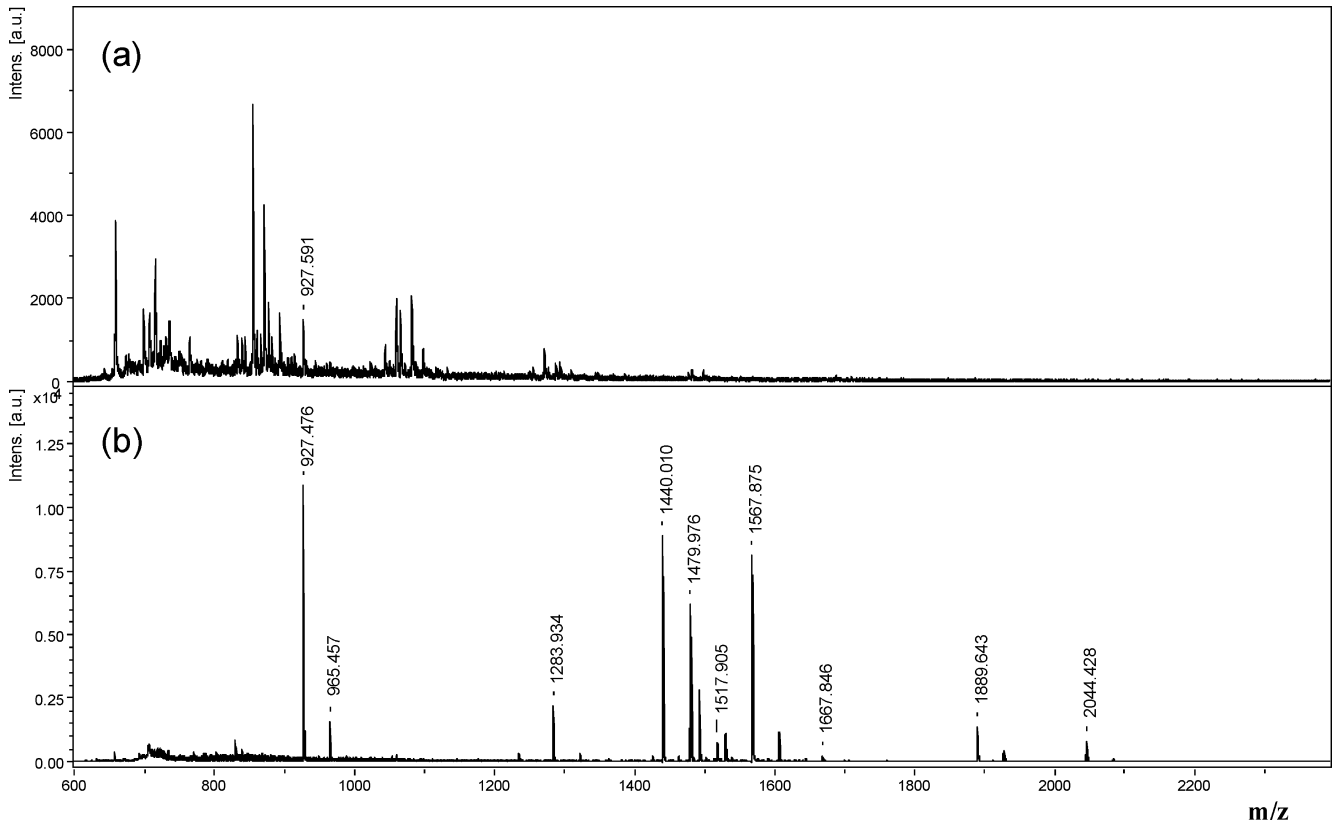


**Figure 3.** MALDI mass spectra of tryptic digest of 0.36 $\mu$M of BSA in 25 mM Tris and 2 M urea (a) before and (b) after LLME. Labeled peaks correspond to the matched peptides of BSA.

Several factors, e.g., sonication time, temperature, and the pH value, that affect the liquid−liquid extraction process were examined. The recovery rate of Ang I did not depend strongly on the sonication time when the times were shorter than 10 min. Because of the formation of emulsion after sonication, the surface area between the aqueous phase and extraction solvent was extremely large; in addition, the time for extracting the analyte into extraction solvent could be as little as 30 s. However, the recovery rates worsened when extraction times exceeded 30 min (see Figure S1 in the Supporting Information). With longer extraction times, the extraction solvent dissolved into the aqueous solution owing to the ionic nature of the extraction solvent. Notably, 2 min of sonication was performed.

The recovery rates of Ang I at extraction temperatures of 25, 35, 45, and 55 °C were 62, 73, 71, and 85%, respectively (Figure S2 in the Supporting Information). A high temperature favored, to some extent, the extraction of the peptide. However, the recovery rate dropped significantly to 49% at 65 °C. At this temperature, the loss of the extraction solvent due to the dissolution into the aqueous phase was observed, subsequently decreasing the recovery rate. No additional heating was applied during extraction in this work. In general, the pH value of sample solutions is an important factor during extraction. When the recovery rate of Ang I was measured at various pH values of aqueous sample solutions, the extraction is optimum at the pH around the isoelectric point (pH 6.9) of the peptide (Figure S3 in the Supporting Information), where the hydrophobic interaction between the extraction solvent and the analyte is the strongest. Interestingly, the peptide recovery is good over pH values ranging from 4 to 10. The recovery is in the range from 58 to 77%. The ionic nature of the extraction solvent appears to play an important role when the peptide is above or below its p*I* value. The peptide carries a positive charge falling below its p*I* value and a negative charge surpassing its p*I* value. The charge on the peptide allows the ionic solvent to extract the peptide according to the "like dissolves like" rule. The recovery rate of Ang I is lower than 20% at the extreme pH value of 2. When the solution is too acidic or too basic, solubility of the extraction solvent in the aqueous solution increases, subsequently decreasing the recovery rate of Ang I from the aqueous phase.

The detection limits of peptides in aqueous solutions were determined using the proposed liquid−liquid microextraction method followed by in situ MALDI analysis. Figure 2 shows the MALDI mass spectra of Ang I in water obtained before and after LLME. When 0.5 $\mu$L of the 5 nM analyte was mixed with the same volume of matrix solution of CHCA and analyzed by MALDI-MS, no signal of the peptide ions was detected (Figure 2a). However, when 20 $\mu$L of the 5 nM Ang I solution was extracted using 0.5 $\mu$L of CHCA/3-AQ/Q, in situ MALDI analysis clearly yielded a detectable signal (Figure 2b). The minimum detectable concentration of Ang I in the aqueous solution using LLME is 1.25 nM, which generated a signal with S/N of 4.7 (Figure 2c). The same approach was applied to several other peptides (including ACTH (18−39) and sub P), yielding detection limits of 2.5 and 1.25 nM, respectively (data not shown). Experimental results indicated that an excellent sample concentration was achieved using CHCA/3-AQ/Q as an extraction solvent.

The digestion of proteins often requires adding denaturants and buffers such as urea and Tris. Therefore, sample cleanup procedures are necessary to avoid signal suppression and produce

**Table 1. Peptide Mass Fingerprints Obtained from Direct MALDI Analysis and LLME Followed by in Situ MALDI Analysis**

| protein | method | no. of matched peptides | sequence coverage | Mascot score[c] | *E*-value in Mascot |
|---|---|---|---|---|---|
| BSA | solid matrix[a] | 18 | 32 | 173 | $1.6 \times 10^{-11}$ |
| | LLME[b] | 14 | 25 | 161 | $2.6 \times 10^{-10}$ |
| lysozyme | solid matrix | 14 | 76 | 208 | $5.1 \times 10^{-15}$ |
| | LLME | 9 | 59 | 143 | $1.6 \times 10^{-8}$ |
| myoglobin | solid matrix | 14 | 87 | 189 | $4.1 \times 10^{-13}$ |
| | LLME | 10 | 80 | 131 | $2.6 \times 10^{-7}$ |
| ovalbumin | solid matrix | 13 | 54 | 144 | $1.3 \times 10^{-8}$ |
| | LLME | 8 | 36 | 118 | $5.1 \times 10^{-6}$ |

[a] A total of 1 pmol of proteins was analyzed. [b] Proteins (0.1 $\mu$M, 20 $\mu$L) following trypsin digestion were extracted using 0.5 $\mu$L of CHCA/3-AQ/Q prior to in situ MALDI analysis. [c] The significance threshold is 78.

quality mass spectra. The proposed approach was applied to extract BSA digest from a solution containing Tris buffer and urea. Sample cleanup and concentration were thus completed in one step. Figure 3 shows the MALDI mass spectra of tryptic digest of 0.36 $\mu$M of BSA in 25 mM Tris and 3.6 M urea before and after LLME. Direct analysis of the protein digest by MALDI using a CHCA matrix before the extraction was seriously interfered with by the sample matrix (Figure 3a). Following extraction of 20 $\mu$L of the digest using 0.5 $\mu$L of CHCA/3-AQ/Q, the peptide signals derived from BSA were easily detected (Figure 3b). The 20 $\mu$L volume is used because it is a reasonable working volume for protein digestion.

Table 1 summarizes the peptide mass fingerprints obtained after LLME of tryptic digests of several proteins, including BSA, lysozyme, myoglobin, and ovalbumin. This table compares the number of matched peptides, sequence coverage, and MASCOT search score for protein identification using direct conventional MALDI analysis and LLME followed by in situ MALDI analysis. The sequence coverages and Mascot scores of the proteins analyzed using the LLME approach are all satisfactory. Overall, the number of matched peptides obtained from the LLME approach is somewhat less than that obtained from direct MALDI analysis. The results are not unexpected because some peptides may have a low ion abundance or low solubility in the liquid matrix and are not detectable by MS.

## CONCLUSIONS

This study demonstrates the feasibility of applying a liquid matrix to the concentration and analyzing peptides in aqueous solutions by integrating LLME and MALDI-MS. The proposed scheme provides in situ MALDI-MS analysis of peptides concentrated by a liquid matrix CHCA/3-AQ/Q (1:4:4). The hydrophobic liquid mixture has an ionic nature owing to the reaction between the acidic CHCA and basic 3-AQ. The properties of this liquid matrix make it an effective extraction solvent for peptides. The detection limit of peptides in aqueous solutions was in the nanomolar range. This method has been successfully adopted to analyze protein digests in the presence of signal suppressing buffers and denaturants. Sample concentration and cleanup were performed in one step, followed by in situ MALDI analysis. Efforts are currently underway to use this liquid matrix for the extraction and detection of small organic compounds, such as pesticides or other environmental pollutants.

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## SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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